Manual for Marine Monitoring in the

COMBINE

Programme of HELCOM

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PART A. GENERAL ASPECTS

A.1. INTRODUCTION

Monitoring is since long a well established function of the Helsinki Convention. Monitoring of physical, chemical and biological variables of the open sea started in 1979, monitoring of radioactive substances in the Baltic Sea started in 1984.

Until 1992 monitoring of coastal waters was considered as a national obligation and only assessment of such data had to be reported to the Commission. However, under the revised Helsinki Convention, 1992, it is an obligation to conduct also monitoring of the coastal waters and to report the data to the Commission. This programme will also cater for the needs of monitoring in the Baltic Sea Protected Areas (BSPA).

The Environment Committee decided that for management reasons the different program should be integrated into a common structure and thus the Cooperative Monitoring in the Baltic Marine Environment - COMBINE - was instituted in 1992.

This Manual is directed to all performing monitoring in the COMBINE Programme. The Manual defines the contributions made by all Contracting Parties and regulates all methods used.

The document will be revised when there is a need for changes in the Programme content or for updating of technical annexes.

The official version of the Manual for Marine Monitoring in the COMBINE Programme of HELCOM is always available electronically via the HELCOM home page. The validity of copies must always at all times be controlled against the official version by end users.

This Manual is updated once a year. Changes to be included in the Manual should be considered by the Monitoring and Assessment Group and after its endorsement submitted to the Secretariat **not later than 1 June**. These changes will then be valid from **1 January the following year**. All changes are highlighted by a separate note, section by section.

The Manual has last been updated in January 2008 according to the decisions by HELCOM MONAS 10/2008.

A.2. AIMS FOR THE MONITORING

The aims of COMBINE, as decided by HELCOM (HELCOM 14/18, Paragraph 5.27) and further elaborated by BMP-WS 2/96, are:

- To identify and quantify the effects of anthropogenic discharges/activities in the Baltic Sea, in the context of the natural variations in the system, and
- To identify and quantify the changes in the environment as a result of regulatory actions.

This general statement, which is equally valid for monitoring of inputs as well as monitoring of environmental conditions, is then converted into more specific aims for the different types of monitoring. More specifically the aims of COMBINE mean:

For the open sea and coastal area monitoring:

- Hydrographic variations: to set the background for all other measurements related to the identification and quantification of the effects of anthropogenic discharges/activities, the parameters providing an indication of natural fluctuations in the hydrographic regime of the Baltic Sea must be monitored on a continuous basis.

Problems related to eutrophication:

- To determine the extent and the effects of anthropogenic inputs of nutrients on marine biota, the following variables must be measured:

    a) concentrations of nutrients,
    b) the response of the different biological compartments and
    c) Integration and evaluation of results

For contaminants:

- To compare the level of contaminants in selected species of biota (including different parts of their tissues) from different geographical regions of the Baltic Sea in order to detect possible contamination patterns, including areas of special concern (or ´hot spots´).
- To measure levels of contaminants in selected species of biota at specific locations over time in order to detect whether levels are changing in response to the changes in inputs of contaminants to the Baltic Sea.
- To measure levels of contaminants in selected species of biota at different locations within the Baltic Sea, particularly in areas of special concern, in order to assess whether the levels pose a threat to these species and/or to higher trophic levels, including marine mammals and seabirds.

For the effects of contaminants:

- To carry out biological effects measurements at selected locations in the Baltic Sea, particularly at sites of special concern, in order to assess whether the levels of contaminants in sea water and/or suspended particulate matter and/or sediments and/or in the organisms themselves are causing detrimental effects on biota (e.g., changes in community structure)."

In more explicit terms this requires several types of investigations.

For the study of eutrophication and its effects:

- long-term trend studies,
- studies with the budget approach (i.e. budgets or "mass balances" for main nutrients),
- studies of effects on biota,
- studies providing 'online' information on sudden events,
- studies giving background information including baseline studies and joint studies.

For the study of contaminants and their effects:

- studies of temporal trends of contaminants,
- studies of spatial variations in contaminant concentrations and patterns,
- studies providing information on episodic events,
- studies of effects on biota as well as risk evaluations for target species,
- studies of environmental fate of contaminants

A.3. NATIONAL COMMITMENTS

Given that the data obtained in the monitoring programme are needed to conduct periodic assessments of the state of the Baltic marine environment, the variables included in the programme have been classified into three categories to ensure that basic information is obtained for all regions of the Baltic Sea, but that specific regional requirements are taken into account as well as resource levels, different competences available, and the desirability and necessity of sharing the workload among the Contracting Parties. The categories also take account of the need for different types of supporting studies on an occasional basis. The three categories are:

Category 1: Core variables

Explanation: Core variables comprise measurements that have to be carried out on a routine basis to produce comparable and accurate results from all regions of the Baltic Sea as a basic information for an assessment.

Category 2: Main variables

Explanation: Main variables are of equal importance as the core variables for the Baltic Sea Periodic Assessments and have to be measured on a regular basis.

However, for reasons of regional requirements as well as of competence and/or resources not all CPs will be required to carry out all measurements but all measurements will need to be covered on a work-sharing basis.

Category 3: Supporting studies

Explanation: Supporting studies provide information that facilitates the interpretation of monitoring data collected in Category 1 and Category 2 or provide additional information as required.

These investigations are carried out by individual CPs or groups of CPs often in a project- or campaign-like manner. These investigations include, e.g. baseline studies, special monitoring studies, process studies and tests of new methods and techniques.

The success of the monitoring programme depends entirely on the willingness of Contracting Parties to commit themselves to carry out the various parts, particularly variables in Category 1 and Category 2, and that they allocate the resources needed. In this context the following table explaining the regional responsibilities for the Contracting Parties should be considered.

The main responsibilities are as follows:

Baltic Proper: Estonia, Finland, Germany, Latvia, Lithuania, Poland, Sweden and Russia

Gulf of Bothnia: Finland and Sweden

Gulf of Finland: Estonia, Finland and Russia

Gulf of Riga: Estonia and Latvia

Sound and the Kattegat: Denmark and Sweden

Great Belt: Denmark

Bay of Kiel and Bay of Mecklenburg: Germany

Apart from their main responsibilities, however, the Contracting Parties are encouraged to participate in the programme in other regions of the Baltic Sea Area whenever practicable.

Each Contracting Party has offered to carry out a certain combination of variables, sampling stations and frequencies as regards to Category 1 and Category 2, and often also offered special studies as in Category 3. These contributions are regarded as mandatory for the Contracting Party in question with the understanding that future national decisions on priorities and resource allocation may change their contributions to the programme.

A.4. DIVISION OF THE BALTIC AND ADJACENT WATERS

Sea area			Description
1. BALTIC SEA			The waters bordered by the Swedish, Finnish, Estonian, Latvian, Lithuanian, Russian, Polish, German and Danish coasts to the lines FALSTERBO, STEVN KLINT and GEDSER - DARSSER ORT.
	1.1 GULF OF BOTHNIA		The waters north of a line between SIMPNÄS KLUBB - SÖDERARM - SVENSKA BJÖRN - KÖKARSÖREN HANGÖ PENINSULA.
		A 1.1.1 Bothnian Bay	Gulf of Bothnia north of the line RATAN - ST. FJÄDERÄGG - HÄLSINGKALLAN - STUBBEN - MONÄS.
		B 1.1.2 The Quark	Gulf of Bothnia between the lines RATAN - MONÄS as above and HÖRNEFORS - VAASA.
		C 1.1.3 Bothnian Sea	Gulf of Bothnia between the lines HÖRNEFORS - VAASA and ORMÖN - UNDERSTEN - EMSKÄR - ECKERÖ - SÄTSKÄR - UUSIKAUPUNKI.
		D 1.1.4 Åland Sea	Gulf of Bothnia between Sweden and Åland, bordered to the north by a line ORMÖN - UNDERSTEN- EMSKÄR - ECKERÖ and to the south by a line SIMPNÄS KLUBB - SÖDERARM - SVENSKA BJÖRN - KÖKARSÖREN - NYHAMN.
		E 1.1.5 Archipelago Sea	Gulf of Bothnia between Åland and Finland, bordered to the north by a line SÄLSKÄR - UUSIKAUPUNKI and to the south by a line NYHAMN - KÖKARSÖREN - HANGÖ PENINSULA.
	F 1.2 GULF OF FINLAND		Baltic Sea east of the line HANGÖ PENINSULA - PÕÕSASPEA.
	G 1.3 GULF OF RIGA		Baltic Sea east of the lines OVISI- SÕRVE - PAMMANA - SÕRU - TAHKUNA - PÕÕSAPEA.

	1.4 BALTIC PROPER		Baltic Sea within the lines SIMPNÄS KLUBB - SÖDERARM - SVENSKA BJÖRN - KÖKASÖREN - HANGÖ PENINSULA - PÕÕSAPEA - TAHKUNA - SÕRU - PAMMANA SÕRVE - OVISI - Estonian, Latvian, Lithuanian, Russian, Polish and German coasts up to DARSSER ORT - GEDSER - Danish coast to STEVNS KLINT - FALSTERBO - Swedish coast to SIMPNÄS KLUBB
		H 1.4.1 Northern Baltic Proper	Baltic Proper north of a line ARKÖSUND - GOTSKA SANDÖN - VILSANDI - SAAREMAA.
		1.4.2 Central Baltic Proper	Baltic Proper between the lines ARKÖSUND - GOTSKA SANDÖN - VILSANDI - SAAREMAA and UTLÄNGAN - southern end of ÖLAND - PAPE.
		I 1.4.2.1 Western Gotland Basin	1.4.2.1 The division lines between the Eastern and Western Central Basins
		J 1.4.2.2 Eastern Gotland Basin	1.4.2.2 (Gotland Basins) are GOTSKA SANDÖN - FÅRÖ and HOBURG to the coordinate N 56o 11.00' E 18o09.00'.
		K 1.4.3 Southern Baltic Proper	Baltic Proper south of the line UTLÄNGÄN - southern end of ÖLAND to PAPE.
		L 1.4.3.1 Gulf of Gdansk	Baltic Proper south of the line ROSEWIE - TARAN (Brusterort).
2. BELT SEA			The waters between the lines HASENÖRE - GNIBEN in the north and GEDSER - DARSSER ORT in the south.
	M 2.1 BAY OF MECKLENBURG		Baltic Sea between the lines GEDSER - DARSSER ORT and HYLLEKROG - MARIENLEUCHTE.
	N 2.2 KIEL BAY		The waters between the lines FALSHÖFT - VEJSNÄS NAKKE - GULSTAF - KAPPELS CHURCH and MARIENLEUCHTE - HYLLEKROG.

	O 2.3 LITTLE BELT		The waters between the lines FALSHÖFT - VAJSNÄS NAKKE - GULSTAF in the south to the line between AEBELÖ - BJÖRNS KNUDE in the north.
	P 2.4 GREAT BELT		The waters between the line HASENÖRE - GNIBEN in the north, and in the south the line GULSTAF - KAPPELS CHURCH.
Q 3. THE SOUND			The waters between the Danish and Swedish coasts between the lines STEVNS KLINT - FALSTERBO and GILLEJE - KULLEN.
R 4. KATTEGAT			The waters between the Danish and Swedish coasts from the lines HASENORE - GNIBEN and GILLEJE - KULLEN to a line SKAGEN - MARSTRAND.
S 5. SKAGERRAK			The waters between the Danish, Swedish and Norwegian coasts from the line SKAGEN - MARSTRAND to the line LINDESNES - HANSTHOLM.

A map of the division of the Baltic Sea and adjacent waters (Figure A.1.) can be found in the HELCOM Monitoring and Assessment Strategy

The COMBINE sampling stations presented by the Contracting Parties are accessible in Excel table format or via HELCOM's new interactive Combine Monitoring GIS map service.

Furthermore, the sampling stations and sampling frequencies for different parameters can be viewed in the following maps:

Figure A.2. All monitoring stations
Figure A.3. Hydrograpahy stations
Figure A.4. Nutrient stations
Figuer A.5. Chlorophyll-a stations
Figure A.6. Phytoplankton stations
Figure A.7. Productivity stations
Figure A.8. Zooplankton stations
Figure A.9. Zoobenthos stations
Figure A.10. Microbiology stations

A.5. DATA REPORTING

Results of measurements carried out according to the agreed monitoring programme shall be reported and exchanged as follows:

Data should be submitted to the ICES the year after sampling (http://ocean.ices.dk/Submission/Default.aspx). The deadline for the submission of data to the Secretariat is **1 May for hydrographic and hydrochemical data and 1 September for biological data and harmful substances**.

Data reporting should be in accordance with the latest ICES reporting formats. Together with the data a national data report is to be provided containing the following information (EC MON 2/97, 12/1, Annex 9):

I Data identification in the reporting format

- type of samples
- sample identification

II Results

1. Compliance with the programme

2. The reporting institute should state if there is a quality management system established or not

3. Chemical data should have an uncertainty value and a method of calculating the uncertainty

4. Internal QA information

- methods (possible deviations from the manual)
- detection limits (on voluntary basis)
- equipment
- conditions during sampling and analysis

5. External QA information

- certified reference material used (mean values; voluntary if an uncertainty value and a method of calculating the uncertainty is reported)
- participation in ring tests (voluntary if an uncertainty value and a method of calculating the uncertainty is reported)
- participation in taxonomic workshops

III Acivity report

- stations
- variables
- basic statistics on data aggregated by sub-region, season and variable/species with full scientific name (mean, range and number of samples, for phytoplankton range of cell

volumes)
- comments on concentrations/values found

IV Information about corrections made on the data delivered in previous years

V Description of exceptional natural conditions, possible events etc. in the sub-regions

VI Short description on the environmental state of the sub-regions

For further information about data collection and use, please refer to the **HELCOM Data and Information Strategy**.

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A.6. CONTINUOUS INFORMATION BETWEEN MONITORING INSTITUTES

The Internet/World Wide Web provides a co-ordinated, but decentralised, mean to disseminate and to get access to information and data of relevance for the COMBINE. Most institutes responsible for data collection in the COMBINE Programme have a World Wide Web server of their own or have at least an access to Internet.

The internet/www links should be basis for fast and continuos dissemination of information.

To improve the information flow the HELCOM Web site should be further developed. The basic information should, however, be provided by various research institutes. At the first stage, the institutes should publish information at their Web sites on

- cruise plans relevant for the monitoring programmes
- cruise reports
- exceptional environmental events
- other relevant information on the Baltic Sea environment.

At the second stage, systems for regular information dissemination on results relevant to monitoring programme and for data exchange should be developed.

The cruise reports should contain a table listing the stations visited and the determinands measured at each station, together with a short description of the cruise in general and the most important findings. The cruise report could also contain a map showing the cruise track and stations visited, and maps or drawings showing interesting observations, e.g., areas with H_2 S or low 0_2 concentrations.

In case the Internet/www is not available, the cruise plans and cruise reports should be mailed directly to the following addresses:

Denmark

National Environmental Research Institute
Department of Marine Ecology and Microbiology
Gunni Ærtebjerg Nielsen
Frederiksborgvej 399
P.O. Box 358
DK-4000 Roskilde
e-mail: gae@dmu.dk

Estonia
Estonian Marine Institute
Marine Research Centre
Urmas Lips
Paldiski St. 1
EE-0001 Tallinn
e-mail: urmas@phys.sea.ee

Finland

Finnish Environment Institute
Marine Centre
P O Box 140
FI-00251 Helsinki
http://www.ymparisto.fi/default.asp?node=24453&lan=en

Germany

Federal Research Institute for Rural Areas, Forestry and Fisheries
Mr. Michael Haarich

Institute of Fishery Ecology
Marckmannstr. 129 b Haus 4
D-20539 Hamburg
e-mail: michael.haarich@vti.bund.de

Latvia

Latvian Institute of Aquatic Ecology
8 Daugavgrivas str.
LV-1007 Riga
e-mail: juris.aigars@lhei.gov.lv
http://www.lhei.lv/en/index.php

Lithuania

Marine Research Centre
Taikos ave. 26
LT-91149 Klaipeda
e-mail: jtc@jtc.am.lt

Poland

Institute of Meteorology and Water
Management, Maritime Branch
Waszyngtona 42
PL-81 342 Gdynia
e-mail: krzymins@stratus.imgw.gdynia.pl

Russian

State Oceanographic Institute
6 Kropotkinski per.
119 288 Moscow
Russia

Sweden

Stockholm Marine Sciences Centre
Stockholm University
S-106 91 Stockholm
e-mail: smf@smf.su.se

Gothenburg Marine Sciences Centre
Gothenburg University
S-413 81 Gothenburg
e-mail: robert.engstrom@matnat.gu.se

Umeå Marine Sciences Centre
Umeå University
Norrbyn
S-910 20 Hörnefors
e-mail: erik.bonsdorff@umf.umu.se,
johan.wikner@umf.umu.se

Swedish Meteorological and Hydrological Institute
Oceanographical Laboratory
Building 31, Nya Varvet
S-426 71 Västra Frölunda
e-mail: postmaster@smhi.se

Swedish Meteorological and Hydrological Institute
Mr. Hans Dahlin
S-601 76 Norrköping
e-mail: hdahlin@smhi.se

International Organisations

International Council for the Exploration of the Sea (ICES)
H.C. Andersens Boulevard 44-46
DK-1553 Copenhagen V
e-mail: info@ices.dk

Helsinki Commission - Baltic Marine Environment Protection Commission
Katajanokanlaituri 6 B
FI-00160 Helsinki
e-mail: helcom@helcom.fi

The Contracting Parties are also encouraged to send the usual ROSCOP cruise reports as a file copy or as a paper copy to ICES (info@ices.dk). Cruise reports can also be submitted via the SeaDataNet website at: http://www.seadatanet.org/Metadata/CSRl.

For the CMP neither cruise reports nor ROSCOP reports must be given.

A.7. CONDITIONS REQUIRED FOR CARRYING OUT MONITORING/SCIENTIFIC RESEARCH IN THE FISHING/ECONOMIC ZONE (FOR PROGRAMMES ADOPTED BY THE HELSINKI COMMISSION)

HELCOM Recommendation 12/1 concerning procedures for granting permits for monitoring and research activities in the territorial waters and exclusive economic zones, fishing zones or continental shelves was adopted by the Comission in 1992. The recommendation "urges the Contracting Parties to grant one year permits for planned research activities in the exclusive economic zones, fishing zones or continental shelfs, in the framework of the BMP, during which period the coastal state is only to be notified in advance for each individual cruise. Also the Contracting Parties are urged to facilitate and without unnecessary delay grant the permits in connection with the monitoring cruises and for research vessels for all Baltic Sea States to carry out joint scientific studies of common interest, and to submit to the Environment Committee information about their efforts made in this respect."

The conditions (status in August 1997) required for carrying out scientific research in the fishing/economic zone are given in Table A.1.

TABLE A.1. Conditions required for carrying out scientific research in the fishing/economic zone (for programmes adopted by the Helsinki Commission)

Party	Territorial zone (T_z) (nautical miles)	Which one is defined: Exclusive economic zone (Ez) or fishing zone (Fz)	Is permission or notification needed for		Time limit for the application	Notification/request for a permission to be addressed to
			T_z	Ez / Fz		
Denmark	3	E_z, F_z	1) Permission 2) In case of 1 year permits: Permission/ notification	1) Permission 2) In case of 1 year permits: Permission/ notification	30 days	Ministry of Foreign Affairs (via diplomatic channels)
Estonia	12 *	E_z	yes	yes	6 months	Ministry for Foreign Affairs
Finland	12 **	F_z	yes	F_z	6 working days	Ministry for Foreign Affairs (via diplomatic channels)

Germany	up to 12	E_z = Sea Limit chart No. 2921 (various distances), Fz = up to 12 nm	yes	yes	6 weeks	from the country's embassy in Germany to the Auswärtiges Amt
Latvia	12	E_z	yes	yes (E_z)	3 months	Ministry for Foreign Affairs
Lithuania	12	E_z	yes	yes	6 weeks	Ministry for Foreign Affairs
Poland	12	E_z, F_z	yes	yes	3 months	Ministry for Foreign Affairs
Russia	12	E_z	permission	permission	5 months	Ministry of Science and Technical Policy of the Russian Federation
Sweden	12	E_z	yes	yes (E_z)	4-6 weeks	Coast Guard (via the country's embassy in Sweden)

*The maximum 12 nm. The actual zone varies due to the adjacent territorial zone of Finland, Russia and Latvia.

** The maximum 12 nm. The actual zone varies due to the adjacent territorial zone of Estonia, Russia and Sweden.

A.8. FORMAT FOR NOTIFICATION OF PROPOSED MONITORING AND RESEARCH CRUISES

NOTIFICATION OF PROPOSED RESEARCH CRUISE, GENERAL, PART A

1. Name of research ship

Cruise No.

2. Dates of cruises From To

3. Operating authority

Telephone
Telefax

4. Owner (if different from para 3)

5. Particulars of ship:

Name
Nationality
Overall length metres
Maximum draught metres
Net tonnage
Propulsion
Call sign

6. Crew

Name of master
No. of crew

7. Scientific personnel

Name and address of scientist in charge
Telephone
Telefax
No. of scientists

8. Geographical area in which ship will operate (with reference in latitude and longitude)

9. Brief description of purpose of cruise

10. Dates and names of intended ports of call

11. Any special logistic requirements at ports of call

Notification of proposed research cruise, Detail, Part B

1. Name of research ship

Cruise No.

2. Dates of cruise From To

3. Purpose of research and general operational methods

4. Attach chart showing (on an appropriate scale) the geographical area work, positions of intended stations, tracks of survey lines, positions of moored/seabed equipment

5. Types of samples required, e.g. geological/water/plankton/fish/radioactivity/isotope ... and methods by which samples will be obtained (including dredging/coring/drilling)

6. Details of moored equipment:

Dates
Laying Recovery Description Latitude Longitude

7. Explosives:

(a) Type and trade name
(b) Chemical content
(c) Depth and trade class and storage
(d) Size
(e) Depth of detonation
(f) Frequency of detonation
(g) Position in latitude and longitude
(h) Dates of detonation

8. Detail and reference of

(a) Any relevant previous/future cruises
(b) Any previously published research data relating to the proposed cruise
(Attach separate sheet if necessary)

9. Names and addresses of such scientists with whom previous contact has been made in the coastal state in which the waters where the proposed cruise is to take place are

10. State:

(a) Whether visits to the ship in port by scientists of the coastal state concerned will be acceptable
(b) Whether it will be acceptable to carry on board an observer from the coastal state for any part of the cruise and dates and ports of embarkation/disembarkation
(c) When research data from intended cruise is likely to be made available to the coastal state and if so by what means

SCIENTIFIC EQUIPMENT

11. Complete the following table - SEPARATE COPY FOR EACH COASTAL STATE
(indicate "YES" or "NO")

List of all major Marine Scientific Equipment proposed to be used and indicate waters in which it will be deployed	Within fishing limits	On continental shelf	Distance from / coast			
			Within 3 NM	Between 3-12 NM	Between 12-50 NM	Between 50-200 NM

PART B. GENERAL GUIDELINES ON QUALITY ASSURANCE FOR MONITORING IN THE BALTIC SEA

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B.1. Introduction
  B.1.1 Need for Quality Assurance of Analytical Procedures in Marine Monitoring
  B.1.2 Objective
  B.1.3. Topics of Quality Assurance
  B.1.4 Units and Conversions

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  B.2.3 Organization, Management and Staff
    B.2.3.1 Organization
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  B.5.7. Selection of suitable control charts
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B.6. External quality assessment

B.7. Definitions

References

Annexes

Foreword

This document provides an introduction to quality issues, in general, and quality assurance in Baltic marine monitoring laboratories, in particular. The guidelines are intended to assist laboratories in starting up and operating their quality assurance systems. For laboratories with existing quality systems, the guidelines may give inspiration for issues that can be improved. The guidelines contain information for all levels of staff in the marine laboratory.

Sections 1, 2, 3 and 6 together with Annexes B-1 (Quality manual) and B-3 (Quality audit) give guidance on organizational technical quality assurance principles that are relevant to *administrative managers*.

Sections 1, 2, 5 and 6 with Annexes B-1 (Quality manual), B-7 (Reference materials), and B-3 (Quality audit), regarding the implementation and operation of a quality system, are the main sections of relevance for *quality managers*.

For *technical managers* all sections in the main part of the document are relevant. The guidelines provide technical managers with a description of the principles concerning how to introduce and maintain the technical aspects of quality assurance.

It is believed that *analysts* will find all of the guidelines and annexes relevant regarding optimization of their analytical work. The applicability of Annexes B-6 (Sampling), B-10 (Technical notes on nutrients) and B-12 (Technical notes on contaminants) will, however, depend on the specific job description of each analyst.

It is the intention of the guidelines that other members of the *staff connected to the Monitoring Programme* can find use for specific parts of the guidelines.

B.1. INTRODUCTION

B.1.1 NEED FOR QUALITY ASSURANCE OF ANALYTICAL PROCEDURES IN MARINE MONITORING

It has been seen that, although there has been considerable improvement in analytical procedures over the past two decades, it has been obvious that a large number of European laboratories which still had difficulties in providing reliable data in routine work (Topping, 1992; HELCOM, 1991; ICES, 1997). Topping based his conclusion on the results of a series of external quality assessments of analysis (generally referred to as intercomparison exercises), organized over the last twenty years by the International Council for the Exploration of the Sea (ICES), and which have shown that there are large interlaboratory differences.

As a consequence of improperly applied measures to assure the quality of analytical data, information about variations of levels both in space and time is often uncertain or misleading, and the effects of political measures to improve the quality of the marine environment cannot be adequately assessed. Therefore, the acquisition of relevant and reliable data is an essential component of any research and monitoring programme associated with marine environmental protection. To obtain such data, the whole analytical process must proceed under a well-established Quality Assurance (QA) programme. Consequently, the HELCOM Environment Committee (EC) at its fifth meeting (HELCOM, 1994) recommended that: 'all institutes reporting data to BMP/CMP shall introduce in-house quality assurance procedures'.

In addition, the following principles of a quality assurance policy were formulated:

QUALITY ASSURANCE POLICY OF THE HELSINKI COMMISSION (HELCOM, 1995)

1. Contracting Parties acknowledge that only reliable information can provide the basis for effective and economic environmental policy and management regarding the Convention area;
2. Contracting Parties acknowledge that environmental information is the product of a chain of activities, constituting programme design, execution, evaluation and reporting, and that each activity has to meet certain quality requirements;
3. Contracting Parties agree that quality assurance requirements be set for each of these activities;
4. Contracting Parties agree to make sure that suitable resources are available nationally (e.g., ships, laboratories) in order to achieve this goal;
5. Contracting Parties fully commit themselves to following the guidelines, protocols, etc., adopted by the Commission and its Committees in accordance with this procedure of quality assurance.

The Contracting Parties shall clearly declare, in relevant data reports, if they fail to fulfil the recommendations of the Manual. If alternative methods are being used, proof shall be given that the results are comparable with results generated from methods described in the Manual. The supplier of the data has then the responsibility to proof the comparability of the methods. HELCOM Monitoring and Assessment Group (HELCOM MONAS) will ultimately decide if the data could be incorporated in the HELCOM Database. If agreement could not be reached, HELCOM/ICES Steering Group on Quality Assurance of Chemical Measurement (SGQAC) will be given the task to evaluate the method and give advice to HELCOM MONAS.

All Contracting Parties have nominated persons responsible for quality assurance in all laboratories reporting to the monitoring programmes.

All institutes/laboratories should participate in regular (annual) intercomparison exercises, arranged in the Baltic community and laboratories should take part in proficiency testing schemes, e.g. the QUASIMEME-II. As new certified reference materials become (commercially) available these might be used by all participating institutes or laboratories.

The results of intercomparison exercises and the analyses of certified standards should be reported together with the monitoring data according to procedures to be decided by EC MON. It should also be noted that it is possible for laboratories to authorize the QUASIMEME and the BEQUALM office to report the individual laboratories performance data directly to the data host for HELCOM.

The monitoring laboratories should have a QA/QC system that follows the requirements of EN ISO/IEC 17025 "General requirements for the competence of testing and calibration laboratories" (formerly EN 45001 and ISO Guide 25). Participating laboratories are encouraged to endeavour the obtainment of official accreditation (or certification) for the variables on which they report data in accordance with COMBINE.

In order to assist laboratories in setting up their quality assurance system the general advice that follows in this chapter is applicable.

B.1.2 OBJECTIVE

The objective of the Manual outlined here is to support laboratories working in marine monitoring to produce analytical data of the required quality. The Manual may also help to establish or improve quality assurance management in the laboratories concerned. The technical part of the Manual provides advice on more practical matters. The Manual will, for chemical variables, not focus on sampling in detail, since this will be dealt with at a later stage. The details for biological sampling are found in Annexes C-4 to C-12.

B.1.3 TOPICS OF QUALITY ASSURANCE

In practice, Quality Assurance applies to all aspects of analytical investigation, and includes the following principal elements:

- A knowledge of the purpose of the investigation is essential to establish the required data quality.
- Provision and optimization of appropriate laboratory facilities and analytical equipment.
- Selection and training of staff for the analytical task in question.
- Establishment of definitive directions for appropriate collection, preservation, storage and transport procedures to maintain the integrity of samples prior to analysis.
- Use of suitable pre-treatment procedures prior to analysis of samples, to prevent uncontrolled contamination and loss of the determinand in the samples.

- Validation of appropriate analytical methods to ensure that measurements are of the required quality to meet the needs of the investigations.

- Conduct of regular intralaboratory checks on the accuracy of routine measurements, by the analysis of appropriate reference materials, to assess whether the analytical methods are remaining under control, and the documentation and interpretation of the results on control charts.

- Participation in interlaboratory quality assessments (proficiency testing schemes, ring-tests, training courses) to provide an independent assessment of the laboratory's capability of producing reliable measurements.

- The preparation and use of written instructions, laboratory protocols, laboratory journals, etc., so that specific analytical data can be traced to the relevant samples and *vice versa*.

B.1.4 UNITS AND CONVERSIONS

This notes summarizes the units that should be used for data submission within the COMBINE programme, and also gives the relevant formulas for conversion between different commonly used units.

References are made to the appropriate annexes of the COMBINE Manual.

Please note that the units dm^3 and cm^3 are used throughout the note, although the units l (litre) and ml (millilitre) would be equally correct.

Part 1: Units

Parameter	Symbol	Unit	Comment
Temperature	t	C	see Annex C-2
Salinity	S		see Annex C-2 according to the current definition of the Practical Salinity Scale of 1978 (PSS78)
Secchi depth (light attenuation)		m	see Annex C-2

Current speed		cm/s	see Annex C-2
Current direction			report as compass directions; see Annex C-2
Dissolved Oxygen	DO	cm^3/dm^3	see Annex C-2
Oxygen saturation			reported as fraction (%), see Annex C-2
Hydrogen Sulphide		µmol/dm^3	see Annex C-2
Nutrients		µmol/dm^3	as N, P or Si; see Annex C-2
Total P and N	TP/TN	µmol/dm^3	see Annex C-2
pH			NBS-scale; see Annex C-2
Alkalinity		mmol/dm^3	as carbonate; see Annex C-2
Particulate and dissolved organic matter (TOC, POC, DOC and PON)		µmol/dm^3	as C or N; see Annex C-2
Humic matter			depending on way of calibration; see Annex C-2
Heavy metals in water		ng/dm^3 or pg/dm^3	dissolved
Halogenated organics in water		ng/dm^3	

PAHs in water		ng/dm^3	
Heavy metals in biota		µg/kg	wet weight
Halogenated organics in biota		µg/kg or ng/kg	wet weight, reported together with lipid content
Total suspended matter load		mg/dm^3	
Chlorophyll a	Chl-a	mg/m^3	see Annex C-4
Primary production (as carbon uptake)		mg/m^3*h	see Annex C-5
Phytoplankton species			see Annex C-6
abundance		Counting units/dm^3	
biomass		mm^3/dm^3	
Mesozooplankton			see Annex C-7
abundance		Individuals/m^3	
biomass		mm^3/m^3; mg/m^3	
Macrozoobenthos			see Annex C-8
abundance		Counting units/m^2	

biomass		g/m^2	dry or wet weight

Part 2: Conversions

Parameter	From	To	Formula or multiplication factor
Any compound	g/dm^3	mol/dm^3	(g/dm^3)/molar weight
	mol/dm^3	g/dm^3	(mol/dm^3)* molar weight
	µmol/kg	µmol/dm^3	(µmol/kg)*density; density determined from salinity, temperature and pressure
	µmol/dm^3	µmol/kg	(µmol/dm^3)/density; density determined from salinity, temperature and pressure
Dissolved oxygen	mg/dm^3	cm^3/dm^3	0.7
	cm^3/dm^3	mg/dm^3	1.429
	µmol/dm^3	cm^3/dm^3	11.196
	cm^3/dm^3	µmol/dm^3	0.0893
	mg/dm^3	µmol/dm^3	0.06251
	µmol/dm^3	mg/dm^3	15.997

	DO	Oxygen saturation	see Grasshoff et al., Methods of Seawater Analysis, 2nd or 3rd edition
	Oxygen saturation	DO	see Grasshoff et al., Methods of Seawater Analysis, 2nd or 3rd edition
Hydrogen sulphide	µmol/dm^3	Negative oxygen	– 0.044001 (multiplication factor)
	Negative oxygen	µmol/dm^3	– 22.727 (multiplication factor)

B.2. THE QUALITY SYSTEM

B.2.1 GENERAL

'Quality system' is a term used to describe measures which ensure that a laboratory fulfills the requirements for its analytical tasks on a continuing basis. A laboratory should establish and operate a Quality System adequate for the range of activities, i.e., for the type and extent of investigations, for which it has been employed.

The Quality System must be formalized in a Quality Manual which must be maintained and up-to-date. A suggested outline of a Quality Manual is given in Annex B-1. Some comments and explanations are given in this section.

The person responsible for authorization and compilation of the Quality Manual must be identified, and an identification of holders of controlled copies should be listed in the manual.

The Quality System must contain a statement of the intentions of the laboratory top management in relation to quality in all aspects of its work (statement on Quality Policy).

For chemical variables guidance on the interpretation of ISO/IEC/EN 17025 'General Requirements for the Competence of Testing and Calibration of Laboratories' (formerly EN 45001 and ISO Guide 25) was given by a joint international EURACHEM/WELAC Working Group (EURACHEM/WELAC, 1992). Specific guidance to Analytical Quality Control for Water Analysis was elaborated by European - CEN/TC 230 (EN 14996) - as well as by international - ISO/TC 147 SC 7 (ISO/TR 13530) - standardisation authorities. All these publications have been taken into consideration when drafting these guidelines. References, which deal with specific aspects of quality assurance of chemical measurements, are cited in the text.

B.2.2 SCOPE

The laboratory's scope should be formulated in terms of:

- the range of products, materials or sample types tested or analysed;
- the types of tests or analyses carried out;
- the specification of method/equipment/technique used;
- the concentration range and accuracy of each test and analysis.

B.2.3 ORGANIZATION, MANAGEMENT AND STAFF

B.2.3.1 ORGANIZATION

The Quality System should provide general information on the identity and legal status of the laboratory and should include a statement of the technical role of the laboratory (e.g., employed in marine environmental monitoring).

The following information must be included in an organizational chart:

- Technical Manager, Quality Manager, and any deputies;
- general lines of responsibility within the laboratory (including the relationship between management, technical operations, quality control and support services);
- the lines of responsibility within individual sections of the laboratory;
- the relationship between the laboratory and any parent or sister organizations.
- The appropriate chart should show that, for matters related to quality, the Quality Manager has direct access to the highest level of management at which decisions are taken on laboratory policy and resources, and to the Technical Manager.

B.2.3.2 MANAGEMENT

Job descriptions, qualifications, training and experience are necessary for:

- Technical Manager,
- Quality Manager,
- other key laboratory managerial and technical posts.

Job descriptions should include:

- title of job and brief summary of function,
- person or functions to whom jobholder reports,
- person or functions that report to jobholder,

- key tasks that jobholder performs in the laboratory,
- limits of authority and responsibility.

The Technical Manager. The Quality System should include a statement that the post-holder has overall responsibility for the technical operation of the laboratory and for ensuring that the Quality System requirements are met.

The Quality Manager. The Quality System should include a statement that the post-holder has responsibility for ensuring that the requirements for the Quality System are met continuously and that the post-holder has direct access to the highest level of management at which decisions are taken on laboratory policy or resources, and to the Technical Manager.

The Quality System should state explicitly the Quality Manager's duties in relation to control and maintenance of documentation, including the Quality Manual, and of specific procedures for the control, distribution, amendment, updating, retrieval, review and approval of all documentation relating to the calibration and testing work of the laboratory.

B.2.3.3 STAFF

The laboratory management should define the minimum levels of qualification and experience necessary for engagement of staff and their assignment to respective duties.

Members of staff authorized to use equipment or perform specific calibrations and tests should be identified.

The laboratory should ensure that all staff receive training adequate to the competent performance of the tests/methods and operation of equipment. A record should be maintained which provides evidence that individual members of staff have been adequately trained and their competence to carry out specific tests/methods or techniques has been assessed. Laboratory managers should be aware that a change of staff might jeopardize the continuation of quality.

B.2.4 DOCUMENTATION

Necessary documentation includes:

- a clear description of sampling equipment;
- a clear description of all steps in the sampling procedure;
- a clear description of the analytical methods;
- a strict keeping of ship and laboratory journals;
- instrument journals;
- protocols for sample identification;

- clear labelling of samples, reference materials, chemicals, reagents, volumetric equipment, stating date, calibration status, concentration or content as appropriate and signature of the person responsible.

B.2.5 LABORATORY TESTING ENVIRONMENT

Samples, reagents and standards should be stored and labelled so as to ensure their integrity. The laboratory should guard against deterioration, contamination and loss of identity.

The laboratory should provide appropriate environmental conditions and special areas for particular investigations.

Staff should be aware of:

- the intended use of particular areas,
- the restrictions imposed on working within such areas,
- the reasons for imposing such restrictions.

B.2.6 EQUIPMENT

As part of its quality system, a laboratory is required to operate a programme for the necessary maintenance and calibration of equipment used in the field and in the laboratory to ensure against bias of results.

General service equipment should be maintained by appropriate cleaning and operational checks where necessary. Calibrations will be necessary where the equipment can significantly affect the analytical result.

The correct use of equipment is critical to analytical measurements and this equipment must be maintained, calibrated and used in a manner consistent with the accuracy required of data. For certain chemical analysis, one should consider that measurements can often be made by mass rather than by volume.

Particularly for trace analyses, contamination through desorption of impurities from, or uncontrolled determinand losses through sorption on, surfaces of volumetric flasks can be significant. Therefore, special attention should be paid to the selection of appropriate types of material (quartz, PTFE, etc.) used for volumetric equipment and its proper cleaning and conditioning prior to analysis.

Periodic performance checks should be carried out at specific intervals on measuring instruments (e.g., for response, stability and linearity of sources, sensors and detectors, the separating efficiency of chromatographic systems).

The frequency of such performance checks will be determined by experience and based on the need, type and previous performance of the equipment. Intervals between checks should be shorter than the time the equipment has been found to take to drift outside acceptable limits and should be given in the equipment list.

B.2.7 QUALITY AUDIT

The ISO 9000 and ISO 14000 series of International Standards emphasize the importance of audits as a management tool for monitoring and verifying the effective implementation of an organizations quality and/or environmental policy. Audits are also an essential part of conformity assessment activities such as accreditation.

So it is stated in ISO/IEC 17025:2005 General Requirements for the Competence of Testing and Calibration Laboratories that the laboratory shall periodically and in accordance with a predetermined schedule and procedure conduct

- internal audits of its activity to verify that its operations continue comply with the requirements of the quality system and the International Standard,
- review about the laboratory's quality system testing and/or calibration activities: ensure their continuing suitability effectiveness, and to introduce necessary changes or improvements.

The audit is a systematic, independent and documented process for obtaining audit evidence and evaluating it objectively to determine the extent to which the audit criteria are fulfilled (EN ISO 19011:2002).

Internal audits, sometimes called first-party audits, are conducted by the organization itself for management review and other internal purposes, and may form the basis for an organization’s self-declaration of conformity. External audits include those generally termed second- and third-party audits. Second-party audits are conducted by parties having an interest in the organization, such as customers. Third-party audits are conducted by external, independent auditing organizations, such as accreditation bodies.

Further information on auditing is available in EN ISO 19011:2002.

Arrangements for implementing a program for internal audits may be based upon a check list developed by APLAC (APLAC, 2004), which is attached as Annex B-3 to these Guidelines.

B.3. SPECIFYING ANALYTICAL REQUIREMENTS

B.3.1 GENERAL

The objective of analytical investigations in chemistry is to obtain information about materials or systems concerning their specific qualitative and quantitative composition and structure (Danzer, 1992).

The objectives of analytical investigations in biology are to measure rates in activity concentrations of biological variables and to make taxonomical determinations.

Before the analyst starts an analytical investigation, the intended use of the data must be explicitly stated. That is, the minimum quality requirement the data must meet to make it useful for a given purpose should be established for every measurement situation. Careful specification of analytical requirements and critical consideration of data quality objectives are vital when designing analytical programmes.

Environmental analytical measurements are developed for a variety of purposes, such as the determination of the fate of a component in the context of biogeochemical studies, or the determination of the environmental concentration of a component for use in environmental risk assessment.

The broad range of applications of analytical data requires different analytical strategies, and the accuracy of the data obtained must be adequate for each use. A failure to pay proper attention to this topic can endanger the validity of an analytical programme, since the analytical results obtained may be inadequately accurate and lead to false conclusions.

Based on these considerations, the following parameters should be discussed and evaluated before an investigation is carried out:

- the variable of interest,
- the type and nature of the sample,
- the concentration range of interest,
- the permissible tolerances in analytical error.

B.3.2 VARIABLE OF INTEREST

Frequently, particularly for chemical variables, a single method may be used for analysis of a variable in a wide variety of matrices. However, one has to recognize that many variables exist in different matrices in a variety of chemico-physical forms, and most analytical methods provide a different response to the various forms. Therefore, particular care must be exercised that the variable of interest is clearly defined and the experimental conditions selected allow its unambiguous measurement.

B.3.3 TYPE AND NATURE OF THE SAMPLE AND ITS ENVIRONMENT

A precise description of the type and nature of the sample is essential before the analytical method can be selected. Suitable measures and precautions can only be taken during sampling, sample storage, sample pretreatment and analysis, if sufficient knowledge about the basic properties of the sample is available. There may be other, non-analytical factors to consider, including the nature of the area under investigation.

B.3.4 CONCENTRATION RANGE OF INTEREST

It is important that samples of a definite type and nature have been characterized by the concentration range of the variable. If such information is not given, needless analytical effort may be expended or, vice versa, insufficient effort may jeopardize the validity of the analytical information gained.

B.3.5 PERMISSIBLE TOLERANCES IN ANALYTICAL ERROR

Taylor (1981) pointed out that 'the tolerance limits for the property to be measured are the first condition to be determined. These are based upon considered judgement of the end user of the data and present the best estimate of the limits within which the measured property must be known, to be useful for its intended purpose'...'Once one has determined the tolerance limits for the measured property, the permissible tolerances in measurement error may be established'.

In the whole analytical chain, there are systematic errors (biases) and random errors, as indicated by the standard deviation. The bounds representing the sum of both must be less than the tolerance limits defined for the property to be measured, if the analytical data are to be useful.

B.3.6 TECHNICAL NOTE ON THE QA OF THE DETERMINATION OF CO-FACTORS

Co-factors, Definition, and Use

A co-factor is a property in an investigated sample, which may vary between different samples of the same kind, and by varying may affect the reported concentration of the determinand. Thus, the concentration of the co-factor has to be established in order to compare the determinand concentrations between the different samples (e.g., for the purpose of establishing trends in time or spatial distribution) by normalization to the co-factor.

By the definition given above, it is understood that the correct establishment of the co-factor concentration is just as vital to the final result and the conclusions as is the correct establishment of the determinand concentration. Thus, the co-factor determination has to work under the same QA system, with the same QA requirements and the same QC procedures, as any other parts of the analytical chain. It is also vital that QA information supporting the data contains information on the establishment and use of any co-factors.

Co-factors in Biota Analysis

Dry weight

Freeze-drying or heat drying at 105 °C can be used. Dry to constant weight in both cases. By constant weight is meant a difference small enough not to significantly add to the measurement uncertainty.

Lipid content

The method by Smedes (1999), which uses non-chlorinated solvents and has been demonstrated to have high performance, is recommended. This method is a modification of the Bligh and Dyer (1959) method, and can be performed using the same equipment. The two methods have been shown to give comparable results.

Physiological factors

Age, sex, gonad maturity, length, weight, liver weight, etc., are important co-factors for species of, for example, fish. For more information, see Section D.5 of the COMBINE Manual.

Co-factors in Water Analysis

Particulate material

Determined by filtration through a filter according to the ISO 11923:1997 standard.

Organic carbon

The method recommended is described in Annex C-2 of the COMBINE Manual.

Salinity

Salinity (and temperature) may be defined as a co-factor in investigations where mixing of different water masses is studied or takes place. The same standard oceanographic equipment as described in the Technical Note on Salinity is used, and the performance requirements will also be the same.

QA Information to Support the Data

When reporting data that have been normalized to a co-factor, or where the co-factor data are reported along with the results, always supply the following information:

- type of co-factor (parameter),
- analytical method for the co-factor,
- uncertainty in the co-factor determination,
- how the co-factor has been used (if it has),
- results from CRMs and intercomparison exercises (on the co-factor).

References

Smedes, F. 1999. Determination of total lipid using non-chlorinated solvents. The Analyst, 124: 1711.

Bligh, E.G., and Dyer, W.J. 1959. Canadian Journal of Biochemical Physiology, 37: 911.

ISO. 1997. Water quality—Determination of suspended solids by filtration through glass-fibre filters. ISO 11923:1997.

B.4. VALIDATION OF ANALYTICAL METHODS

B.4.1 GENERAL

On the basis of the specifications developed in the items under Section 3, the method must now be examined to determine whether it actually can produce the degree of specificity and confidence required. Accordingly, the objective of the validation process is to identify the performance of the analytical method and to demonstrate that the analytical system is operating in a state of statistical control.

When analytical measurements are 'in a state of statistical control', it means that all causes of errors remain the same and have been characterized statistically.

B.4.2 VALIDATION

Validation of an analytical method is the procedure that 'establishes, by laboratory studies, that the performance characteristics of the method meet the specifications related to the intended use of the analytical results' (Wilson, 1970; EURACHEM/WELAC, 1992).

Performance characteristics include:

- selectivity,
- sensitivity,
- range,
- limit of detection,
- accuracy (precision, bias).

These parameters should be clearly stated in the documented method description so that the suitability of the method for a particular application can be assessed.

In the following, a brief explanation and, where appropriate, guidance on the estimation of these parameters is given.

B.4.2.1 SELECTIVITY

Selectivity refers to the extent to which a particular component in a material can be determined without interference from the other components in the material. A method which is indisputably selective for a variable is regarded as specific.

Few analytical methods are completely specific for a particular variable. This is because both the variable and other substances contribute to the analytical signal and cannot be differentiated. The effect of this interference on the signal may be positive or negative depending upon the type of interaction between variable and interfering substances.

The applicability of the method should be investigated using various materials, ranging from pure standards to mixtures with complex matrices.

- Each substance suspected to interfere should be tested separately at a concentration approximately twice the maximum expected in the sample (use Student's t-test to evaluate).
- Knowledge of the physical and chemical mechanisms of interference operative in the particular method will often help to decide for which substances tests should be made.

Interference effects causing restrictions in the applicability of the analytical method should be documented.

B.4.2.2 SENSITIVITY

Sensitivity is the difference in variable concentration corresponding to the smallest difference in the response by the method that can be detected at a certain probability level. It can be calculated from the slope of the calibration curve.

Most analytical methods require the establishment of a calibration curve for the determination of the (unknown) variable concentration. Such a curve is obtained by plotting the instrumental response, y, versus the variable concentration, x. The relationship between y and x can be formulated by performing a linear regression analysis on the data. The analytical calibration function can be expressed by the equation $y = a + bx$, where b is the slope or response and a is the intercept on the y-axis.

As long as the calibration curve is within the linear response range of the method, the more points obtained to construct the calibration curve the better defined the b value will be. A factor especially important in defining the slope is that the measurement matrix must physically and chemically be identical both for samples to be analysed and standards used to establish the calibration curve.

B.4.2.3 LIMIT OF DETECTION, LIMIT OF QUANTIFICATION

General

Limit of detection, quantification or application are validation parameters which describe the sensitivity of an analytical methods with regard to the detection and quantification of a certain analyte. Therefore, a number of publications recently provided different approaches to define and calculate these measures by instrumental or mathematical approaches (DIN 32645, 1994; EURACHEM, 1992; Geiß and Einax, 2000; ICH, 1996; ISO 11843, 1997-2003; ISO/CD 13530, 2003; IUPAC, 1997, 2002).

Definitions

In broad terms, the limit of detection (LOD) is the smallest amount or concentration of an analyte in the test sample that can be reliably distinguished from zero (IUPAC, 2002). For analytical systems where the application range does not include or approach it, the LOD does not need to be part of a validation.

There has been much diversity in the way in which the limit of detection of an analytical system is defined. Most approaches are based on multiplication of the within-batch standard deviation of results of blanks by a certain factor. These statistical inferences depend on the assumption of normality, which is at least questionable at low concentrations (ISO/WD 13530, 2003).

Limit of quantification (LOQ) is a performance characteristic that marks the ability of an analytical method to adequately “quantify” the analyte. Sometimes that LOD is arbitrarily defined as a relative standard deviation RSD (commonly RSD = 10%), sometimes the limit is arbitrarily taken as a fixed multiple (typically 2-3) of the detection limit. This quite arbitrary setting of LOQ does not consider that measurements below such a limit are not devoid of information content and may well be fit for purpose. Hence, it is preferable to try to express the uncertainty of measurement as a function of concentration and compare that function with a criterion of fitness for purpose agreed between the laboratory and the client or end-user of the data (IUPAC, 2002).

Lower Limit of Application (LLOA) is an agreed criterion of the fitness for purpose for the monitoring of priority hazardous substances within the EU Water Framework Directive (2000/60/EC). The LLOA shall be defined: LLOA ≥ LOQ. The LLOA refers to the lowest concentrations for which a method has been validated with specified accuracy (AMPS, 2004). For methods which need calibration the lowest possible LLOA is equal to the lowest standard concentration (ISO/CD 13530, 2003).

The LLOA is required to be equal or lower than 30% of the defined Environmental Quality Standards (EQS). This ensures that priority hazardous substance concentrations around the proposed EQS can be measured with an acceptable measurement uncertainty of ≤50% (AMPS, 2004).

Calculation of LOD for methods with normally distributed blank values

The LOD shall be calculated as:

LOD = 3 s_0

where

s_0 standard deviation of the outlier-free results of a blank sample

The precision estimate s_0 shall be based on at least 10 independent complete determinations of analyte concentration in a typical matrix blank or low-level material, with no censoring of zero or negative results. For that number of determinations the factor of 3 corresponds to a significance level of a = 0,01.

Note that with the recommended minimum degrees of freedom, the value of the limit of detection is quite uncertain, and may easily vary by a factor of 2. Where more rigorous estimates are required more complex calculations should be applied ISO 11843 (1997-2003).

Calculation of LOD for chromatographic methods

There are several options for the determination of LOD/LOQ for chromatographic methods:

- The LOD is defined as the concentration of the analyte at a signal/noise ratio S/N=3.
- Measure concentrations in a very low level sample e.g.10 times and calculate standard deviation
- Spike analyte-free sample and measure e.g. 10 times, and then calculate standard deviation
- Dilute natural low level sample extract to achieve the required concentration. Then measure e.g. 10 times and calculate standard deviation

The proposed options are arranged according to their appropriateness.

Calculation of LOQ

The limit of quantification (LOQ) is the smallest amount or concentration of analyte in the test sample which can be determined with a fixed precision, e.g. relative standard deviation s_{rel} = 33,3 %. Usually it is arbitrarily taken as a fixed multiple of the detection limit (IUPAC, 2002).

For method validation the LOQ shall be calculated as: LOQ = 3 LOD

The factor of 3 corresponds to a relative standard deviation s_{rel} = 33,3 %.

For verification of the LOQ a spiked sample at this concentration level shall be analysed in the same manner as real samples. The analytical result must be in the range of LOQ ± 33,3%.

References

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ISO 11843 (1997-2003) Capability of detection, Part 1-4

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B.4.2.4 RANGE

The range of the method is defined by the smallest and greatest variable concentrations for which experimental tests have actually achieved the degree of accuracy required.

The concentrations of the calibration standards must bracket the expected concentration of the variable in the samples.

It is recommended to locate the lower limit of the useful range at $x_B + 10s_B$, where x_B is the measured value for the blank, and s_B is the standard deviation for this measurement.

The range extends from this lower limit to an upper value (upper limit) where the response/variable concentration relationship is no longer linear.

B.4.2.5 ACCURACY

The term 'accuracy' is used to describe the difference between the expected or true value and the actual value obtained. Generally, accuracy represents the sum of random error and systematic error or bias (Taylor, 1981).

Random errors arise from uncontrolled and unpredictable variations in the conditions of the analytical system during different analyses. Fluctuations in instrumental conditions, variations of the physical and chemical properties of sample or reagent taken on different occasions, and analyst-dependent variations in reading scales are typical sources causing random errors.

The term 'precision' should be used when speaking generally of the degree of agreement among repeated analyses. For numerical definition of this degree of agreement, the parameter standard deviation or relative standard deviation should be used.

Systematic errors or biases originate from the following sources:

a) instability of samples between sample collection and analysis

Effective sample storage, sample stabilization and sample preservation, respectively, are essential to ensure that no losses or changes of the physical and chemical properties of the variable occur prior to analysis. Effective sample stabilization methods exist for many variables and matrices, but they must be compatible with the analytical system being employed, and with the particular sample type being analysed.

b) deficiencies in the ability to determine all relevant forms of the variable

Many variables exist in different matrices in a variety of physical and/or chemical forms ('species'). The inability of the analytical system to determine some of the forms of interest will give rise to systematic negative deviations from the true value, if those forms are present in the sample.

c) biased calibration

Most instrumental methods require the use of a calibration function to convert the primary analytical signal (response) to the corresponding variable concentration. Generally, calibration means the establishment of a function by mathematically modelling the relationship between the concentrations of a variable and the corresponding experimentally measured values.
An essential prerequisite when establishing a calibration function is that the sample and calibration standards have similar matrices and are subject to the same operational steps of the analytical method, and that identical concentrations of the variable in standards and sample give the same analytical response.

d) incorrect estimation of the blank

It is common practice to correct quantitative analytical results for a constant systematic offset, denoted the 'blank'. A definite answer must be found to what the true blank in an analysis is, in order to make correction for the blank satisfactory.
A good review of several kinds of 'blank' and their use in quantitative chemical analysis was given by Cardone (1986a, 1986b).
Principally, it is important to realize that a 'blank' is the response from a solution containing all constituents of the sample, except the variable, processed through all procedural steps of the method under study. The analyst must know that the size of the blank and its influence on the analytical result can only be assessed if the sample matrix has been adequately approximated and the whole analytical process has been considered.

B.4.2.5.1 ESTIMATING RANDOM ERRORS

The within-batch standard deviation, s_w, represents the best precision achievable with the given experimental conditions, and is of interest when the analyst is concerned with the smallest concentration difference detectable between two samples.

The between-batch standard deviation, s_b, is a measure of the mutual approximation of analytical results obtained from sequentially performed investigations of the same material in the same laboratory.

The total standard deviation, s_t, is calculated from the formula $s_w^2 + s_b^2$. It is of interest to analysts concerned with the regular analysis of samples of a particular type in order to detect changes in concentration.

A realistic approach to estimate s_w and s_b is to perform n determinations on a representative group of control samples in each of $m$consecutive batches of analysis.

The experimental design recommended to estimate s_w, s_b and s_t is to make n replicate analyses per batch in a series of m different batches. The design should be modified according to practical experience gained from the analytical method tested. In particular, whens_w is assumed to be dominant, n=4 to 6 could be chosen. The product $n \cdot m$ should not be less than 10 and should preferably be 20 or more.

Analysis of variance (ANOVA) allows identification of the different sources of variation and calculation of the total standard deviation s_t. A general scheme of ANOVA (after Doerffel, 1989) is given in the following paragraphs.

Source of variability	Sum of squares	Degrees of freedom	Mean squares (variances)	Variance components
Between batches	$QS_1= n_j(x_j-x)^2$	$f_1=m-1$	$s_{bm}^2=QS_1/m-1$	$s_{bm}^2 = n_j s_b^2 + s_w^2$
Within batches (analytical error)	$QS_2= (xi_j-x^j)^2$	$f_2=m(n_j-1)$	$s_w^2=QS^2/m(n_j-1)$	
Total	$QS_1 + QS_2$	$f = mn_j - 1$		

m = number of batches of analysis;
n_j = number of replicate analyses within a batch;
x_j = mean of jth batch;
x = overall mean;
x_{ij} = jth replicate analytical value in ith batch
s_w^2 = estimate of within batch variance
s_{bm}^2 = estimate of the variance of the batch means
$F = s_{bm}^2/s_w^2$ is tested against the tabled value $F(P = 0.05; f_1;f_2)$.

If the test is significant, i.e., $F > F(P=0.05; f_1;f_2)$, the between batch variance s_b^2 can be estimated as $s_b^2 = (s_{bm}^2-s_w^2)/n_j$

Carry out F-test to see if s_b is significantly larger than s_w.

If the testing value $s_b^2/s_w^2 < F(f_b,f_w,95\ \%)$, one can conclude that s_b is only randomly larger than s_w. In this case $s_t = s_w$.

If the testing value $s_b^2/s_w^2 > F(f_b,f_w,95\ \%)$, one can conclude that s_b significantly influences the total standard deviation.

Accordingly, the estimate of the total variance of a single determination is $s_t^2 = s_b^2+s_w^2$.

For routine analysis, it is recommended that s_b does not exceed the value of s_w by more than a factor of two.

A step-wise approach to scrutinize experimental design and to optimize analytical performance may be necessary. This process might be repeated iteratively until target values of s_w, s_b and s_t , respectively, are attained.

B.4.2.5.2 ESTIMATING SYSTEMATIC ERRORS (BIASES)

a) Using an independent analytical method

The analyst can test for systematic errors in the analytical procedure under investigation by using a second, independent analytical method (Stoeppler, 1991). A t-test can be carried out to check for differences in the measured values obtained (on condition that the precision of both methods applied is comparable). A significant difference between the results obtained by both procedures indicates that one of them contains a systematic error. Without further information, however, it is not possible to say which one.

b) Using Certified Reference Material (CRM)

An analytical procedure should be capable of producing results for a certified reference material (CRM) that do not differ from the certified value more than can be accounted for by within-laboratory statistical fluctuations.

In practice, when performing tests on CRM, one should ensure that the material to be analysed and the certified reference material selected have a similar macrocomposition (a similar matrix) and approximately similar variable concentrations.

c) Participation in intercomparison exercises

In an intercomparison exercise, the bias of the participating laboratory's analytical method is estimated with respect to the assigned value X for the concentration of the variable in the sample which was distributed to participants. The assigned value X is an estimate of the true value and is predetermined by some 'expert' laboratories. In some instances, X is a consensus value established by the coordinator after critical evaluation of the results returned by the participants. The bias is equal to the difference between the variable concentration x reported by the participant and the variable concentration X assigned by the coordinator.

If a target standard deviation s representing the maximum allowed variation consistent with valid data can be estimated, the quotient $z = (x - X)/s$ is a valuable tool for appropriate data interpretation.
If z exceeds the value of 2, there is only a 5 percent probability that the participating laboratory can produce accurate data (Berman, 1992).

B.4.2.6. MEASUREMENT UNCERTAINTY

Introduction

The principal aim of analytical work is to gain information on the material under investigation. This information always constitutes a probability distribution determined by a random error and a systematic error inherent in the analytical procedure used. A systematic error can act as an additive or as a multiplicative shift. Systematic errors are superimposed by the random error. Analytical practice shows that there is always some doubt about the correctness of a stated result, even when all the suspected sources of error have been taken into account and the appropriate corrections have been applied. This is due to the uncertainty regarding the correction factors and the uncertainty arising from random effects, which cannot be eliminated, although they can be reduced by increasing the number of observations. Consequently, a measurement cannot be properly interpreted without the knowledge of the uncertainty associated with the result.

The concept of expressing or estimating the uncertainty of measurements was developed to inform the final users of the analytical data concerning how much allowance must be made for the possibility that repetition of the test will give a different value (Horwitz, 1998). This information is particularly necessary when analytical results are not used by the data originator, as is the regular case in the assessment of data from environmental monitoring program. This technical note provides information on how the uncertainty of measurement of the analytical methods used in the COMBINE program of HELCOM can be estimated, so that it would be possible to judge whether or not the accuracy (trueness and precision) of the method meets the requirements of this program. It should be taken into account that the requirements on accuracy depend on the aims and the purpose of the monitoring program.

Definitions

The “Guide to the expression of uncertainty in measurement” (JCGM 100: 2008) defines Measurement Uncertainty as a parameter, associated with the result of a measurement, which characterises the dispersion of the values that could reasonably be attributed to the measurand.

The following definitions apply (EURACHEM/CITAC, 2000):

Standard uncertainty

$u(x_i)$ uncertainty of the result x_i of a measurement expressed as a standard deviation

Combined standard uncertainty

$u_c(y)$ standard uncertainty of the result y of a measurement when the result is obtained from the values of a number of other quantities, equal to the positive square root of a sum of terms, the terms being the variances or covariances of these other quantities weighed according to how the measurement results varies with these quantities

Expanded standard uncertainty

U quantity defining an interval about the result of a measurement that may be expected to encompass a large fraction of the distribution of values that could reasonably be attributed to the measurand, an expanded uncertainty is calculated from a combined standard uncertainty u_c and a coverage factor k using
$U = k * u_c$

Coverage factor

k numerical factor used as a multiplier of the combined standard uncertainty in order to obtain an expanded uncertainty, the choice of the factor k is based on the level of confidence desired, $k = 2$ for an approximate level of confidence of 95 %

Procedures to Estimate Uncertainty

Generally, there are two main approaches to estimate the uncertainty of analytical measurements or an analytical procedure, respectively.

-"*bottom-up*" approach

Using the error budget model (JCGM 100: 2008), the combined standard uncertainty can be calculated as the square root of the sum of squares of all individual error components in the form of standard deviations. This "*bottom-up*" approach assumes that an analytical method can be structured into small, simple steps, and that an individual standard uncertainty can be attributed to all of these steps, sometimes based on a best guess of experienced analysts.

NOTE:
The "*bottom-up*" approach to estimate the uncertainty of analytical measurements seems to be rather impractical (Horwitz, 1998), because it does not use the results from analytical quality control, e.g. control charts, analysis of reference materials or participation in proficiency testing schemes. Therefore, if information on the uncertainty of analytical data generated in the COMBINE programme is needed, the "*top-down*" approach according to ISO 11352 should be preferred.

- "*top-down*" approach

A „*top-down*" view on estimating the measurement uncertainty is described in ISO 11352 (2012-07) based on the NORDTEST Report TR 537 (2004). According to this standard, the combined standard uncertainty u_c is characterised as the within-laboratory reproducibilityu_{Rw} (e.g. from control charts) combined with the method and laboratory bias u_{bias} (e.g. from analysis of suitable reference materials, results from interlaboratory comparisons or recovery experiments):

$$u_c = \sqrt{u_{Rw}^2 + u_{bias}^2}$$

NOTE:
Annex B of the ISO 11352 (2012) "Water quality - Estimation of measurement uncertainty based on validation and quality control data" gives detailed examples for the calculation of the measurement uncertainty from validation data (e.g. using reference material, using data from proficiency tests and using a standard solution as quality control sample).

Uncertainty in dependence on analyte levels

For results near the limit of quantification, the uncertainty is often found to be constant and can therefore be expressed as an absolute value. When results are well above the limit of quantification the uncertainty is often proportional to the analyte concentration and can therefore be expressed as a relative value.

To allow for both proportionality of uncertainty and the possibility of an essentially constant value with level, the following general expression is used (EURACHEM/CITAC, 2000):

$$u(x) = \sqrt{s_0^2 + (x * s_1)^2}$$

where

s_0 represents a constant contribution to the overall uncertainty and

s_1 is a proportionality constant.

When the result is far from zero (i.e. well above the limit of quantification) and there is clear evidence that the uncertainty changes proportionally with the level of analyte, the term $x*s_1$ dominates. Under these circumstances s_0 may reasonably be recorded as zero and s_1 is simply the uncertainty expressed as a relative standard deviation.

Reporting of Uncertainty

The expanded uncertainty $U = k * u_c$ (usually with $k = 2$) should be reported for individual monitoring parameters as percent relative uncertainty or as absolute uncertainty (in the form of a standard deviation) together with information on how it was determined.

B.4.3 VALIDATION OF DATA (NEW CHAPTER)

Data validation is defined as the inspection of all the collected data for completeness and reasonableness, and the elimination of erroneous values. To validate, correct and evaluate data a broad range of different tools are provided. To each time series a set of so-called plausibility checks can be assigned to. These plausibility checks can be defined for a particular time range or season.

This step of data validation transforms raw data into validated data. The validated data are then processed to produce the summary reports you require for data assessment and reporting. In principle, it is necessary that persons who are involved in data validation have enough experiences and knowledge of measurements, expected results and environmental conditions.

There are essentially two parts to data validation, data screening and the treatment of suspect and missing data.

B.4.3.1 DATA SCREENING

The first part uses a series of validation routines or algorithms to screen all the data for suspect (questionable and erroneous) values. A suspect value deserves scrutiny but is not necessarily erroneous. The result of this part could be a data validation report that lists the suspect values and which validation routine each value failed.

B.4.3.2 TREATMENT OF SUSPECT AND MISSING DATA

The second part requires a case-by-case decision on what to do with the suspect values, retain them as valid, reject them as invalid, or replace them with redundant, valid values (if available). This part is where judgment by a qualified person familiar with the monitoring equipment and local conditions is needed.

Before proceeding to the following sections, you should first understand the limitations of data validation. There are many possible causes of erroneous data. The goal of data validation is to detect as many significant errors from as many causes as possible. Catching all the subtle ones is impossible. Therefore, slight deviations in the data can escape detection. Properly exercising the other quality assurance components of the monitoring program will also reduce the chances of data problems.

A. Data screening

To screen data the following list shows a selection of supported plausibility checks:

1. Check the completeness of the collected data. Check if there are any missing data values. Check if the number of data fields is equal to the expected number of measured parameters.
2. Check the correctness of the defined data format, expected interval range and measurement units.
3. Range tests. The measured data are compared to allowable upper and lower limiting values.
4. Relational tests. This comparison is based on expected relationship between various 30 ICES STGQAC Report 2006 parameters.
5. Trend tests. These tests are based on the rate of change in a value over time.

B. Treatment of suspect and missing data

After the raw data are subjected to all the validation checks, what should be done with suspect data? Some suspect values may be real, unusual occurrences while others may be truly bad. Here are some guidelines for handling suspect data:

1. Generate a validation report that lists all suspect data. For each data value, the report should give the reported value, the date and time of occurrence, and the validation criteria that it failed.
2. A qualified person should examine the suspect data to determine their acceptability.
3. If there are suspect values go back to the raw data in the laboratory and check all analytical steps and quality assurance tools for relevant investigations.
4. Compare suspect values with earlier data from the database or with other information
5. Repeat the analysis if it is possible.

References

AWS Scientific, Inc. (1997) Data Validation, Processing, and Reporting. In: Wind Resource Assessment Handbook.www.awsscientific.com

B.5. ROUTINE QUALITY CONTROL (USE OF CONTROL CHARTS)

B.5.1 GENERAL

According to international standard, e.g. ISO 17025, a defined analytical quality must be achieved, maintained, and proven by documentation. The establishment of a system of control charts is a basic principle applied in this context. For further information for control charts refer to ISO/TR 13530 (1997).

B.5.2 CONTROL OF TRUENESS

As a routine procedure for controlling systematic error, the use of Shewhart control charts based on the mean, spiking recovery and analysis of blanks is recommended.

B.5.2.1 X-CHARTS

Synonyms for X-chart are X-control chart, mean control chart, average control chart or xbar control chart.

For trueness control, standard solutions, synthetic samples or certified real samples may be analysed using a Shewhart chart of mean values.

The analysis of standard solutions serves only as a check on calibration. If, however, solutions with a synthetic or real matrix are used as control samples, the specificity of the analytical system under examination can be checked, provided an independent estimation of the true value for the determinant is available.
A simple X-chart is constructed in the following way:

- The respective control sample should be analysed later on a regular basis with each batch of unknown environmental samples or, if a large number of unknowns is run in a batch, one control sample for each 10 or 20 unknowns.

- Analyse the control sample at least ten times for the given variable. The analyses should be done on different days spread over a period of time. This enables a calculation of the total standard deviation (s_t).

- It is advisable to analyse certified reference samples (if suitable ones are available and are not too expensive) with routine samples as a check on trueness. A restricted check on systematic error by means of recovery control charts is often made instead (5.2.2).

- Calculate the mean value ($\bar{x}$), the standard deviation (s) and the following values: $x + 2s$, $x - 2s$, $x + 3s$, $x - 3s$. Use these data to produce the plot.

If the data follow a normal distribution, 95 % of them should fall within $x \pm 2s$ (between the Upper Warning Limit and Lower Warning Limit) and 99.7 % should fall within $x \pm 3s$ (between the Upper Action Limit and Lower Action Limit).

B.5.2.2 BLANK CONTROL CHART

The blank control chart represents a special application of the X-chart (mean control chart). The following (constant) systematic error sources may be identified by the blank control chart:

- contamination of container for sampling, sample storage and sample pre-treatment;

- contamination of reagents, reaction vessels or laboratory equipment used during analysis.

Generally, the simultaneous determination of the blank value would be required for each analysis. Since this requirement can seldom be met due to the considerable effort, it appears reasonable to determine a minimum of two blank values during the series of analyses (at the beginning and at the end of each batch of samples).

B.5.2.3 RECOVERY CONTROL CHART

Synonyms of recovery control charts are Control Charts for Spiked Sample Recovery, Spiked-sample (control) chart or Accuracy charts.

In marine chemistry the control charts for spiked sample recovery are especially useful when the sample matrix can be suspected of causing interferences that have an influence on the analytical response. They are useful in trace metal analysis and in nutrient analysis where the sample matrix can affect the chemical reaction of the signal response.
The control chart for spiked sample recovery can be constructed as follows:

- Use the same spike concentration in all series of the same variable, concentration range and matrix.

- Select and analyse a natural sample in each analytical series.

- Spike by adding to the sample a known concentration of the analyte to be determined, and re-analyse. If possible, use a CRM concentrate.
- Calculate the measured difference in concentration by subtraction and correction for dilution from spiking.
- Calculate the percent recovery (%R) for each spiked sample for a given test, matrix, range.
- Calculate the mean %R by taking the %R`s and dividing by the total number (n) of %R`s (outlier excluded).
- Calculate the total standard deviation (s_t) on the basis of at least ten analytical series.
- Calculate the following values: $R + 2s_t$, $R - 2s_t$, $R + 3s_t$, $R - 3s_t$. Use these data to produce the plot.

With the presumption that the measured recoveries are normally distributed, the data should be distributed within the same limits as described for the X-charts (see B.5.2.1).

The recovery control chart, however, provides only a limited check on trueness because the recovery tests will identify only systematic errors which are proportional to determinant concentration; bias of constant size may go undetected.

B.5.3 CONTROL OF PRECISION

There are four ways of controlling the precision of analytical results in routine analysis:

- use of the mean control chart (5.2.1);
- use of a range control chart (5.3.1);
- estimation of precision with replicate analysis (5.3.2);
- standard addition (5.3.3).

B.5.3.1 R-CHART

Synonyms for R-chart are Range (control) chart or Precision chart.

R-charts are used for graphing the range or the relative percent difference (RPD) of analytical replicate or matrix spike duplicate results.

It is common practice in analytical laboratories to run duplicate analyses at frequent intervals as a means of monitoring the precision of analyses and detecting out-of-control situations in R-charts. This is often done for determinants for which there are no suitable control samples or reference materials available.

The R-chart can be constructed using the following method:

- Calculate the relative range (R_{rel}) for each replicate analysis for a given natural sample of the same matrix:

- $R_{rel} = \frac{x_{max} - x_{min}}{\bar{x}} * 100\%$ with the mean value ($\bar{x}$) of the replicate set $\bar{x} = \frac{1}{n} * \sum_{i=1}^{n} x_i$
- Calculate the mean relative range $\overline{R_{rel}}$ by summing all R_{rel} and dividing by the total number (n) of replicate sets (outliers excluded).
- Calculate the Upper Action Limit (UA): $UA = \overline{R_{rel}} \times D_{UA}$
- The R_{rel} for each replicate analysis and the UA are drawn on the chart.

When performing replicate determinations (duplicate to six-fold), the lower action limit (LA) is identical with the abscissa (zero-line).

The numerical values for the factor D_{UA} are:

	Duplicate determination	Three-fold determination	Four-fold determination	Five-fold determination
D_{UA}(P=99,7%)	3,267	2,575	2,282	2,115

NOTE: For further numerical values for the factor UA refer to Funk et al. (1992).

B.5.4. CONTROL CHARTS WITH FIXED QUALITY CRITERIONS (TARGET CONTROL CHARTS)

In the contrary to the under clauses B.5.2 and B.5.3 described classical control charts of the SHEWHART type the target control charts operate without statistically evaluated values. The bounds for this type of control charts are given by external prescribed and independent quality criterions. A target control chart (for the mean, the true value, the blank value, the recovery rate, the range) is appropriate if:

- there is no normal distribution of the values from the control sample (i.e. blank values)
- the Shewhart or range control charts show persisting out of control situations
- there are not enough data available for the statistical evaluation of the bounds
- there are external prescribed bounds which should be applied to ensure the quality of analytical values.

The control samples for the target control charts are the same as for the classical control charts as described in clauses B.5.2 and B.5.3.

The bounds are given by:

- requirements from legislation
- standards of analytical methods and requirements for internal quality control (IQC)
- the (at least) laboratory-specific precision and trueness of the analytical value, which had to be ensured
- the valuation of laboratory-intern known data of the same sample type.

The chart is constructed with an upper and lower bound. A pre-period is inapplicable. The target control chart of the range needs only the upper bound.

The analytical method is out-of-control if the analytical value is higher or lower than the respective prescribed bounds. The measures are the same as described in clauses 5.2 and 5.3.

B.5.5. CONTROL CHARTS FOR BIOLOGICAL MEASUREMENTS

For the quality control while measuring biological variables the Shewhart charts (in these cases R-charts where the criteria for evaluation of testing results is based on statistically calculated values are used.

The control chart for duplicate samples can be constructed as follows:

- For bacterioplankton, phytoplankton and mesozooplankton, run every 10th sample or at least one sample per batch as duplicate, counting two sub samples from the same sample (ca 10% of all samples).
- For chlorophyll-a run one duplicate sample within every batch of samples and calculate the range (R) with $R = |x_1 - x_2|$,

  where x_1 and x_2 are concentrations of chlorophyll-a in duplicate samples. For other biological variables the difference in abundance of organisms and/or biomass is calculated.
- Calculate the relative range (R_{rel}) for each duplicate analysis
- $R_{rel} = \frac{x_{max} - x_{min}}{\bar{x}} * 100\%$ with the mean value ($\bar{x}$) $\bar{x} = \frac{1}{n} * \sum_{i=1}^{n} x_i$ of the replicate set
- Calculate the mean relative range $\overline{R_{rel}}$ by summing all R_{rel} and dividing by the total number (n) of replicate sets (outliers excluded).
- Calculate the Upper Action Limit (UA): $UA = \overline{R_{rel}} \times D_{UA}$ in the same way as in chapter 5.3.1
- For duplicate determination the Upper Action Limit (UA) is then: UA= 3,267 R_{rel}
- The lower action limit (LA) is identical with the abscissa (zero-line).
- The R_{rel} for each replicate analysis and the UA are drawn on the chart.

The blank control chart can also be used for biological measurements, e.g. for the determination of chlorophyll *a*.

B.5.6. INTERPRETATION OF CONTROL CHARTS, OUT-OF-CONTROL SITUATIONS

The results of analyses of reference material analysed with each batch of environmental samples indicate whether the errors fall within acceptable limits.

The quality control charts is intended to identify changes in random or systematic error.

The following criteria for out-of-control situations are recommended for use with Shewhart charts:

- 1 control value being outside the action limit UA or LA; or
- 2 consecutive values outside warning limit UW or LW; or
- 7 consecutive control values with rising tendency; or
- 7 consecutive control values with falling tendency; or
- 10 out of 11 consecutive control values being on one side of the central line.

The following out-of control situations apply to the R-chart if:

- a range RPD falls outside the upper action limit; or
- a range RPD falls below the lower action limit (valid only for LA>0); or
- 7 consecutive control values show an ascending/descending tendency; or
- 7 consecutive control values lie above the mean range RPD.

For control charts with fixed quality criterions (target control charts) the analytical method is out-of-control if the analytical value is higher or lower than the respective prescribed bounds.

A cyclic variation of ranges may be observed, for example, by a regularly scheduled maintenance of an analytical instrument or by re-preparation of reagents.

B.5.7. SELECTION OF SUITABLE CONTROL CHARTS

The table shows which control samples are suitable for checking trueness and precision.

Type of control chart	Trueness	Precision
X-chart with standard solution	Restricted	Yes

X-chart with Certified reference materials	Yes	Yes
X-chart with Laboratory reference materials/intercomparison samples	Yes	Yes
Blank control chart	Restricted	No
Recovery control chart with real sample	Yes	Yes
R-chart	No	Yes

Control charts for the determination of sum parameters:

- R-chart as Shewhart or as target control chart for the whole range of the method whereas it should be worked with various matrices and concentration levels
- Blank control charts as Shewhart chart for an (approximated) normal distribution or otherwise as a target control chart

Control charts for the determination of single parameters:

- X-chart as Shewhart or as target control chart for the whole range of the method whereas the control sample concentration should be in the middle of the range
- Blank control charts as Shewhart chart for an (approximated) normal distribution or otherwise as a target control chart

Control charts for multi-parameter methods:

- The selection of suitable control charts for multi-parameter procedures such as gas chromatography and optical emission spectrometry should depend on whether the selected measured variable is particularly problematic, representative or relevant.

B.5.8. REFERENCES

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B.6. EXTERNAL QUALITY ASSESSMENT

For marine environmental monitoring programmes, it is essential that the data provided by the laboratories involved are comparable. Therefore, activities like participation in an external quality assessment schemes, ring tests, taxonomical workshops and use of external specialists by the laboratories concerned should be considered indispensable.

While the use of a validated analytical method and routine quality control (see above) will ensure accurate results within a laboratory, participation in an external quality assessment or proficiency testing scheme provides an independent and continuous means of detecting and guarding against undiscovered sources of errors and acting as a demonstration that the analytical quality control of the laboratory is effective.

Generally, proficiency testing, ring tests, etc. are useful to obtain information about the comparability of results, and ensures that each of the participating laboratories achieves an acceptable level of analytical accuracy.

Details of the development and operation of proficiency testing schemes are outlined in ISO Guide 43. An overview of the structure and an assessment of the objectives of proficiency testing have been given by the Analytical Methods Committee (1992).

An approach known as the paired sample technique, which has been described by Youden and Steiner (1975), provides a valuable means of summarizing and interpreting in graphical form the results of interlaboratory comparison exercises.

Most ring tests and proficiency testing schemes are based on the distribution of samples or identical sub-samples (test materials) from a uniform bulk material to the participating laboratories. The test material must be homogeneous and stable for the duration of the testing period. Amounts of the material should be submitted that are sufficient for the respective determinations.

The samples are analysed by the different laboratories independently of one another, each under repeatable conditions. Participants are free to select the validated method of their choice. It is important that the test material is not treated in any way different from the treatment of samples ordinarily analysed in the laboratory. In this way, the performance established by the proficiency testing results will reflect the actual performance of the laboratory.

Analytical results obtained in the respective laboratories are returned to the organizer where the data are collated, analysed statistically, and reports issued to the participants.

B.7. DEFINITIONS

In the following, a summary of the technical/scientific terms used in this document is given. Sections are mentioned when the terms have been explained in the text. Definitions are provided for terms not explained in the text.

Accuracy. See Section 4.2.5.

Analytical method. The set of written instructions completely defining the procedure to be adopted by the analyst in order to obtain the required analytical result (Wilson, 1970).

An **analytical system** comprises all components involved in producing results from the analysis of samples, i.e., the sampling technique, the 'method', the analyst, the laboratory facilities, the instrumental equipment, the nature (matrix, origin) of the sample, and the calibration procedure used.

Biological variables are chlorophyll *a*, primary production measurements, bacterioplankton, phytoplankton, zooplankton, macrozoobenthos, phytobenthos and fish.

HELCOM BMP. Baltic Monitoring Programme.

Blank control chart. See Section 5.5.

Calibration is the set of operations which establish, under specified conditions, the relationship between values indicated by a measuring instrument or measuring system, or values represented by a material measure, and the corresponding known values.

HELCOM CMP. Coastal Monitoring Programme.

CRM (Certified Reference Material) is a material one or more of whose property values are certified by a technically valid procedure, accompanied by or traceable to a certificate or other documentation which is issued by a certifying body.

Cusum Charts. See Section 5.4.

Detection limit. See Section 4.2.3.

Errors. See Sections 4.2.5, 4.2.5.1 and 4.2.5.2.

External quality assessment. See Section 6.

LCL. Lower control limit.

LRM. Laboratory Reference Material.

Matrix. The totality of all components of a material including their chemical, physical and biological properties.

Performance characteristics of an analytical method used under given experimental conditions are a set of quantitative and experimentally determined values for parameters of fundamental importance in assessing the suitability of the method for any given purpose (Wilson, 1970).

Proficiency testing is the determination of the laboratory calibration or testing performance by means of interlaboratory comparisons. **Quality**. Characteristic features and properties of an analytical method/analytical system in relation to their suitability to fulfill specific requirements.

The term **Quality Assurance** involves two concepts: **Quality control** and **Quality assessment**.

- **Quality control** is 'the mechanism established to control errors', and **quality assessment** is 'the system used to verify that the analytical process is operating within acceptable limits' (ACS Committee, 1983; Taylor, 1981).
- **Quality assessments** of analyses, generally referred to as intercomparison exercises, have been organized over the last twenty years by the International Council for the Exploration of the Sea (ICES).
- **Quality audits** are carried out in order to ensure that the laboratory's policies and procedures, as formulated in the Quality Manual, are being followed.
- **Quality Manual** is a document stating the quality policy and describing the quality system of an organization.
- **Quality policy** forms one element of the corporate policy and is authorized by top management.
- **Quality system** is a term used to describe measures which ensure that a laboratory fulfills the requirements for its analytical tasks on a continuing basis.

Range. See Section 4.2.4., Ring test - See proficiency testing

Selectivity. See Section 4.2.1.

Quality Manager. The Quality System should include a statement that the post-holder has responsibility for ensuring that the requirements for the Quality System are met continuously and that the post-holder has direct access to the highest level of management at which decisions are taken on laboratory policy or resources, and to the Technical Manager.

Technical Manager. The Quality System should include a statement that the post-holder has overall responsibility for the technical operation of the laboratory and for ensuring that the Quality System requirements are met.

Traceability. Results obtained from an analytical investigation can only be accurate if they are traceable. Traceability of a measurement is achieved by an unbroken chain of calibrations connecting the measurement process to the fundamental units. In most instances, when analyses are carried out, the chain is broken because due to the sample pretreatment and preparation the original material is destroyed. In order to approach full traceability, it is necessary to demonstrate that no loss or contamination has occurred during the analytical procedure.

Traceability to national or international standards can be achieved by comparison with certified reference standards or certified reference materials, respectively, the composition of which must simulate to a high degree the sample to be analysed. Consequently, if analytical results for a certified reference material are in agreement with the certified values, it should be realized that owing to discrepancies in composition between certified reference material and sample, there is still a risk that the results on real samples may be wrong.

UCL. Upper control limit.

Validation of an analytical method is the procedure that 'establishes, by laboratory studies, that the performance characteristics of the method meet the specifications related to the intended use of the analytical results' (EURACHEM/WELAC, 1992).

X-charts. See Section 5.2.1.

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ANNEX B-1 PRINCIPLE COMPONENTS OF A QUALITY MANUAL

The quality system should be formalized in a quality manual which must be maintained and kept up-to-date.

The person responsible for authorization and compilation of the quality manual should be identified. A distribution list of the quality manual and identification of holders of controlled copies of the quality manual should be included.

The quality manual should contain, for example, the following items or their equivalent:

1) Scope.
2) References.
3) Definitions.
4) Statement of quality policy.
5) Organization and management.
6) Quality system audit and review.
7) Personnel.
8) Accommodation and environment.
9) Equipment and reference material.
10) Measurement, traceability and calibration.
11) Calibration and test methods.
12) Handling of calibration and test items.
13) Records.
14) Certificates and reports.
15) Sub-contracting of calibration or testing.
16) Outside support services and supplies.
17) Complaints.

ANNEX B-2. VALIDATION OF ESTABLISHED ANALAYTICAL METHODS

By means of the experimental design described in this Annex, data are obtained for calculation of the following:

- Range.
- Linearity of calibration on the day when N2 and N6 are analysed.
- Sensitivity.
- Verification of the precision at the limit of detection.
- Accuracy of synthetic samples at two concentration levels (N2 and N5) and of a spiked sample (N4 minus N3).
- Repeatability at four levels for synthetic and natural samples.
- Standard deviation between series for synthetic and natural samples at four concentration levels.

Start by performing six replicate measurements of a blank sample (or a sample with a concentration close to the expected detection limit), and calculate the detection limit (see Section 4.2.3, in body of report). Wherever possible, use natural samples with a known concentration. As these are rarely available, the experimental design could be as follows:

Concentration level	N1	N2	N3	N4	N5	N6
Measurements in each analytical series	2	2	2	2	2	2
Number of analytical series	6	1	6	6	6	1

Level N1: A synthetic sample, or if possible a natural sample with a concentration near the detection limit.
Level N2: A synthetic sample with concentration between N1 and N3.
Level N3: A natural sample with a concentration in the middle of the concentration range.
Level N4: The natural sample N3 with addition of property/variable (a spiked sample).
Level N5: A synthetic sample with a concentration at the upper level of the range.
Level N6: A synthetic sample with a concentration approximately 20 % higher than N5.

In at least one of the analytical series, a calibration is performed with replicate measurements at all six concentration levels.

If the natural samples are not reference materials and for this reason the true concentrations are unknown, synthetic samples with approximately the same concentrations are also analysed. These samples are only measured in the same series as N2 and N6.

The analyses are performed in randomized order to obtain a realistic value for the standard deviation within a series.

All data are thus obtained by performing six determinations in one series, twelve determinations in another series and eight determinations in each of five further series, which is in total 58 determinations. This design gives sufficient data for determination of the range, the linearity, the selectivity, the detection limit and the accuracy. If a reference material has been applied as a natural sample, the data documentation is of an even higher quality.

If the type and concentration of the samples that have been chosen for the method validation have been chosen appropriately, they can be applied as the first data in the internal quality control charts. This is, of course, presuming that the method validation was satisfactory.

ANNEX B-3 QUALITY AUDIT

1. OBJECTIVES OF INTERNAL AUDITS

1.1 The laboratory or inspection body should conduct internal audits of its activities to verify that its operations continue to comply with the requirements of its quality management system.

1.2 These audits should check that the quality management system fulfils the requirements of ISO/IEC 17025, whichever is applicable, or other relevant criteria documents, i.e. that there is conformity.

1.3 These audits should also check whether or not the requirements stated in the organisation's quality manual and related documents are applied at all levels of work.

1.4 The non-conformities found in internal audits give valuable information for the improvement of the organisation's quality system and should thus be used as input to management reviews.

2. ORGANISATION OF INTERNAL AUDITS

2.1 The internal audits should be carried out according to a documented procedure.

2.2 Internal audits should be programmed such that each element of the quality management system is checked at least once a year. In large laboratories or inspection bodies it may be advantageous to establish a plan whereby the different elements of the quality management system or different sections of the organisation are audited throughout the year.

2.3 The quality manager is normally the audit program manager and may be the lead auditor.

2.4 The quality manager should be responsible for ensuring that the audits are carried out in accordance with the established plan.

2.5 Such audits should be carried out by qualified personnel who have sufficient technical knowledge of the operations they are auditing, and who are trained specifically in auditing techniques and processes.

2.6 The quality manager may delegate the task of performing audits provided that the person used is familiar with the organisation's quality management system and accreditation requirements and meets the requirements set out in 2.5.

2.7 In large organisations carrying out calibration and/or testing and/or inspection over a wide range of technical disciplines, it may be necessary for audits to be carried out by a team of individuals under the control of the quality manager.

2.8 In small organisations audits may be carried out by the quality manager alone. The management should, however, ensure that another person is given the task of auditing the quality manager's activities to ensure that the quality function is carried out satisfactorily.

2.9 Wherever resources permit, the auditor should be independent of the activity to be audited. Personnel should not audit their own activities or activities under their own direct responsibility except where there is no alternative and it can be demonstrated that an effective audit can be carried out. Laboratories and inspection bodies should pay particular attention to checking the effectiveness of an internal audit where it has been carried out by staff members who are not independent of the audited activities.

2.10 here an organisation is accredited for calibration and/or testing and/or inspection at a client's site, or for sampling in the field, these activities should be included in the audit program.

2.11 Audits carried out by other parties, such as customers or an accreditation body, should not be considered as a substitute for internal audits.

3. PLANNING OF INTERNAL AUDITS

3.1 An audit plan including the audit scope, the audit criteria, the audit schedule, reference documents (such as the organisation’s quality manual and audit procedure) and the names of audit team members, should be established by the quality manager.

3.2 Each auditor should be assigned specific quality management system elements or functional departments to audit. These assignments should be made by the lead auditor in consultation with the auditors concerned. Assigned auditors should have some technical knowledge of the departments they are to audit.

3.3 Working documents required to facilitate the auditor's investigations and to document and report results may include: criteria documents such as

- ISO/IEC 17025 and any supplementary documents
- laboratory or inspection body manuals and documents
- checklists used for evaluating quality management system elements (normally prepared by the auditor assigned to audit that specific element)
- forms for reporting audit observations, such as a “non-conformance” form or “correction action request” form. These permit the recording of the nature of the ”nonconformity”, the agreed corrective action, and the eventual confirmation that the action has been taken effectively.

3.4 An audit timetable should be developed by each auditor in conjunction with the auditee to ensure the smooth and systematic progress of the audit.

3.5 Prior to the actual audit, a review of documents, manuals, previous audit reports and records should be carried out to check for conformity with the quality management system requirements and to develop a checklist of key issues to be audited.

4. IMPLEMENTATION OF INTERNAL AUDITS

4.1 The key steps of an audit are planning, investigation, analysis, reporting, follow-up corrective action and close-out.

4.2 The opening meeting should introduce the audit team, confirm the audit criteria, review the audit scope, explain the audit procedure, clarify any relevant details, and confirm the timetable, including the time or date, and attendees for the closing meeting.

4.3 The investigation process for gathering objective evidence involves asking questions, observing activities, examining facilities, and examining records. The auditor examines the conformity of the activities with the quality management system.

4.4 The auditor uses the quality management system documents (quality manual, system procedures, test methods, work instructions, etc.) as references, and compares what is actually happening with what these quality management system documents state should happen.

4.5 At all times during the audit the auditor seeks objective audit evidence that the quality management system requirements are being fulfilled. Evidence should be collected as efficiently and effectively as possible, without prejudice, and without upsetting the auditee.

4.6 Nonconformities should be noted and should be investigated further by the auditor to identify underlying problems.

4.7 All audit findings should be recorded.

4.8 After all activities have been audited, the audit team should carefully review and analyse all of their findings to determine which are to be reported as nonconformities and which can be included as recommendations for improvement.

4.9 The audit team should prepare a clear, concise report, supported by objective audit evidence, of nonconformities and recommendations for improvement.

4.10 Nonconformities should be identified in terms of the specific requirements of the organisation’s quality manual and related documents against which the audit has been conducted.

4.11 The audit team should hold a closing meeting with the senior management of the organisation and those responsible for the functions audited. The main purpose of this meeting is to present audit findings and to report to senior management in such a manner as to ensure that they clearly understand the results of the audit.

4.12 The lead auditor should present observations, taking into account their perceived significance. Both positive and negative aspects of the operations should be presented.

4.13 The lead auditor should present the audit team's conclusions regarding the quality management system's conformity with audit criteria, and the conformance of the operations to the quality management system.

4.14 Nonconformities identified during an audit should be noted, and the appropriate corrective action and the time frame for correction agreed with the auditee and recorded.

4.15 Records of the closing meeting should be kept.

5. FOLLOW-UP CORRECTIVE ACTION AND CLOSE-OUT

5.1 The implementation of the agreed corrective action is the responsibility of the auditee.

5.2 Whenever a non-conformity that may jeopardise the result of a calibration, test or inspection is discovered, the corresponding activity should be halted until the appropriate corrective action has been taken and has been shown to lead to satisfactory results. In addition, results that may have been affected by the non-conformity should be investigated and customers informed if the validity of corresponding calibration, test or inspection certificates/reports is in doubt.

5.3 The formal corrective action procedure may need to be followed to reveal the root causes of some problems and to implement effective corrective and preventive actions.

5.4 The auditor should check the effectiveness of corrective actions as soon as possible after the agreed time frame has elapsed. The quality manager should have the ultimate responsibility for confirming the clearance of nonconformities by the auditee and then closing them out.

6. RECORDS AND REPORTS OF INTERNAL AUDITS

6.1 A complete record of the audit should be maintained even where no nonconformities have been found.

6.2 Each of the nonconformities that have been identified should be recorded, detailing their nature, their possible cause(s), corrective action(s) required.

6.3 Following the audit close-out, a final report should be prepared which should summarise the outcome of the audit and include the following information:

- the name(s) of the auditor(s);
- the date of the audit;
- the areas audited;
- the details of all areas examined;
- the positive or good aspects of the operations;
- any nonconformity identified, linked to references to relevant documents;
- any recommendations for improvement;
- corrective action agreed, the time frame agreed for completion, and the person responsible for carrying out the action;
- corrective actions taken;
- the date of confirmation of completion of corrective action;
- the signature of the quality manager confirming close-out of corrective actions.

6.4 All records of audits should be stored for an agreed period of time.

6.5 The quality manager should ensure that the audit report and, where appropriate, individual nonconformities, are brought to the attention of the organisation’s senior management.

6.6 The trends in results of internal audits and corrective actions should be analysed by the quality manager and a report prepared for review by senior management at the next management review meeting.

6.7 The purpose of such reviews is to ensure that the audits and the corrective actions are contributing to the continuing effectiveness of the quality management system as a whole.

7. REFERENCES

APLAC Asia Pacific Laboratory Accreditation Body (2002) Internal Audits for Laboratories and Inspection Bodies – APLAC TC 002 Issue No. 2

ISO/IEC 17025 (2005) General requirements for the competence of testing and calibration laboratories

ISO 19011 (2002) Guidelines for quality and/or environmental management systems auditing

ANNEX B-4 STANDARD OPERATING PROCEDURES

HELCOM/OSPAR COMBINE/JAMP manual ***Part L*** **Annex L-00**	*Title of Standard Operating Procedure*	**Issue no.: 1** **Revision 0: 0** **Page no: 1 (19)** **Issue date: 07-02-15** **Approved by:** Signature:

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1. INTRODUCTION

Presentation of the method and its relevance as an environmental quality factor. Relevant references are provided.

1.1 Background

General description of the basis for the method for non-experts with some detail and references.

1.2 Principle

Detiled description of the principle behind the method for e.g. technicians.

1.3 Extent

Present under what environmental conditions the method is applicable. For example present limitation due to salinity, temperature, concentrations, rates or other factors.

1.4 Disturbances

Present factors that may cause erroneous results by the method

1.5 Contamination risk

Present compounds that may contaminate the sample, distort specimens or hamper growth.

1.6 Safety

Present hazardous substances used in the method and precautions that should be applied.

2. PREPARATIONS

2.1 Cleaning and purification

2.2 Identification of sample

2.3 Reagents

2.4 Before cruise/sampling

2.5 Protocol

Identify protocols for e.g. logistic data, incubation conditions that should be used.

3. SAMPLING

3.1 Sampling

3.1.1 Sampling strategy

Provide advice on sampling frequency in time and spatial coverage of stations that is recommended based on statistical considerations.

3.1.2 Sampling method

3.2 Preservation/processing

Give time limits within which sample processing or analysis should have been completed.

3.3 Storage

4. METHOD DESCRIPTION

4.1 Reagents

4.2 Calibration solutions

4.3 Processing

4.4 Calibration

Define calibrations of instruments that should be performed routinely.

4.5 Analysis

5. CALCULATIONS

5.1 Calculation functions

5.2 Calculations

Present how calculations are best performed by e.g. manually, by data bases or distributed software.

5.3 Measurement uncertainty

Provide extended measurement uncertainty estimates of the method and detection limit according to current international standards and guidelines.

6. QUALITY ASSURANCE AND EVALUATION

6.1 Control charts

Recommend suitable control charts according to international scientific guidelines.

6.2 Evaluation

Describe quality criteria for approval of a measurement value.

7. Reporting

Present variables and other information that should be supplied with measurement values of the operating procedure.

Table 2. Primary database variables and units.

Parameter	SI-unit	Valid digits	Calculation function	Category	Database acronyme	Value example

† footnote.

Define what primary (raw) data that should be reported.

Table 3. Calculated parameters.

Parameter	SI-unit	Valid digits	Calculation function	Category	Database acronyme	Value example

Define what secondary (calculated) data that should be possible to derive from the primary data and used in status assessment.

8. Equipment

Detailed presentation of equipment, its performance, model number and brand.

9. Chemicals and solutions

Detailed description of solutions and chemicals, their quality requirements, distributor, concentrations, preparations procedure and storage.

10. References

Give full traceable reference to scientific literature, reports or links to web sites.

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ANNEX B-5 GENERAL REMARKS ON SAMPLING

Sampling for the performance of analytical investigation has to be oriented towards the particular analytical task. Different aspects of sampling programmes are comprehensively dealt with in articles by Kratochvil and Taylor (1981), the ACS Committee on Environmental Improvement (ACS, 1983), and Garfield (1989).

Based on information provided by the above-mentioned authors, an acceptable sampling programme should include the following:

1) a predetermined sampling plan that takes into account the specific purpose of the investigations, including the contaminants to be determined, their expected concentration range, and the type of matrix to be analysed;

2) sample collection by personnel trained in the sampling techniques and procedures specified;

3) maintenance of the sample integrity by

- using sampling devices that have been found to be suitable for the particular purpose,
- avoiding contamination of samples from the use of unclean equipment,
- using transportation procedures that ensure that the composition of the sample or the concentrations of the variables are not altered;

4) instructions for labelling the sample specifying its identity;

5) a record that demonstrates an unbroken control over the sample from collection to its final disposition.

Detailed guidelines on sampling will be dealt with at a later time. Recommendations from other bodies or working groups will be taken into consideration when available. Detailed information on sampling for biological measurements is found with each separate variable.

References

ACS (American Chemical Society Committee on Environmental Improvement. Keith, L., Crummet, W., Deegan, J., Libby, R., Taylor, J., Wentler, G.) 1983. Principles of environmental analysis. Analytical Chemistry, 55: 2210-2218.

Garfield, F. 1989. Sampling in the analytical scheme. Journal of the Association of Official Analytical Chemists, 72: 405-411.

Kratochvil, B., and Taylor, K. 1981. Sampling for chemical analysis. Analytical Chemistry, 53: 924A-938A.

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ANNEX B-6 EXAMPLES OF REFERENCE MATERIALS FOR INTERNAL QUALITY CONTROL

Currently available (January 2001) CRMs for marine monitoring programmes are listed in Table B-6.1. Information on matrix composition and analyte concentrations can be found on the Internet:

BCR (Belgium)	www.irmm.jrc.be/mrm.html
DHI (Denmark)	www.refmat.dhi.dk
NRC (Canada)	www.cm.inms.nrc.ca/ems1.htm
NIST (USA)	http://ts.nist.gov/ts/htdocs/230/232/232.htm
IAEA (Austria)	www.iaea.org/programmes/nahunet/e4/nmrm/index.htm
LGC (UK)	www.lgc.co.uk/
NIES (Japan)	www.nies.go.jp

The ICES Marine Chemistry Working Group (MCWG) regularly publishes comprehensive lists of suitable CRMs for marine monitoring programmes including certified determinand concentrations (the MCWG 2000 report is available at: http://www.ices.dk/sites/pub/Publication%20Reports/Expert%20Group%20Report/mhc/2000/mcwg00.pdf).

Table B-7.1. Currently available CRMs for marine monitoring programmes (January 2001).

Matrix	Certified values for	Material	Name	Manufacturer
Sediment	New batch in prep.	BCR 277R	Trace elements in estuarine sediment	BCR (Belgium)
Sediment	2 Sn-species	BCR 462	Coastal sediment	BCR (Belgium)
Sediment	2 Hg-species	BCR 580	Estuarine sediment	BCR (Belgium)

Sediment	10 Metals	MURST-ISS-A1	Antarctic sediment	BCR (Belgium)
Sediment	14 Metals	HISS-1	Marine sediment	NRC (Canada)
Sediment	20 Metals	MESS-3	Marine sediment	NRC (Canada)
Sediment	19 Metals, 3 Sn-species	PACS-2	Marine sediment	NRC (Canada)
Sediment	18 Metals	SRM 1646A	Estuarine sediment	NIST (USA)
Sediment	9 Metals	SRM 1944	New York/New Jersey sediment	NIST (USA)
Sediment	39 Metals	IAEA 356	Marine sediment	IAEA (Austria)
Sediment	23 Metals	GBW 07313	Marine sediment	NRCCRM (China)
Sediment	9 Metals	GBW 07314	Offshore marine sediment	NRCCRM (China)
Sediment	56 Metals	GBW 07315	Marine sediment	NRCCRM (China)
Sediment	56 Metals	GBW 07316	Marine sediment	NRCCRM (China)
Sediment	19 Metals	LGC6137	Estuarine sediment	LGC (UK)
Sediment	20 Metals	LGC6156	Harbour sediment	LGC (UK)

Sediment	in prep. (PAHs)	SRM 1941b	Organics in marine sediment	NIST (USA)
Sediment	24 PAHs, 29 PCBs	SRM 1944	New York/New Jersey sediment	NIST (USA)
Sediment	7 PCBs	LGC6114	Harbour sediment	LGC (UK)
Sediment	PAHs, Organo-Cl	IAEA 383	Marine sediment	IAEA (Austria)
Sediment	PAHs, Organo-Cl	IAEA 408	Marine sediment	IAEA (Austria)
Sediment	10 PCBs	NRCC-HS-1	Marine sediment	NRC (Canada)
Sediment	10 PCBs	NRCC-HS-2	Marine sediment	NRC (Canada)
Sediment	20 PAHs	NRCC-HS-3B	Harbour sediment	NRC (Canada)
Sediment	20 PAHs	NRCC-HS-4B	Harbour sediment	NRC (Canada)
Sediment	16 PAHs	NRCC-HS-5	Marine sediment	NRC (Canada)
Sediment	16 PAHs	NRCC-HS-6	Marine sediment	NRC (Canada)
Sediment	13 PAHs	SES-1	Spiked estuarine sediment	NRC (Canada)
Sediment	PCBs	CS-1	Marine sediment	NRC (Canada)
Biota	9 Metals	BCR 278R	Mussel tissue	BCR (Belgium)
Biota	6 Metals	BCR 279	Sea lettuce	BCR (Belgium)

Biota	11 Metals	BCR 414	Plankton	BCR (Belgium)
Biota	10 Metals	BCR 422	Cod muscle	BCR (Belgium)
Biota	2 Hg-species	BCR 463	Tuna fish	BCR (Belgium)
Biota	2 Hg-species	BCR 464	Tuna fish	BCR (Belgium)
Biota	3 Sn-species	BCR 477	Mussel tissue	BCR (Belgium)
Biota	3 As-species	BCR 627	Tuna fish tissue	BCR (Belgium)
Biota	10 Metals	MURST-ISS-A2	Antarctic krill	BCR (Belgium)
Biota	17 Metals and species	DOLT-2	Dogfish liver	NRC (Canada)
Biota	14 Metals, Methyl-Hg	DORM-2	Dogfish muscle	NRC (Canada)
Biota	17 Metals	LUTS-1	Lobster hepatopancreas	NRC (Canada)
Biota	15 Metals, Methyl-Hg	TORT-2	Lobster hepatopancreas	NRC (Canada)
Biota	21 Metals	SRM 1566b	Oyster tissue	NIST (USA)
Biota	2 Hg-species	SRM 1974a	Organics in mussel tissue	NIST (USA)
Biota	6 Metals, Methyl-Hg	SRM 2977	Mussel tissue	NIST (USA)

Biota	25 Metals	IAEA-140/TM	Fucus (sea plant homogenate)	IAEA (Austria)
Biota	20 Metals	GBW08571	Mussel	NRCCRM (China)
Biota	19 Metals	GBW08572	Prawn	NRCCRM (China)
Biota	27 Metals	NIES-CRM-09	Sargasso seaweed	NIES (Japan)
Biota	3 Sn-species	NIES-CRM-11	Fish tissue	NIES (Japan)
Biota	6 PCBs	BCR 349	Cod liver oil	BCR (Belgium)
Biota	6 PCBs	BCR 350	Mackerel oil	BCR (Belgium)
Biota	18 PCDDs, PCDFs, PCBs	CARP-1	Fish (carp)	NRC (Canada)
Biota	24 PCBs, 14 Pesticides	SRM 1588a	Organics in cod liver oil	NIST (USA)
Biota	27 PCBs, 15 Pesticides	SRM 1945	Organics in whale blubber	NIST (USA)
Biota	PAHs, PCBs, Pesticides	SRM 1974a	Organics in mussel tissue	NIST (USA)
Biota	PAHs, PCBs, Pesticides	SRM 2974	Organics in freeze-dried mussel tissue	NIST (USA)

Biota	PAHs, PCBs, Pesticides	SRM 2977	Mussel tissue	NIST (USA)
Biota	PAHs, PCBs, Pesticides	SRM 2978	Mussel tissue	NIST (USA)
Biota	PAHs, PCBs, Pesticides	IAEA-140/OC	Fucus (sea plant homogenate)	IAEA (Austria)
Water	6 Metals	BCR 403	Trace elements in seawater	BCR (Belgium)
Water	4 Metals	BCR 505	Trace elements in estuarine water	BCR (Belgium)
Water	Hg	BCR 579	Coastal seawater	BCR (Belgium)
Water	12 Metals	CASS-4	Nearshore seawater	NRC (Canada)
Water	10 Metals	NASS-5	Open ocean seawater	NRC (Canada)
Water	11 Metals	SLEW-3	Estuarine water	NRC (Canada)
Water	6 Metals	LGC6016	Estuarine water - metals	LGC (UK)
Water	NO_3, NH_4, PO_4	QCWW1A	Nutrients (Concentrate in Ampoule)	DHI (Denmark)
Water	NH_4, PO_4	QCWW2.1	Nutrients (Concentrate in Ampoule)	DHI (Denmark)
Water	NO_3	QCWW2.2	Nutrients (Concentrate in Ampoule)	DHI (Denmark)

Water	TN, TP	QCWW3	Nutrients (Concentrate in Ampoule)	DHI (Denmark)
Water	DOC, TOC	QCWW4	Nutrients (Concentrate in Ampoule)	DHI (Denmark)
Water	DOC, TOC	QCWW4a	Nutrients (Concentrate in Ampoule)	DHI (Denmark)
Water	BOD	QCWW5	Nutrients (Concentrate in Ampoule)	DHI (Denmark)
Water	NO_3, NH_4, PO_4	QCRW1	Nutrients (Concentrate in Ampoule)	DHI (Denmark)
Water	TN, TP	QCRW2	Nutrients (Concentrate in Ampoule)	DHI (Denmark)

Last updated: 29.10.2012 (Annex number changed from Annex B 7 to Annex B 6)

ANNEX B-7 UNITS AND CONVERSION

This notes summarises the units that should be used for data submission within the COMBINE programme, and also gives the relevant formulas for conversion between different commonly used units.

References are made to the appropriate sections of the COMBINE Manual.

Please note that the units dm^3 and cm^3 are used throughout the note, although the units l (litre) and ml (millilitre) would be equally correct.

Part 1: Units

Parameter	Symbol	Unit	Comment
Temperature	t	C	See Annex C2
Salinity	S		See Annex C2
Secchi depth (light attenuation)		m	according to the current definition of the Practical Salinity Scale of 1978 (PSS78) See Annex C2
Current cpeed		cm/s	report as compass directions; see Annex C2
Dissolved oxygen	DO	cm^3/dm^3	See Annex C2
Oxygen saturation			reported as fraction (%); see Annex C2

Hydrogen sulphide		µmol/dm^3	See Annex C2
Nutrients		µmol/dm^3	as N, P or Si; see Annex C2
Total P and N	TP/TN	µmol/dm^3	See Annex C2
pH			NBS-scale; see Annex C2
Alkalinity		mmol/dm^3	as carbonate, see Annex C2
Particulate and dissolved organic matter (TOC, POC,DOC and PON)		µmol/dm^3	as C or N; see Annex C2
Humic matter			depending on way of calibration; see Annex C2
Heavy metals in water		ng/dm^3 or pg/dm^3	dissolved
Halogenated organics in water		ng/dm^3	
PAH in water		ng/dm^3	
Heavy metals in biota		µg/kg	wet weight
Halogenated organics in biota		µg/kg or ng/kg	wet weight, reported together with lipid content

Total suspended matter load		mg/dm^3	
Chlorophyll-a	Chl-a	mg/m^3	See Annex C2
Primary production (as carbon uptake)		mg/m^3*h	See Annex C5
Phytoplankton species			See Annex C6
- *abundance*		Counting units/dm^3	
- *biomass*		mm^3/dm^3	
Mesozooplankton			See Annex C7
- *abundance*		Individuals/m^3	
- *biomass*		mm^3/m^3; mg/m^3	
Macrozoobenthos			See Annex C8
- *abundance*		Counting units/m^2	
- *biomass*		g/m^2	Dry or wet weight

ANNEX B-8: TECHNICAL NOTE ON THE DETERMINATION OF HYDROGRAPHIC PARAMETERS:

- Appendix 1. Technical note on the determination of salinity and temperature of seawater
- Appendix 2. Technical note on the determination of dissolved oxygen in seawater
- Appendix 3. Recommended equations for the calculation of solubility and saturation of dissolved oxygen in seawater
- Appendix 4. Technical note on the determination of hydrogen sulphide in seawater

ANNEX B-8 APPENDIX 1: TECHNICAL NOTE ON THE DETERMINATION OF SALINITY AND TEMPERATURE OF SEAWATER

PART I: TECHNICAL NOTE ON THE DETERMINATION OF SALINITY OF SEAWATER (LABORATORY SALINOMETER)

1 INTRODUCTION

Many investigations have been performed to present salinity and chlorinity of sea water and the connection between salinity and chlorinity since 1884. Those investigations are thoroughly reviewed by Müller (1999). These guidelines describe the determination of the salinity (S) of seawater samples which is based on measuring conductivity with a laboratory salinometer.

Salinity values have been reported as p.s.u. (practical salinity units), parts per thousand, ppt or U. Numeric values of them (e.g., ppt, psu, per mille) are equal. However, salinity values according to the current definition of the Practical Salinity Scale of 1978 (PSS78) are dimensionless with no units.

2 METHODS

A laboratory salinometer measures the conductivity of sea water relative to a reference standard sea water. The principle of the operation of a salinometer is described in more detail by Müller (1999). General specifications and maintenance of a salinometer are presented in the manual of each manufacturer.

3 SAMPLING

See the Part III: Technical Notes on the Determination of Temperature and Salinity using a CTD Probe.

4 ANALYTICAL PROCEDURE

4.1 CALIBRATION AND STABILITY OF CALIBRATION

Calibration of a salinometer with internationally accepted IAPSO (International Association for the Physical Sciences of the Ocean) Standard Seawater:

S= 35 ‘Normal Standard Seawater’ (suitable for oceans)

S=10 ‘Low Standard Seawater’ (suitable for Baltic Sea conditions)

The conductivity of a laboratory salinometer is calibrated by using standard sea water under controlled temperature conditions. Calibration of the salinometer is always performed after changing temperature or temperature-dependent values, after malfunction of the salinometer or when the range of the measured conductivity is dramatically changed (while changing from brackish water to oceanic sea water or vice versa). The calibration intervals depend strongly on the equipment used, and can vary from daily calibration to calibrations twice a year. Consult the manufacturer’s recommendations. In any case, the stability of the calibration has to be checked using a control seawater sample in every sample batch (see Section 5.1, below).

The stability of temperature during measurement is controlled and documented during conductivity/salinity measurements. Make sure that the temperature reading of the thermometer used is traceable to the respective national reference laboratory for temperature. The thermometer(s) have to be checked against a reference thermometer at least twice a year.

4.2 SALINITY MEASUREMENT WITH SALINOMETER

Follow the instructions of the salinometer’s manufacturer during the procedures of measurement.

Check the stability of the temperature of the thermostatted water bath before measurement. Do not start the measurements until the temperature has stabilized.

Avoid air bubbles in the cell during the measurement. If the sample measurement is performed one or more weeks after sampling, mix the bottle and let it rest for at least one hour before the measurement. In case of any deposits, these either have to be removed or settled down before the measurement.

Check that the parallel results of the seawater sample are within accepted limits (0.05; Annex C-2 of the COMBINE Manual).

4.3 DOCUMENTATION

For every sample run, document in a logbook/sheet (in addition to the measured values):

- date, and identity of the analyst;
- identity of the equipment used;
- temperature of the measurement environment and the samples;
- thermometer identity (if separate from the instrument);
- batch number and result of standard seawater and control samples;
- instrument constants (if applicable to the equipment).

5 QUALITY ASSURANCE

5.1 CALIBRATION AND TRACEABILITY

Regular calibrations and checks of the salinometer are described under Section 4.1, above.

Solutions for calibration and control

Calibration of the salinometer is performed with internationally accepted IAPSO Standard Seawater. A control seawater sample (Laboratory Reference Material) should be included in every sample series and the results plotted in control charts.

It is possible to prepare a control seawater solution from sea water which is filtered, aged, bottled in several bottles with tight caps and stored in a cool room for a maximum of one year. The salinity of this control sea water should be measured daily during at least ten days and the average value for the salinity calculated. Salinity values of control sea water that is measured before the sample series may vary within certain accepted limits. If the salinity values are outside the accepted limits, the salinometer should be calibrated with IAPSO Standard Seawater. Standard Seawater is an alternative as a control sample (e.g., calibrating at 35 and use a control of 10).

Calculation

The calculation procedures used should be checked at least once a year by calculating the salinity of IAPSO Standard Seawater using three different temperature values.

6 REPORTING

Calculate the final results according to the formula recommended by the Joint Panel on Oceanographic Tables and Standards for *in situ*measurements with conductivity instruments (UNESCO, 1981), unless this calculation is carried out automatically in the salinometer. The effect of temperature on the conductivity is discussed by Müller (1999). Data should be reported according to the ICES data format (2 decimals).

7 REFERENCES

Müller, T.J. 1999. *In* Methods of Seawater Analysis. Ed. by Grasshoff *et al*. Wiley-VCH, Germany.

UNESCO. 1981. Background papers and supporting data on the Practical Salinity Scale 1978. UNESCO Technical Papers in Marine Science, 37.

PART II: TECHNICAL NOTES ON THE DETERMINATION OF TEMPERATURE WITH REVERSING THERMOMETERS

1 INTRODUCTION

Measurement of temperature using reversing thermometers is carried out for two purposes: (1) to obtain the correct temperature associated with each sample when samples are collected using separate bottles on a wire (in contrast to rosette samplers), and (2) to verify the temperature reading of a CTD probe.

2 METHODS

Reversing thermometers of two main types are available, mercury (Hg) thermometers and digital, electronic, thermometers. The performance of the two types is similar, even though digital thermometers are generally easier to handle and calibrate. It is important to remember that the mercury thermometers should only be handled by skilled and experienced staff. For the monitoring Z

3 MEASUREMENT PROCEDURE

3.1 TEMPERATURE MEASUREMENT

The correct handling of the thermometers should be described in the manufacturer's manual. Some important points have to be stressed concerning Hg thermometers:

The thermometers must be given enough time to equilibrate with the surrounding water before they are locked. Usually this means waiting 5–10 minutes at the correct depths before reversing the thermometers. Digital thermometers normally equilibrate much faster.

When reading the temperature, it is of utmost importance to ensure that the eye is level with the top of the Hg column in order to avoid refraction errors.

3.2 DOCUMENTATION

For every temperature reading, document:

- the name of the person reading the thermometer;
- the identity of the thermometer;
- for Hg-thermometers, the reading of the supporting thermometer.

4 QUALITY ASSURANCE

All reversing thermometers, regardless of type, have to be calibrated against a reference thermometer at least every second year. The reference thermometer in turn has to be calibrated with traceability to the international temperature scale. Mercury reference thermometers are calibrated every 5 years, digital reference thermometers every second year.

The calibration has to be carried out in a thermostatted water bath, capable of being thermostatted to all temperatures within the measured range. Calibration in air does not produce results of the required quality. The calibration of thermometers used in the COMBINE programme has to cover temperatures from approximately −2 °C to 25 °C. Note that special procedures for correcting mercury reference thermometers have to be applied (Theisen, 1947).

The laboratory or the data bank must uphold routines for correcting:

a) the calibrations for deviations from the true temperature of the reference thermometer;

b) the measured temperatures for the calibration results, using an individual calibration curve for each reversing thermometer.

For digital thermometers, the laboratory must uphold routines for changing batteries at regular intervals, or when needed.

5 REPORTING

5.1 DATA PROCESSING

The temperature readings of mercury reversing thermometers have to be corrected for the temperature of the mercury column when reading the temperature. This temperature is given by the supporting thermometer. Correction also has to be made for the calibration. The correction is carried out according to (Anderson, 1974; Theisen, 1947):

$$T_{corr} = \frac{(T_{obs} + I + V_0) \times (T_{obs} + I - t)}{(1/\beta) - (T_{obs} + I + V_0 + (T_{obs} + I - t)/2)}$$

where

Tcorr = the corrected, final, temperature,

Tobs = the observed temperature from the main thermometer,

I = correction according to the calibration,

V0 = a constant, specific for each mercury thermometer (the volume of the mercury),

1/b = a constant, depending on the quality of the glass (approximately = 6000)

t = the temperature of the supporting thermometer (i.e., the temperature of the mercury column when reading the Tobs).

5.2 DATA ACCURACY

The main causes for inaccuracy are usually the calibration and temperature correction procedures. Applying the suggested procedures carefully, an accuracy of at least 0.02 °C is possible. The temperature should be reported according to the ICES data format (two decimals).

6 REFERENCES

Anderson, L. 1974. Correction of reversing thermometers and related depth calculations in Baltic water. Meddelande 166 from Havsfiskelaboratoriet i Lysekil, Hydrografiska avdelningen i Göteborg (SMHI Oceanographical Laboratory, Göteborg, Sweden).

Theisen, E. 1947. Correction of temperatures and a handy way of making correction charts for reversing thermometers. Fiskeridirektoratets Skrifter: Report on Norwegian Fishery and Marine Investigations, Vol. VIII, No. 9.

PART III: TECHNICAL NOTES ON THE DETERMINATION OF TEMPERATURE AND SALINITY USING A CTD PROBE

1 INTRODUCTION

Temperature and salinity are among the most important parameters in physical oceanography. At present, *in situ* measurements of temperature and salinity are possible using automatic temperature and salinity systems (CTD systems), the configuration of which are formed by **C**onductivity, **T**emperature, and pressure (**D**epth) sensors. In order to assure the functioning of a CTD system, it is useful to make comparisons during every cruise by taking water samples with a sampler that is connected to the CTD system for further analysis with a salinometer, and by verifying temperature values with reversing thermometers attached to water samplers of the CTD system. Pressure values obtained from the sensor of the CTD system can be compared with a digital pressure sensor.

2 METHODS

General specifications and maintenance of conductivity, temperature, and pressure sensors that are used in CTD systems are presented in the manual of each manufacturer. For CTD profiling, all parameters are usually measured several times per second.

3 SAMPLING EQUIPMENT

A CTD probe equipped with sensors for temperature, conductivity, and pressure.

Reversing thermometers, the temperature of which is traceable to the national reference laboratory.

A Rosette multisampler for taking water samples.

4 ANALYTICAL PROCEDURE

There are many protocols available for CTD measurements (WOCE, 1991; UNESCO, 1988, 1994). Based on a combination of the previous protocols and field measurement experience from the COMBINE Programme, the following protocol is proposed.

4.1 SENSOR QUALITY CONTROL

It is useful to control the function of the CTD conductivity sensors by analysing water samples, that have been taken from homogeneous water masses during a CTD cast, with a laboratory salinometer that is calibrated under controlled conditions with internationally accepted standard sea water (see Part I of this Technical Note).

Temperature values measured by a CTD system can be controlled by using a pair of reversing thermometers during a CTD cast and comparing those values with each other (see Part II of this Technical Note).

The functioning of the pressure sensor is checked by verifying the measured value with a value from a separate reference probe.

These three procedures should be carried out on every cruise, and the results documented properly so that any drift in the sensor can be traced.

4.2 CTD CAST

1. Stabilization

The CTD and Rosette package are lowered a few metres below the sea surface for at least two minutes before starting the measurements.

2. Starting of CTD cast

The CTD is brought back to near the sea surface. The measurement is started. If the sea state is rough, it is recommended to start the down-cast from a few metres below the sea surface to prevent the bubbles of the breaking waves from entering the conductivity cell.

3. The speed of lowering

It is recommended to keep the lowering speed as constant as possible and between 40 cm s–1 and 120 cm s–1.

4. Documentation

The CTD depth, sonic depth, and all the other information required by the CTD logbook are documented.

5. Water samples

The Rosette bottles should preferably be fired at the selected depths during the up-cast in order to obtain an undisturbed CTD profile during the down-cast and undisturbed water samples on the way up.

Make sure that the Rosette sampling bottles are not leaking. Water for salinity determination should be sub-sampled into clearly identified glass or plastic bottles with screw caps. Plastic under-stoppers are recommended. Water sampling bottles—as well as caps and under-stoppers—are rinsed with the sample water at least two times before bottling. Fill the sample bottle with the sample water by taking into account the thermal expansion of water, e.g., do not fill the glass bottles completely. Store the water samples at room temperature before measurement of salinity with a salinometer (see Part I of this Technical Note).

Flush the CTD and Rosette sampler with fresh water after sampling.

4.3 DOCUMENTATION

Make sure that sufficient, confident, and traceable documentation of the samples and measurements is available for further data handling. One example of data documentation is presented in UNESCO (1988).

5 QUALITY ASSURANCE

5.1 CALIBRATION AND TRACEABILITY SENSORS

The general specifications of the CTD sensors, for example, range, response time, resolution, initial accuracy, settling time, stability, and drift, are presented in the manual of the manufacturer.

Calibration of the CTD sensor via the system provider or in another competent calibration laboratory is necessary every second year or on special request to assure traceability of conductivity, pressure, and temperature. The COMBINE requirements for temperature and salinity accuracy are given in Annex C-2 of the COMBINE Manual.

5.2 MAINTENANCE

Exchangeable, pre-calibrated, spare temperature, conductivity, and pressure sensor modules are recommended to be available on board in case of a breakdown. Note that cleaning of the sensors could be carried out with fresh water and a soft brush, e.g., a tooth brush, or a similar gentle technique. By no means should the sensor be cleaned with hydrochloric acid.

6 REPORTING

6.1 DATA PROCESSING

The modern salinity measurement is based on the high accurate measurement of temperature, conductivity, and pressure. Salinity is calculated according to the Practical Salinity Scale 1978 (PSS-78). Guidelines for CTD data handling are presented by UNESCO (1988, 1991).

6.2 REQUIREMENTS FOR DATA QUALITY

COMBINE Programme requirements for the accuracy of salinity, temperature, and pressure data are presented in the table below.

	Accuracy
Salinity	0.05 (BMP)
	0.1 (CMP)
Temperature	0.05 °C (BMP)
	0.05 °C (CMP)

Pressure	not specified in the manual

7 REFERENCES

UNESCO. 1988. The acquisition, calibration, and analysis of CTD data. A report of SCOR Working Group 51. Technical Papers in Marine Science, 54.

UNESCO. 1991. Processing of oceanographic station data. JPOTS editorial panel.

UNESCO. 1994. Protocols for Joint Global Flux Study (JGOFS) Core Measurements. Manual and Guides, 29. WOCE. 1991. WOCE Operational Manual, Vol. 3.

WOCE Report 68/91, July 1991.

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ANNEX B-8 APPENDIX 2: TECHNICAL NOTE ON THE DETERMINATION OF DISSOLVED OXYGEN IN SEAWATER

1. INTRODUCTION

The dissolved oxygen (DO) content in sea water is controlled by several unrelated processes including exchange with air, metabolism of plants and animals, microbial and chemical decomposition of organic matter, and hydrodynamic features such as mixing, advection, convection, and up- or down-welling. The DO content is always the result of multifactorial influences and the reasons for changes may be difficult to assess.

In stratified Baltic waters, DO depletion occurs regularly below the halocline.

2. METHODS

The reference method for the determination of DO is the Winkler titration to the iodine endpoint. It is based on the reaction of DO with iodide ion to iodine in alkaline solution in the presence of manganese (II) ion. Iodine is back titrated with standardized thiosulphate in acid solution. The endpoint can either be detected visually (see EN 25813: 1993 and ISO 5813: 1983) or in automated methods, by spectrometric or electrochemical means.

Electrochemical probes for DO exploit the reduction of oxygen to produce a current that is expressed in DO equivalents. Sensors on a polarographic or galvanic basis also exist (see EN 25814: 1992 and ISO 5814: 1990). In connection with a CTD probe, continuous profiling is feasible. Hysteresis between down- and up-profiling is possible and depends on the response times of the sensors. Many of these sensors are poisoned by hydrogen sulphide and not suited for use in anoxic waters.

3. TECHNICAL ASPECTS OF SAMPLING

It should first of all be noted that the subsampling of oxygen samples is the most critical step of the total analysis. It is of utmost importance that this step is carried out by trained and experienced staff. Samplers suitable for other hydrochemical investigations can be employed for oxygen. A special bottom water sampler could be useful for studying the oxygen conditions in the near-bottom water layer.

DO samples should be the first to be drawn from the hydrocast bottles. For subsampling and titration, only glass bottles with conical-shaped tops and with glass ground stoppers meet the requirements of the Winkler method. Subsample bottles must be calibrated and identified with their stoppers since they must not be interchanged. Subsamples are drawn with a flexible plastic tube attached to the hydrocast bottle reaching to the bottom of the glass bottle. Fill and overflow each bottle with at least three volumes. Make sure not to draw any air bubbles into the sample. Reagents are added with the dispenser tip submerged at least 1 cm below the neck of the vial. The inserted stopper displaces the excess of water. Carefully avoid contact with reagent and trapping bubbles. The sample is mixed by thoroughly shaking, as this is a very critical step in the fixation of the oxygen. Some laboratories prefer to mix a second time after a few minutes to maximize the contact between the sample and the reagents.

4. STORAGE AND PRE-TREATMENT

DO samples may be stored in the dark for 24 hours, and under water for a maximum of 4 weeks after the reagents have been added and the fixation is completed. Bottles should be kept free of change of temperature.

5. ANALYTICAL PROCEDURES

The standard procedure for the determination of DO in water is the Winkler method in several modifications (e.g., Carpenter, 1965; Hansen, 1999; ICES, 1997).

If sensors for DO are used (at fixed stations or attached to the CTD), regular checks and calibrations have to be made by titration of water samples by the Winkler method. If sulphide is positive, discard the oxygen results.

6. ANALYTICAL QUALITY ASSURANCE

There is no Certified Reference Material for oxygen in water. The reference method is the properly performed Winkler method (Hansen, 1999). The quality assurance relies to a very high degree on good practice applied by experienced staff.

Essential procedures include:

1. calibration and identification of sample bottles and their respective stoppers;
2. calibration of volumetric flasks and dispensers;
3. control charts for reagent and titration blanks;

4. control charts of precision by replicate samples;
5. in case automated titration is used, check the accuracy of the addition of the titrand.

Replicate samples can be taken from the same sampler, but ideally from different samplers triggered at the same depth in deep water.

Blanks can be checked by adding double or triple amounts of reagents to identical samples.

Several publications contain descriptions of how the calibration should be performed and quality assurance can be achieved (WOCE, 1994; ICES, 1997). The demands of the COMBINE programme are exceeded by WOCE (World Ocean Circulation Experiment) standards.

Water stored with air contact for several weeks at a stable temperature can be used as a Laboratory Reference Material for control charts.

7. REPORTING OF RESULTS

DO concentrations should be reported in cm3/dm3 (ml/l) O2 at NTP and/or in % of saturation (Weiss, 1970).

The calculation of saturation also requires the *in situ* temperature known to ±0.1 EC and salinity within 0.2 (PSS 78). To allow conversion between different units, the sample temperature at the addition of the reagents should be reported, if significantly different from the *in situ* sample temperature.

Conversion factors for other units are:

- cm^3/dm^3 ´ 1.429 = mg/dm^3;
- mg/dm^3 ´ 0.700 = cm^3/dm^3;
- cm^3/dm^3 ´ 0.0893 = µM O_2;
- µM O_2 ´ 11.20 = cm^3/dm^3.

8. PRECISION

With the Winkler method, a repeatability of 0.1 % can be achieved in the upper concentration range.

9. REFERENCES

Carpenter, J.H. 1965. The Chesapeake Bay Institute technique for the Winkler dissolved oxygen method. Limnology and Oceanography, 10: 141–143.

Hansen, H.-P. 1999. Determination of oxygen. *In* Methods of seawater analysis, 3rd edition, pp. 75–89. Ed. by K. Grasshoff *et al*. Wiley-VCH, Germany.

ICES. 1997. Report of the Advisory Committee on the Marine Environment, 1997. ICES Cooperative Research Report, 222: 129–136.

ISO. 1983. Water quality; determination of dissolved oxygen; iodometric method. ISO 5813.

ISO. 1990. Water quality; determination of dissolved oxygen; electrochemical probe method. ISO 5814.

Weiss, R.F. 1970. The solubility of nitrogen, oxygen, and argon in water and sea water. Deep Sea Research, 17: 721–735.

WOCE. 1994. Operational Manual. Volume 3: The Observational Programme.

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ANNEX B-8 APPENDIX 3: RECOMMENDED EQUATIONS FOR THE CALCULATION OF SOLUBILITY AND SATURATION OF DISSOLVED OXYGEN IN SEA WATER

1. INTRODUCTION

When only physical processes are involved, the dissolved oxygen (DO) concentration in water is governed by the laws of solubility, i.e., it is a function of atmospheric pressure, water temperature, and salinity. The corresponding equilibrium concentration is generally called solubility. It is an essential reference for the interpretation of DO data. Precise solubility data, tables, and mathematical functions have been established (Carpenter, 1966; Murray and Riley, 1969; Weiss, 1970) and adopted by the international community (UNESCO, 1973). However, Weiss (1981) drew attention to an error in the international tables in which the values are low by 0.10 % since they are based on ideal gas molar volumeinstead of actual dioxygen molar volume. Later, the Joint Panel on Oceanographic Tables and Standards (JPOTS) recommended that the oxygen solubility equation of Benson and Krause (1984), which incorporated improved solubility measurements, be adopted and the tables updated (UNESCO, 1986). However, the UNESCO paper only referred to the equation that gives concentrations in the unit “micromole per kilogram”.

The present document repeats the equations that should be used for the computation of solubility values of dissolved oxygen, in various units, according to the UNESCO recommendation. These equations (so-called B & K equations) are directly taken from the paper of Benson and Krause (1984), who provided two equations for calculation either in “micromole per kilogram” or in “micromole per litre”, and the conversion factors for data in “milligram per litre” and “millilitre per litre”.

2. B & K SOLUBILITY EQUATIONS

Two equations of the same type have been established for DO solubility, to obtain concentrations either in “micromole per kilogram” or in “micromole per litre”.

Two points should be clear:

1. in these equations, the species under consideration is dioxygen (O2), therefore, “micromole” means “micromole of O2”;
2. 1 litre = 1 cubic decimetre, exactly.

The following symbols are used:

t : Celsius temperature (°C),

T : Kelvin temperature (K), T (K) = t (°C) + 273.15,

S : salinity on the Practical Salinity Scale 1978 (PSS78),

Cs : DO solubility concentration (the unit is mentioned using subscripts).

The equations can be expressed as follows:

$\ln Cs_{(\mu mol\ kg^{-1})} = A + B/T + C/T^2 + D/T^3 + E/T^4 - S \times (F + G/T + H/T^2)$,

and

$\ln Cs_{(\mu mol\ l^{-1})} = I + J/T + K/T^2 + L/T^3 + M/T^4 - S \times (N + P/T + Q/T^2)$.

The constants A to Q are the following:

Unit:

micromole per kilogram	micromole per litre
$A = -135.29996$	$I = -135.90205$
$B = +1.572288 \times 10^5$	$J = +1.575701 \times 10^5$
$C = -6.637149 \times 10^7$	$K = -6.642308 \times 10^7$
$D = +1.243678 \times 10^{10}$	$L = +1.243800 \times 10^{10}$
$E = -8.621061 \times 10^{11}$	$M = -8.621949 \times 10^{11}$
$F = +0.020573$	$N = +0.017674$
$G = -12.142$	$P = -10.754$
$H = +2363.1$	$Q = +2140.7$

Application domain: t = 0–40 °C; S = 0–40.

Cs is obtained as:

$Cs = \exp(\ln Cs)$,

i.e., when developing the equation:

$Cs_{(\mu mol\ kg^{-1})} = \exp\ [-135.29996 + (1.572288 \times 10^5) / (t + 273.15) - (6.637149 \times 107) / (t + 273.15)^2 + (1.243678 \times 10^{10}) /$

$(t + 273.15)^3 - (8.621061 \times 10^{11}) / (t + 273.15)^4 - S \times (0.020573 - 12.142 / (t + 273.15) + 2363.1 / (t + 273.15)^2)].$

and

$Cs_{(\mu mol\ l^{-1})} = \exp\ [-35.90205 + (1.575701 \times 10^5)/(t + 273.15) - (6.642308 \times 10^7) / (t + 273.15)^2 + (1.243800 \times 10^{10})$

$/ (t + 273.15)^3 - (8.621949 \times 10^{11}) / (t + 273.15)^4 - \times (0.017674 - 10.754 / (t + 273.15) + 2140.7 / (t + 273.15)^2)].$

3. SOLUBILITY DATA IN "MILLIGRAM PER LITRE" AND "MILLILITRE PER LITRE"

Solubility in **milligram per litre** is obtained from the value in micromole per litre by multiplying by the molar mass of dioxygen (O_2) and 10−3 for unit consistency, that is:

$Cs_{(mg\ l^{-1})} = Cs_{(\mu mol\ l^{-1})} \times 0.0319988.$

Solubility in **millilitre per litre** is obtained from the value in micromole per litre by multiplying by the molar volume of the gas at standard temperature and pressure (STP; 0 °C, 1 atmosphere). For that conversion, some data previously published refer to the molar volume (STP) of dioxygen (O_2; 0.0223916 ml per micromole), like those of Weiss (1970), while others refer to that of an ideal gas (0.022414 ml µmol−1), like those of the UNESCO tables and Benson and Krause (1984). Referring to exact O_2 molar volume:

$Cs_{(ml\ l^{-1})} = Cs_{(\mu mol\ l^{-1})} \times 0.0223916.$

4. OXYGEN SATURATION

The percentage of oxygen saturation in the water is calculated from the following equation at temperatures between 0–40 ºC and salinity between 0–40:

$$\%\,O_2 = \frac{O_2}{O_2'} \times 100$$

where:
O_2 is the oxygen concentration in the sample,

O_2' is the oxygen solubility in sea water at the same temperature and salinity, as the sample, calculated according to Section 2, above.

5. CHECKING THE CALCULATIONS

After programming the computer with the new equations, the calculations can be checked against the ICES oceanographic calculator, which uses the same equations. It is available on the ICES website at http://www.ices.dk/ocean.

6. ACKNOWLEDGEMENT

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ANNEX B-8 APPENDIX 4: TECHNICAL NOTE ON THE DETERMINATION OF HYDROGEN SULFIDE IN SEAWATER

1. INTRODUCTION

Hydrogen sulphide is a poisonous gas that readily dissolves in water. The sulphide is formed in stagnant waters, where the oxygen has been consumed by bacteria oxidizing organic matter to carbon dioxide, water, and inorganic ions. Sulphate-reducing bacteria then use the oxygen bound in sulphate ions as an electron acceptor while reducing the sulphate ions to sulphide. No higher life forms can exist in water containing hydrogen sulphide, and these areas are thus turned into oceanic deserts. Hydrogen sulphide in a water sample is easily detected by its characteristic smell, even at concentrations lower than those measurable with the method below.

2. METHODS

The reference method for sampling and determination of hydrogen sulphide in the Baltic area is the spectrophotometric method described in Fonselius *et al*. (1999). This book should be consulted for exact reagent compositions and procedures. For concentrations up to approximately 250 µM, the method by Fonselius *et al*. (1999) is recommended. Samples with higher concentrations can be treated in two different ways. Samples containing higher concentrations may be diluted after precipitation with a zinc acetate solution containing 2 g l^{-1} of gelatin (Grasshoff and Chan, 1971). This solution can be homogenized and diluted. However, higher levels of sulphide are better quantified using the method by Cline (1969).

3. SAMPLING

Samples are taken from ordinary hydrocast bottles immediately after the oxygen samples, using the same sampling technique (cf. 'Technical Notes on the Determination of Dissolved Oxygen in Sea Water'). If no oxygen is present, the sulphide samples should be taken first. Sulphide reacts with many metals, and the samplers should thus preferably be all-plastic. 50–100 ml oxygen bottles are recommended.

The two reagents are added simultaneously using piston pipettes or dispensers. The tips of the pipetting devices should be close to the bottom of the bottle. No air bubbles should be trapped in the bottle. Note that the amount of reagents added have to be adjusted according to the size of the bottles used. As concentrations rather than amounts are measured, no exact knowledge of the bottle volume is required.

Samples that cannot be analysed within 48 hours may be preserved with zinc acetate, which precipitates the sulphide as zinc sulphide. The preserved samples can be stored for a few months, if light and temperature changes are avoided. Prior to analysis, the reagents are added in the same way as for unpreserved samples. When the bottle is turned, the precipitate dissolves easily, and the colour develops normally.

4. ANALYTICAL PROCEDURES

Absorbances are measured in a spectrophotometer or a filter photometer at 670 nm. Measurements should be performed no sooner than 1 hour and no later than 48 hours after the reagent addition.

5. ANALYTICAL QUALITY ASSURANCE

The following QA elements must be satisfied:

The performance of the photometer with regard to absorbance and wavelength correctness must be checked and documented using a certified set of filters, or by an equivalent method.

The reagents must be calibrated using the procedure described in Fonselius *et al*. (1999). For measuring volumes in this procedure, only calibrated or class A glassware should be used. It is essential that the working solutions are freshly prepared, and that the sulphide content of the stock solution is measured, not calculated from the weighing of Na_2S (as Na_2S of sufficient purity is not available).

New reagents should be prepared at one-year intervals. The old reagents always must be checked against the newly prepared reagents in order to prove their stability.

No stable solutions are available for control charts. The difference between double samples in a control chart with zero as the reference line provides information on both precision and the validity of the subsampling. Ideally, the result (Sample 1 – Sample 2) should be evenly distributed around zero. Any deviations from this suggest subsampling problems.

6. REPORTING OF RESULTS

The concentration of hydrogen sulphide is usually expressed as µmol l^{-1} (µM), or in some cases as ml l^{-1} H_2S or as negative oxygen.

X µmol l^{-1} S^{2-} = $X \times 22.41 \times 10^{-3}$ ml l^{-1} H_2S

Y ml l^{-1} H_2S = $Y \times 10^{3} / 22.41$ µmol l^{-1} S^{2-}

Z µmol l^{-1} S^{2-} = $-0.044 \times Z$ negative oxygen units (ml l^{-1})

7. PRECISION

Using the method recommended in Fonselius *et al*. (1999), the analytical precision will be approximately ±1 µmol l-1.

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ANNEX B-9 TECHNICAL NOTE ON THE DETERMINATION OF NUTRIENTS

The commonly designated nutrients are inorganic nitrogen compounds (NO_3^-, NO_2^-, NH_4^+), phosphate (PO_4^{3-}) and silicate (SiO_4^{3-}). Total phosphorus (P_{tot}) and total nitrogen (N_{tot}) are also included because of their importance in relation to ecosystem analysis and budgets.

Nutrients in sea water are considered trace determinands and their analysis is liable to various sources of contamination. Sea water for nutrient analysis is usually collected from research vessels or ships of opportunity (e.g., ferry boats, fishing boats, coast guard or navy vessels). The reference method for measuring nutrients in the Baltic Sea (including storage and pretreatment) is Grasshoff (1976) 'Methods of Seawater Analysis'.

1. SAMPLE HANDLING

Special attention must be paid to possible nutrient sample contamination generated by the ship. Wastewater discharged from wash basins, showers and toilets contains significant amounts of phosphorus and nitrogen compounds and, therefore, can contaminate surface waters to be sampled. For this reason, the water sampler must be deployed far from wastewater outlets, even if no sewage is discharged at the time of sampling. Although most modern ships are equipped with special sewage tanks, they are often emptied at sea owing to a lack of appropriate reception facilities in ports. In addition, there are potential problems with kitchen garbage.

Mixing by the ship's propeller can disturb the natural distribution of the determinands in the surface layer, particularly as regards oxygen. These problems, including the exact location of the ship, should be considered along with the natural variability.

Phosphorus and nitrogen compounds are secreted from human skin. However, touching of the sampler and the sample bottles by hands does not cause problems unless the sample comes into contact with the outer surface of the sampler or sample bottle. This is something that should never happen since the outer surfaces cannot be kept free of contamination on-board a ship. In view of the potential for contamination, the analyst should preferably supervise the collection of samples. The attaching of bottles to a hydrowire or the preparation of a rosette and the subsequent removal and transport of samples to the ship's laboratory should be done by trained personnel.

The written instructions for the collection of samples should include the precautions to be taken when a sub-sample is transferred to the storage container. The instructions must include the details of the essential record of the sample: station location, station code, depth of sampling, date, time, etc., and the identity of the person responsible for sampling.

2. STORAGE OF SAMPLES

The stability of nutrients in seawater samples depends strongly on the season and the location from which the samples were taken. Nutrients in seawater samples are generally unstable. Grasshoff (1976) recommends that ammonia and nitrite are measured no later than one hour after sampling. Samples for nitrate, phosphate and silicate should preferably be analysed within six hours after sampling, and no later than ten hours. If for practical reasons samples cannot be analysed within these time limits, the corresponding data should be flagged if stored in databases, unless the storage method has been validated.

Samples should be stored protected from light and refrigerated. Plastic bottles must be used if silicate is measured. New sample bottles sometimes adsorb nutrients onto their walls. The new bottles, if necessary, should be cleaned with phosphate-free detergent, rinsed generously with distilled/deionized water and left filled with sea water containing nutrients for a few days. Then checks for adsorption of nutrients onto the walls or losses due to transformation to another chemical form should be carried out. Sample bottles should always be rinsed with the seawater sample from the sampler before they are filled. As regards ammonia determination, glassware for ammonia should always be cleaned with dilute hydrochloric acid.

If samples cannot be analysed within the above-mentioned time limits, the following methods of storage can be recommended.

Silicate	0-4 °C protected from light. Do **not** freeze (polymerization may occur).
Nitrite	Freezing or 0-4 °C protected from light. Do **not** acidify (rapid decomposition).
Ammonia	No known preservation methods are applicable.
Nitrate	Freezing.
Total nitrogen	Freezing or 0-4 °C protected from light. Do **not** acidify (enhanced risk of contamination).
Phosphate	Freezing or acidification.
Total phosphorus	Freezing or acidification with sulphuric acid with storage at 0-4 °C protected from light.

Addition of mercury or chloroform are alternative preservation methods for all nutrients except ammonia. These chemicals can affect the reaction kinetics, especially with automated methods, and this effect should be evaluated by the laboratory. The same chemical preservation of calibrants and quality controls can compensate for this effect. The use of mercury should be minimized and optimum disposal procedures should be ensured.

These preservation methods are all second choice to immediate analysis. They should, as mentioned, be validated by each laboratory, taking into account the concentration levels, storage time and environment, differences in sample matrices, and the analytical method of the laboratory.

Since no preservation method for nutrients can, at present, be recommended for general use, each laboratory must validate its storage methods for each nutrient before they are used routinely.

3. SAMPLE PRETREATMENT

Sea water contains microorganisms and other suspended matter of different composition. In some cases, these particles bias the measurement of the determinand in the soluble phase. The suspended matter can be removed either by filtration or centrifugation. Unnecessary manipulation of the sample should be avoided, but in particle-rich waters (e.g., coastal waters, during plankton blooms) filtration or centrifugation may become necessary. It is important that the procedure used for filtration/centrifugation has been validated.

For removing algae from the water sample, a GF/C filter is adequate. For work in open oceans with low concentrations of suspended matter, GF/F filters are considered suitable for suspended matter separation from open sea water. Filtration in closed systems with a neutral gas is recommended. Centrifugation is especially advisable for samples destined for ammonia determination.

If a sample containing particles is not filtered, the turbidity causes light scattering which can bias a colorimetric measurement. In this case, a turbidity blank should be carried out by measuring light absorption of the sample before adding the colour-forming reagents.

4. APPROPRIATE CHEMICAL ANALYTICAL METHODS

The choice of an analytical method should be based on the following criteria:

- the method should measure the desired constituent, i.e., be adequately specific, with accuracy sufficient to meet the data needs in the presence of interferences normally encountered in natural samples;
- the method should be sufficiently simple and rapid to permit routine use for the examination of large numbers of samples.

The reference methods used for manual nutrient measurements are described by Grasshoff (1976). Any changes to the reference methodology should be validated before use for routine work (see Annex C).

Apart from manual methods, various automated methods are in use, including different types of continuous flow analysis (CFA, steady state mode, and peak mode) or flow injection analysis (FIA or Reverse Flow Injection). The analyst has to be aware of the effects of the different analytical conditions in automated analysis which might affect accuracy.

5. CALIBRATION AND THE BLANK

Stock standard solutions should be prepared separately for each determinand using analytical grade reagents that can be pretreated to a precise stochiometric composition, e.g., by drying excess moisture. Reagents containing crystal water should be dried at a sufficiently low temperature in order not to remove the crystal water (the drying temperature is compound dependent). Stock standard solutions containing more than 1 mM are stable for long periods (up to one year refrigerated), but working calibration solutions must be prepared daily and used within hours of preparation.

Blank sea water may be prepared from a bulk sample of offshore surface sea water collected in summer, when the nutrients are at low or below-detection concentrations (Kirkwood, 1994). Blank sea water and reagents totally devoid of nutrients are, however, difficult to achieve, especially regarding the content of ammonia. Optimum handling precautions should be taken to minimize the content of nutrients to below approximately 10% of the measuring range. The concentrations of nutrients in the blank and reagents can be assessed by the standard addition method.

For ammonia analysis, the salinity of the samples affects the reaction kinetics, mainly due to the buffer effect of marine water, that results in a sub-optimum end pH. This effect can give biased results, especially with kinetically dependent automated methods. In the Baltic Sea, the salinity ranges from approximately 0 to 30, and therefore the size of this bias will be variable. This kinetic effect should be checked by standard addition, or by checking the pH of the reagent-sample mixture, which should be in the range between 10.5 and 11.

Whenever compensation for this bias is deemed necessary, one of the following methods is suggested:

- If all samples have the same salinity, calibrate using the addition of calibrants to one of the samples. In some situations, low-nutrient sea water can be prepared by aging and filtering natural sea water (as mentioned above).
- Empirical correction in accordance with the measured sample salinity or pH value.

For all photometric nutrient measurements differences in light refraction, caused by differences in the salt concentration, can give rise to shifts in blank/baseline values, especially in light-measuring cells with round windows. This can be compensated by using blanks and calibrants of the same salt concentration as the samples.

Particles can give rise to light-scattering effects that result in interferences in all photometric nutrient analyses. This bias can be avoided by measuring the sample before addition of the colour reagent, or by filtration or centrifugation where this does not cause contamination.

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ANNEX B-10 CHEMICAL ANALYSIS OF ANOXIC WATERS

1. INTRODUCTION

Anoxic seawater is generally found in enclosed areas with restricted water exchange. In most cases, a physical barrier (sill) as well as a pronounced density stratification will prevent oxygen from reaching the deeper parts of the sea area. Anoxic conditions will occur if the rate of oxidation of organic matter by bacteria is greater than the supply of oxygen. Anoxic waters are a natural phenomenon (Richards, 1965; Sarmiento *et al*., 1988), and anoxic waters have occurred during the geological history of the Baltic Sea (Jerbo, 1972; Hallberg, 1974). Recently, there have been some indications that eutrophication has increased the extent of the anoxic areas in, e.g., the Baltic Sea. Primary factors promoting anoxic conditions are stagnant conditions and density stratification (Gerlach, 1994).

Anoxic conditions result from several factors, for example, stagnation periods, inputs of organic material, and strong thermoclines. The bacterial production of sulphide starts in the sediments, where the bacteria find suitable substrates, and then expands into the water column.

When oxygen is depleted in a basin, bacteria first turn to the second-best electron acceptor, which in sea water is nitrate. Denitrification occurs, and the nitrate will be consumed rather rapidly. After reducing some other minor elements, the bacteria will turn to sulphate. The reduction of sulphate occurs according to the reaction:

$(CH_2O)_{106}(NH_3)_{16}H_3PO_4 + 53\ SO_4^{2-}\ 53\ CO_2 + 53\ HCO_3^- + 53\ HS^- +16\ NH_3 + 53\ H_2O + H_3PO_4$

If anoxic sea water becomes reoxygenized, sulphides will be oxidized to sulphate according to:

$HS^- + 2\ O_2\ HSO_4^-$

2. EXPERIMENTAL PROBLEMS ENCOUNTERED

2.1 HYDROGEN SULPHIDE

No ideal method for the determination of hydrogen sulphide in sea water exists today. The presently most widespread method, which is based on the formation of methylene blue and spectrophotometric measurement, although robust and simple to perform in the field, suffers from several weaknesses. The calibration of the reagents is an elaborate procedure requiring, among other things, the availability of oxygen-free water. Another obstacle is that Na_2S, which is used as the sulphide source in the calibration, is not available as a water-free compound of pro analysi quality. Furthermore, the stock and working solutions of sulphide made up for the calibration are extremely unstable, and the working solution will change concentration substantially in a short time (1-2 hours). Sulphides that are commercially available in the pure form generally suffer from extremely low water solubility, and thus are not suitable for this kind of work.

Sampling is carried out using the same technique as for oxygen, and thus is not a general problem for the trained marine scientist. If the samples will not be measured within acceptable time limits, they are generally preserved with zinc acetate (to form zinc sulphide) prior to analysis. The relatively poor precision of the method, often 5-10 %, could probably be attributed to the combined effects of all steps in the sampling and sample pretreatment procedure.

Validation of results is very difficult, since there are no certified reference materials (CRMs) available for sulphide in sea water. The parameter is very rarely included in interlaboratory comparison exercises, mainly due to problems in withdrawing multiple samples with the same sulphide concentration from one sample container.

Very high concentrations of sulphide in certain unusually stagnant areas will cause problems. In some cases, the absorption of the sample will lie outside the working range of the spectrophotometer. Dilution of the sample is possible, but will undoubtedly introduce more uncertainty into the measurement.

2.2 OXYGEN

In cases where sensors are used for measuring the oxygen content of the water column, anoxic layers will poison the sond and quickly deteriorate its performance. The best way to avoid this is not to lower the sond into any anoxic water layers, which will make it rather impractical in many areas of the Baltic Sea.

2.3 SALINITY

The combined effect of mineralization of organic matter and accumulation of nutrients may cause a shift in the salinity measurements by conductivity by no more than 0.02 PSU (Grasshoff, 1975). This difference is caused by differences in ionic composition between the sample and the standard sea water used for calibrating the salinometer. Practical problems may occur, possibly due to particles in the water, causing a certain instability in the conductivity reading. After running a series of anoxic samples, the salinometer has to be rinsed carefully with deionized water and ethanol.

2.4 NUTRIENTS

Of the inorganic nutrients, phosphate is the compound giving rise to special problems in anoxic waters. Both the natural turbidity of anoxic samples and the influence of the sulphide present on the colour reaction may cause biased results or results of low precision. The reduction of the phosphomolybdenum complex to the blue complex is catalysed by antimony. Sulphide could react with the antimony ions to form a yellow-greenish turbidity, which disturbs the photometric measurement (Nehring, 1994). In addition, colloidal sulphur may be formed when the acid molybdate reagent is added (Grasshoff *et al*, 1983). These problems can be overcome by removing the sulphide by oxidation with bromine or degassing under a stream of nitrogen. The wavelength at maximum absorbance of the colour complex also coincides with strong absorption caused by turbidity. The safety limit of interferences has previously been reported to be 2 mg/l of sulphide for phosphorus and ammonia.

In anoxic waters, nitrate will be reduced to ammonia, disappearing rapidly as the oxygen disappears. The presence of small amounts of nitrate in anoxic waters is possible, but only in layers influenced by rapid mixing with overlying water masses (Grasshoff, 1975). In order to find measurable quantities of nitrate in these waters, the speed of mixing has to be higher than the speed of denitrification of the nitrate. The presence of nitrate in anoxic waters should otherwise be treated with care, since it is probably a result of oxidation of ammonia in the sample upon contact with the atmosphere when sampling.

Nitrite is normally not present in detectable amounts in anoxic waters, as it has been reduced to ammonia. However, nitrite has been observed in the presence of large quantities of ammonia in anoxic waters, possibly as a result of rapid oxidation upon contact with the atmosphere. Sulphide has been reported to interfere with the nitrite measurements, and should (if possible) be removed from the sample.

Ammonia accumulates in the anoxic water and remains fairly stable. The oxidizer for the development of the indophenol blue, hypochlorite, is partly consumed by the oxidation of sulphide. It may thus be necessary to increase the amount of hypochlorite added to the sample in strongly sulphidic waters (Nehring, 1994). In all particle-rich waters, including anoxic waters, it is necessary to measure and subtract the seawater blank.

Silicate accumulates in stagnant waters, and the high concentrations make the determination less sensitive to interferences. Sulphide concentrations up to approximately 150 µmol/l will not affect the formation of the colour complex for the determination of silicate, even if the silicomolybdic acid may partly be reduced. At higher concentrations, it may be advisable to remove the sulphide or to dilute the sample.

2.5 TOTAL PHOSPHORUS AND NITROGEN

The hydrogen sulphide is oxidized to elemental sulphur or sulphate by the oxidation reagent used in the analysis of total phosphorus and nitrogen and thus does not interfere directly. In extreme cases, with extraordinarily high sulphide concentrations, all of the sulphide may not be oxidized and may possibly create a problem (see Section I.2.4, above). A high particle content may, as for inorganic phosphorus, give rise to blank problems. In the analysis of total phosphorus, the oxidation and hydrolysis of phosphorus compounds may not be complete, especially when both nitrogen and phosphorus compounds are combusted simultaneously in alkaline media (cf. Koroleff, 1983). It has furthermore been demonstrated that the oxidation of organic phosphorus compounds using potassium peroxodisulphate ($K_2S_2O_8$) is an unsuitable method in the presence of dissolved iron, possibly due to the formation of iron(III) phosphate during the oxidation process (Ichinose *et al.*, 1984).

2.6 ORGANIC CARBON AND NITROGEN

The most modern technique for determining the levels of particulate organic carbon or nitrogen (POC/PON) in sea water starts with filtration of the water through 0.45 µm filters. The filters, with their content of particulate matter, are combusted in an oxygen-rich atmosphere to produce gaseous CO_2 and NO_2. The analysis is very straightforward and robust, and there seem to be no problems (theoretical or practical) involved in the analysis of samples originating from anoxic waters. The samples are characterized by high levels of POC/PON, since anoxic waters are rich in particles, detritus, and other non-living organic material.

2.7 HALOGENATED ORGANIC CONTAMINANTS

The methods used for the determination of halogenated organic contaminants in water are based on extraction of the contaminants from the sea water matrix followed by gas chromatographic separation and some kind of detection. For work in the open sea, the electron capture detector (ECD) is the preferred choice due to its selectivity and sensitivity. The electron capture detector is very selective towards elements with large electron-capturing capability, for example, the halogens. However, the detector also has a certain response towards oxygen and sulphur, and will thus be disturbed by the occurrence of compounds containing these elements. This may give rise to great difficulties in detecting and quantifying, in particular, volatile halogenated compounds in anoxic waters (Krysell *et al*., 1994).

Anoxic environments will cause a breakdown of many halogenated compounds, complicating the distribution patterns and lowering their concentrations. Chlorophenolic compounds have been shown to dehalogenate in anoxic sediments (Abrahamsson and Klick, 1989) and the breakdown of carbon tetrachloride has been observed in anoxic waters (Krysell *et al*., 1994; Tanhua *et al*., 1996).

2.8 METALLIC TRACE ELEMENTS

The concentrations of certain metal ions, most importantly copper (Cu), lead (Pb) and zinc (Zn), and to some extent cadmium (Cd), decrease rapidly in anoxic waters due to the low solubility of their corresponding sulphides. The relatively lower concentrations that follow cause problems mainly when it comes to the correction for blanks, since the blanks become disproportionately high.

In basins with very high sulphide concentrations, elemental sulphur may under some circumstances cause problems in the analysis, since it will be extracted into the same fraction as the metals.

Methods involving ion exchangers for sample work-up and concentration may give a very low yield unless the strength of the ion exchange resin can match that of the strongly bound metal sulphides.

2.9 PH

Electrode deterioration may occur, because in sulphidic waters sulphide will react with the Ag/AgCl electrode, considerably shortening the lifetime of the electrode.

2.10 ALKALINITY

There are no experimental problems, but anoxic waters contain an organic fraction which contributes to the alkalinity. The nature of this organic fraction is still under discussion; it has been suggested that it consists of amino acids or humic substances. Since the true nature of the organic fraction has not been determined, there are still doubts about how it fits into the definition of alkalinity and how the data should be treated and normalized. When determining alkalinity in sulphidic waters, it is more reliable to use a titration method with an indicator because sulphide will react with the Ag/AgCl electrode used in potentiometric titration.

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ANNEX B-11 TECHNICAL NOTE ON THE DETERMINATION OF HEAVY METALS AND PERSISTENT ORGANIC COMPOUNDS IN SEAWATER

- **Appendix 1**. Technical note on the determination of trace metals (Cd, Pb, Cu, Co, Zn, Ni, Fe) including mercury in seawater
- **Appendix 2**. Technical note on the determination of persistent organic compounds in seawater

ANNEX B-11, APPENDIX 1. TECHNICAL NOTE ON THE DETERMINATION OF TRACEMETALS (CD, PB, CU, CO, ZN, NI, FE), INCLUDING MERCURY, IN SEAWATER

Introduction

General techniques which address the questions of water sampling, storage, filtration procedures and determination of trace metals in natural sea water are described by Sturgeon and Berman (1987) and Gill and Fitzgerald (1985, 1987).

For the determination of mercury in sea water, the chemical species of this element are of importance. Therefore, a differentiation between the several Hg species, including ionic, volatile, dissolved (organic) complexes or particulate adsorbed Hg, has to be considered during sample preparation.

Several definitions of mercury compounds are common (Cossa et al., 1996, 1997), for example:

Reactive mercury (HgR): A methodologically defined fraction consisting mostly of inorganic Hg(II).

Total mercury (HgT): Mercury content of an unfiltered sample, after digestion with an oxidizing compound (e.g., K MnO4).

Total dissolved mercury: Mercury content of a filtered sample, after digestion with an oxidizing compound (e.g., K MnO4).

Dissolved gaseous mercury (DGM): This includes elemental mercury (Hg), monomethylmercury (MM-Hg) and dimethylmercury (DM-Hg).

1. CLEAN LABORATORY; CLEAN BENCHES

Particles are everywhere, including dust in the air or on clothes, hair or skin. Owing to the clothes, the person who is working with the samples for trace metal analysis is the main source of contamination because this person is a particle producer. One of the most important things during sample pretreatment for trace metal analysis is to eliminate particles that can contaminate the samples or the sample containers from the laboratory environment.

The best way to eliminate most of this contamination is to work under a laminar flow box with a laminar horizontal flow (sample protection). Recommended conditions for a 'clean bench' or a 'clean lab' are class 100 (US Norm) which means that there are still about one hundred particles present per cubic foot or class 3 (DIN-Norm), which equals 3000 particles per m3 (corresponding to class 100 US Norm).

2. PREPARATIONS

Chemicals

High purity water (e.g., 'Milli-Q water', 18 M cm-1) freshly prepared, is termed 'water' in the following text.

A sub-boiling quartz still is recommended for the distillation of highly purified acids and solvents. A teflon still is recommended for the distillation of HF.

Amalgamation (filtration of oversaturated solutions with goldnet) and volatilization (bubbling with ultrapure argon) are effective methods to purify (clean) chemicals and solutions for mercury analysis.

In order to avoid contamination problems, all plastic ware, bottles and containers must be treated with acids (HCl or HNO3) for several weeks and then rinsed with water and covered in plastic bags until use.

The following procedures (Patterson and Settle, 1976) are suggested:

Laboratory ware

Store in 2M HCl (high purity) for one week, rinse with water, store in water for one week and dry under dust-free conditions (clean bench).

Samplers and bottles

Sampling devices: Fill with 1% HNO3 (high purity), store at room temperature for three weeks, and rinse with water .

Teflon/quartz bottles: Store in warm (40 C ±5 C) 1:1 diluted HCl for one week. Then rinse with water and store with 1M HNO3 (high purity) until the final use (a minimum of three weeks).

Modified cleaning procedures are required for mercury. Glass containers (borosilicate, quartz) used for the collection and storage of samples for the determination of mercury are usually cleaned using an oxidizing procedure described by Sturgeon and Berman (1987). Bottles are filled with a solution of 0.1 % KMnO4, 0.1% K2S2O8 and 2.5 % HNO3 and heated for 2 hours at 80 C. The bottles are then rinsed with water and stored with 2 % HNO3 containing 0.01 % K2Cr2O7 or KMnO4 until ready for use.

Filters

Polycarbonate filters (e.g., Nuclepore) (0.4 m, 47 mm diameter) are recommended for trace metals except mercury. Store the filters in 2M HCl (high purity) for a minimum of three weeks. After rinsing with water, store for one more week in water.

For the determination of mercury, glass microfibre filters (GF/F grade, Millipore type) and teflon filters are recommended for the filtration of natural water samples. Cleaning of these filters is comparable to the procedure used for polycarbonate filters. For GF/F filters, an additional drying step has to be considered (450 C for 12-24 hr) to volatilize gaseous mercury. This procedure is described in detail by Queremais & Cossa (1997).

If trace metals in suspended particulate matter (SPM) are to be determined, filters have to be placed in precleaned plastic dishes, dried in a clean bench for two days, and stored in a desiccator until they are weighed using an electronic microbalance with antistatic properties. Each filter has to be weighed daily for several days until the weight is constant. The same procedure for drying and weighing should be applied to the filters loaded with SPM (Pohl, 1997).

3. SAMPLING AND SAMPLE HANDLING

The basis for the reliable measurement of extremely low concentrations of trace metals in sea water is a well-performed sampling to avoid contamination risk from the ship. Careful handling is recommended because copper and tin are still the main substances used in antifouling paints on ships and there is also a risk of contamination by zinc (anodes of the ship), iron or lead.

In coastal and continental shelf waters, samples are collected using 30 l teflon-coated GO-FLO (General Oceanics, close-open-close system) bottles with teflon O-rings deployed on Kevlar or on a Hostalen coated wire. Niskin bottles deployed on rosettes using standard stainless steel hydrowire are also acceptable. For surface waters, an all-teflon MERCOS-Sampler (Hydrobios) could be chosen.

PVC gloves should be worn during subsampling into the precleaned quartz or teflon bottles (teflon has an extra low content of trace metals). Subsampling should be carried out in a clean lab or a clean-lab container, if available.

Pumping of samples using peristaltic or teflon piston pumps must be carried out using precleaned silicon- or teflon-lined tubes.

In the absence of clean-lab conditions, sampling and sample handling must be carried out in a closed system, or contamination cannot be avoided.

For mercury analysis, it should be noted that the integrity during sampling and storage may be jeopardized by the addition of mercury to the sample as well as by unexpected losses owing to volatilization.

4. FILTRATION PROCEDURE

In the environmental and geochemical scientific community concerned with water analysis, it has generally been accepted that the term 'dissolved' refers to that fraction of water and its constituents which have passed through a 0.45 m membrane filter. This is an operationally defined fraction. Coastal and shelf water samples have to be filtered to eliminate particles from the water. A number of metal species pass through this filter pore size, including metals bound to colloids or clays or to humic, fulvic, amino, and fatty acids.

To prevent desorption of metal ions from particle surfaces or from biological degradation of SPM, separation between the dissolved phase and the particulate phase has to be done immediately after sampling by filtering the water through a 0.45 m polycarbonate filter. This procedure should be carried out under clean conditions (clean benches are recommended on board the ship).

If metals in both the dissolved and particulate phases are to be analysed, pressure filtration with nitrogen is recommended. After filtration the filter should be rinsed with high purity isotonic solution to remove sea salt residues. Only a few millilitres are necessary because a change of pH could cause desorption of metal ions from the particles. In pumping systems, on-line filtration is possible.

5. STORAGE OF SAMPLES

To avoid wall adsorption of metal ions, 1.5 ml HNO3 or HCl (high purity) should be added per litre of seawater sample immediately after filtration for acidification to pH 1.0-1.6. The sample containers should be stored in plastic bags under controlled environmental conditions. The filters should be stored in plastic dishes at -18 C or below. Under these conditions, both water samples and SPM on filters can be stored for at least one year.

Special consideration must be given to samples destined for Hg determinations. It is necessary to add either oxidants (Cr2O72-) in addition to acidification or complexing agents (cysteine) to neutral or alkaline samples to prevent Hg losses during storage.

6. SAMPLE PRETREATMENT

Water samples

Depending on the expected concentration range (10-7-10-9 gkg-1) of trace metals (dissolved) in Baltic Sea water and because of the salt matrix interfering during the measurement process, preconcentration techniques and/or the elimination of sea salt has to be carried out prior to the analytical measurement. Detailed method information is available in the open literature (e.g., Danielsson et al., 1978; Kremling et al., 1983; and Pohl, 1994).

Filters

Different methods to analyse the material on the filter are described by Hovind and Skei (1992) and Loring and Rantala (1991). Pressure decomposition with an acid mixture (HCl, HNO3, HF) is recommended. If the silica content is high due to diatoms, the HF concentration should be increased accordingly. If the organic content increases, it is advisable to work with perchloric acid.

Depending on the digestion system used (high pressure autoclave, microwave digestion, wet ashing in an open system, or dry ashing), the completeness of the digestion is a function of temperature, time, digestion material and pressure, and has to be tested and validated in pilot studies with (certified) reference materials (see the detailed remarks in Annex B-7, Section 4.3).

Digestion of samples for mercury analysis must always be carried out in a closed system to prevent losses by evaporation.

7. INSTRUMENTATION

For the analytical measurements, several analytical techniques can be used, such as GFAAS (graphite furnace atomic absorption spectrometry), electrochemical methods, ICP-MS (inductively coupled plasma-mass spectrometry), ICP-AES (inductively coupled plasma-atomic emission spectrometry), or total-reflection X-ray fluorescence (TXRF).

Because of the very low mercury concentrations in sea water, the most widely used technique for mercury is the cold vapour technique (reduction of mercury with $SnCl_2$ to elemental Hg) and preconcentration of mercury by amalgamation on a gold trap. This is followed by atomic absorption spectrometry or by atomic fluorescence spectrometry, with detection limits adequate for the purpose. In the case of anoxic (sulfur-containing waters), see Annex B-11.

8. QUALITY CONTROL

The internal quality control is described in Chapter B.5 of the Manual.

Blank

Particularly in the case of trace metal analysis, with high contamination risks at each step of the analytical work, a satisfactory blank control is necessary. Therefore, it is important to control the blank daily, for reproducibility and constancy over a longer time. The blank should include all analytical pretreatment procedures, including the addition of the same quantities of chemical substances as for the sample.

Calibration

For calibration purposes, single element standard stock solutions at a concentration of 1000 mg dm^{-3}, purchased from a qualified manufacturer, should be available. Preparation date and concentration should be marked on the bottle. From this stock solution, a multi-element working standard solution can be prepared using dilute HCl or HNO_3 as required (normally 1M acid is used).

Traceability can be ensured by the use of CRMs or participation in intercomparison exercises.

The working standard should be prepared from the stock standard solution for every batch of samples and kept no longer than two weeks. Precleaned teflon containers are preferable for storage.

To evaluate effects from the matrix, the method of standard addition can be used, particularly in connection with the analytical method of voltammetric stripping. For other techniques, the method of standard addition should generally be used with care (Cardone, 1986a, 1986b).

Reference materials

Owing to problems in defining the blank, the use of a low-concentration CRM is important. Regular participation in intercomparison exercises should be considered mandatory.

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ANNEX B-11 APPENDIX 2: TECHNICAL ANNEX ON THE DETERMINATION OF HEAVY METALS AND PERSISTENT ORGANIC COMPOUNDS IN SEAWATER

TECHNICAL NOTE ON THE DETERMINATION OF PERSISTENT ORGANIC POLLUTANTS IN SEAWATER

1. INTRODUCTION

These guidelines concentrate on the sampling and extraction of lipophilic persistent organic pollutants from seawater and special aspects of the sampling matrix. This group of pollutants comprises the group of polycyclic aromatic hydrocarbons (PAHs) and chlorinated hydrocarbons (e.g., HCH, HCB, DDT group, chlorinated biphenyls (PCBs)).

For general aspects and the analytical determination, reference is made to the following guidelines:

- "Determination of Polycyclic Aromatic Hydrocarbons (PAHs) in Sediments: Analytical Methods", ICES ACME Report 1997;
- "Guidelines for the determination of chlorobiphenyls in sediments: Analytical methods", ICES ACME Report 1996;
- "Determination of Polycyclic Aromatic Hydrocarbons (PAH)s in Biota", ICES ACME Report 1998; and
- Annex B-14 (these Guidelines).

As the same analytical methods can be used for the determination of lipophilic pollutants in extracts of water samples as are used for extracts of sediments, it is felt that it is a useful way to unify analytical procedures to refer to these publications only.

However, it should be taken into consideration (e.g., for calibration) that the relative concentrations of the individual pollutants are generally quite different in water and sediment samples. The concentration patterns of the pollutants are mainly influenced by their polarity which can be expressed by their octanol/water coefficient (log K_{ow}; Kow = Concentration in octanol phase / Concentration in aqueous phase). Thus, in water samples the more hydrophilic compounds with log Kow values of 3 to 4 predominate (e.g., 2- and 3-ring aromatics and HCH isomers), while in sediments and biota the pollutants with log Kow values >5 are enriched (4- to 6-ring aromatics, DDT group, PCBs).

These guidelines provide advice on lipophilic persistent organic pollutant (POPs) analyses in total seawater with a log KOW > 3. The analysis of POPs generally includes:

1. • sampling and extraction of the water;
2. • clean-up; and
3. • analytical determination.

The extraction of the POPs simultaneously enables an enrichment of the analytes. Because of the very low concentration range of 10 pg l^{-1} to 10 ng l−1, the enrichment of the contaminants is a very important step in the procedure. Extraction and enrichment can be done by solid phase extraction (SPE) or liquid-liquid extraction (LLE).

Determination depends on the chemical structure of the compounds. PAHs can be determined by high performance liquid chromatography (HPLC) with fluorescence detection or gas chromatographic (GC) separation with flame ionization (FID) or mass spectrometric (MS) detection (Fetzer and Vo-Dinh, 1989; Wise et al., 1995). Chlorinated hydrocarbons are generally analysed by gas chromatographic (GC) separation with electron capture detectors (ECD) or mass spectrometric (MS) detection.

All steps of the procedure are susceptible to insufficient recovery and/or contamination. Therefore, regular quality control procedures must be applied to check the performance of the whole method. These guidelines are intended to encourage and assist analytical chemists to critically reconsider their methods and to improve their procedures and/or the associated quality control measures, where necessary.

These guidelines are not intended as a complete laboratory manual. If necessary, guidance should be sought from specialized laboratories. Whichever procedure is adopted, each laboratory must demonstrate the validity of each step of its procedure. In addition, the use of a second (and different) method, carried out concurrently to the routine procedure, is recommended for validation. The participation in analytical proficiency tests is highly recommended.

2. SAMPLING AND STORAGE

Plastic materials must not be used for sampling and storage owing to possible adsorption on the container material or contamination. Especially the very lipophilic compounds (4- to 6-ring aromatic hydrocarbons, DDT, PCBs) tend to adsorb on every surface. Therefore, the seawater samples should not be stored longer than 2 h and should not be transferred into other containers before extraction. It is highly recommended to extract the water sample as soon as possible after sampling and to use as little manipulation as possible. It is recommended that sampling and extraction should be done in the same device. Extracts in organic solvents are less susceptible to adsorption onto surfaces.

3. BLANKS AND CONTAMINATION

In many cases, the procedural detection limit is determined by the blank value. In order to keep the blank value as low as possible, the compounds to be analysed or other interfering compounds should be removed from all glassware, solvents, chemicals, adsorption materials, etc., that are used in the analysis. The following procedures should be used:

- Glassware should be thoroughly washed with detergents and rinsed with an organic solvent prior to use. Further cleaning of the glassware, other than calibrated instruments, can be carried out by heating at temperatures > 250 °C.

- All solvents should be checked for impurities by concentrating the amount normally used to 10 % of the normal end volume. This concentrate is then analysed in the same way as a sample by HPLC or GC and should not contain significant amounts of the compounds to be analysed or other interfering compounds.

- All chemicals and adsorption materials should be checked for impurities and purified (e.g., by heating or extraction), if necessary. Soxhlet thimbles should be pre-extracted. Glassfiber thimbles are preferred over paper thimbles. Alternatively, full glass Soxhlet thimbles, with a G1 glass filter at the bottom, can be used. The storage of these supercleaned materials for a long period is not recommended, as laboratory air can contain PAHs that will be adsorbed by these materials. Blank values occurring despite all the above-mentioned precautions may be due to contamination from the air. The most volatile compounds will usually show the highest blanks (Gremm and Frimmel, 1990).

As the concentrations of the PAHs and chlorinated hydrocarbons in seawater are very low, possible blank and contamination problems might be even more difficult to control than with sediment samples. Therefore, it is recommended to rewash all equipment (vials, pipettes, glass bottles) with solvent just before use. If possible, critical steps should be done in a clean bench.

The more volatile compounds (especially naphthalene and phenanthrene) show the largest blank problems.

4. PRE-TREATMENT

For the extraction of whole water samples, no pre-treatment is necessary.

If the suspended particulate material (SPM) will be analysed separately from the solute phase, a phase separation has to be done. Because of the necessary additional manipulation step, this is a difficult operation which affords a number of additional quality control procedures (adsorption losses, contamination problems). There are two possible ways for phase separation: filtration and centrifugation.

Filtration is done by GF/F glass fibre filters. As flat-bed filters have a very limited capacity, the use of coiled glass fibre filters is recommended for volumes larger than 10 l and water samples with high amounts of suspended matter. A pump is necessary to force the water through the filter.

Centrifugation needs a high volume centrifuge which must be operable onboard a ship. Such centrifuges with a throughput of 1 m^3 $h^{-1}$and more are commercially available and used for sampling SPM; however, they are expensive and generally not a standard equipment. For centrifugation, blanks and adsorption problems have to be controlled as well as the separation efficiency.

The sampled SPM is analysed like a sediment. The solute phase is analysed like the whole water sample.

Validation of the phase separation procedures is very difficult; thus, it might be wise to analyse the whole water sample for monitoring purposes and to determine separately only the amount of SPM in the water for reference or normalization purposes.

5. EXTRACTION

The volume of the water sample is the most important parameter which influences the limit of determination of the method. As POP concentrations down to 10 pg l^{-1} and less are observed in seawater, large water volumes of 10 l to 100 l have to be sampled and extracted. Large volumes are required not only to obtain a sufficiently high detector signal, but also to discriminate from blank problems.

Principally, there are two different extraction principles in current use: solid phase extraction (SPE) and liquid-liquid extraction (LLE). Unfortunately, the two procedures do not always yield comparable results, as the physical extraction principles are quite different (Sturm et al., 1998, Gomez-Belinchon et al., 1988).

SPE has the advantage of being able to extract very large water volumes (up to 1000 l) and to incorporate a phase separation to obtain separate samples for SPM and the solute phase. The drawbacks of the method are a longer sampling time demand, a more complex instrumentation, and problems with validation and control of the extraction efficiency.

LLE has the advantage that it can be easily validated and controlled, as internal standards can be added before extraction. Also, standard addition techniques can be used for accuracy testing. As LLE is a classical extraction technique, a great deal of experience is available and the robustness of the principle is proven. The limitation in sample volume is only relative, as techniques have been described for sampling 10 l and 100 l on a routine basis (Gaul and Ziebarth, 1993; Theobald et al., 1990). It has been shown that a sampling volume of 100 l is sufficient for nearly all monitoring tasks.

Because of the robustness of the method, there is a preference LLE for routine monitoring purposes for all lipophilic organic contaminants.

5.1 Solid phase extraction

The extraction device consists of a filter holder, an adsorption column filled with an adsorbing material (e.g., XAD resin, C18 modified silica gel), a pump which forces the water sample through the column, a flow meter, an electronic control unit, and a power supply. Sampling can be done either by deploying the whole extraction device into the water (in situ pumping) or by pumping the water with a separate pump onboard a ship and then through the extraction device. A suitable in situ system is described in detail in Patrick et al. (1996). After sampling, the columns are stored at 4 °C and the filters at –20 °C.

The adsorption column is eluted with an organic solvent (acetone or acetonitril). Prior to the extraction, internal standards are added to the solvent. The extract obtained is pre-cleaned and analysed.

Analytical procedures for the use of XAD-2 adsorption resins are published by the IOC (1993), Ehrhardt (1987), and Bruhn and McLachlan (2001).

Although the SPE technique has many advantages, one has to be aware of some problems. Especially for large volume sampling, validation of the method is extremely difficult and has not yet been achieved. Some publications have shown that the extraction efficiency is dependent on, e.g., the amount and kind of humic substances which can complex lipophilic compounds (Johnson et al., 1991; Kulovaara, 1993; Sturm et al., 1998).

5.2 Liquid-liquid extraction

The decision to sample 10 l, 20 l, or 100 l of water depends on the anticipated concentrations of the compounds to be analysed in natural samples . For remote sea areas with expected concentration of 10 pg l−1 or less, a volume of 100 l is recommended. The technique and principle are identical for all volumes, only the sampling bottle and the equipment are different. Details of the sampling and extraction techniques are described in Gaul and Ziebarth (1993) for the 10 l sampler and in Theobald et al. (1990) for the 100 l sampler.

The all-glass bottle sampler fixed in a stainless steel cage is lowered by a hydrographic wire down to the sampling depth and opened under water. After filling, the sampler is brought on deck of the ship and immediately extracted with a non-polar solvent such as pentane or hexane. Prior to extraction, a solution with appropriate internal standards (e.g., deuterated PAHs, e-HCH, PCB 185) is added to the water sample. After phase separation, the organic extract is dried with Na_2SO_4 and carefully concentrated to about 1 ml in a rotary evaporator. Further evaporation is done under a gentle stream of nitrogen.

Extreme care has to be taken to ovoid contamination during sampling, extraction, and work up. Blank samples must be taken in every sampling campaign; this can be done, e.g., by rinsing the cleaned sampling bottle with the extraction solvent and treating this extract like a normal sample. The sampling bottle must be cleaned with detergent, water, and organic solvents (acetone and hexane or pentane) before use. After using in open sea areas, it can be of advantage not to perform the whole cleaning/washing procedure but just to use the sampler directly after emptying the glass bottle from the extracted previous water sample.

Extracts should be stored in the refrigerator and in the dark.

6. CLEAN-UP

Interferences from matrix compounds in seawater samples are generally smaller than in sediment or biota samples. Nevertheless, the crude extracts require a clean-up before chromatographic separation and determination can be done. The clean-up is dependent on the compounds to be analysed, the sample, the determination method used, and the concentration range to be analysed. For all GC methods, it is essential to remove polar and non-volatile compounds in order to protect the GC column from rapid destruction. A detection system with low selectivity (eg., GC-FID) needs a far better clean-up than a detector with a high selectivity such GC-MS or even GC-MS/MS. HPLC with fluorescence detection (for PAH analyses) has a relative high selectivity but the method will fail if petrogenic aromatic compounds (from an oil spill) are present in the sample. GC-ECD (for chlorinated compounds) has a high selectivity but some interferences (e.g., phthalate esters) may disturb the detection; therefore, for GC-ECD a good clean-up is necessary as well.

A clean-up procedure for this is presented here that uses short silica gel chromatography columns that can be applied with any determination technique: HPLC, GC or GC-MS. The method is simple and is sufficient in most cases of PAH and chlorinated hydrocarbon determinations in seawater (ICES, 1996, 1997, 1999).

A 3 ml glass column with glass fibre frit (commercially available for SPE) is filled with 500 mg silica gel (dried for 2 h at 200° C) and subsequently washed with 30 ml CH2Cl2 and 30 ml hexane. The hexane sample extract (concentrated to 500 µl) is applied on top of the column and eluted with 5 ml CH2Cl2/hexane (15/85 v/v) and then with 5 ml of acetone. Fraction 1 contains all lipophilic compounds of interest (PAHs and all chlorinated hydrocarbons (from HCB to HCH)); this fraction can be used for GC-MS determination after concentration to 50–300 µl.

If the water sample has been extremely rich in biological material (algae) or if detection limits far below 10 pg l^{-1} are requested, additional clean-up (HPLC, GPC) might become necessary.

7. CROMATOGRAPHIC DETERMINATION

Details for the chromatographic determinations are comprehensively described in the 1996 ACME report (ICES, 1996) for chlorobiphenyls in sediments (GC-ECD and GC-MS), the 1997 ACME report (ICES, 1997) for PAHs in sediments (HPLC-Fluorescence detection, GC-FID and GC-MS), and the 1998 ACME report (ICES, 1999) for PAHs in biota (HPLC and GC-MS).

As the cleaned extracts from the seawater samples can be analysed in the same way as the extracts from sediments and biota, the above guidelines can be used. When a GC-MS system can be used, all compounds can be determined in one single GC analysis; if not, the samples have to be analysed separately for PAHs (HPLC-F, GC-FID) and chlorinated hydrocarbons (GC-ECD).

7.1 Gas chromatography-mass spectrometry

As GC-MS has the advantage of being both very selective and quite universal, it is strongly recommended to use GC-MS as the determination method. It especially has the advantage that both PAHs and chlorinated hydrocarbons can be determined in one single analysis. This is not possible with any of the other techniques.

Because of the sensitivity required, the mass spectrometric detector must be operated in the selected ion mode (SIM). By this, absolute sensitivities in the range of 1 pg to 10 pg can be achieved for most compounds. Ion-trap instruments can be operated in full-scan mode and are in principle as sensitive as quadrupole detectors; however, with real samples and matrix underground they can lose considerably sensitivity.

With GC-MS, detection limits of 5–30 pg l^{-1} can be reached with water sample volumes of 10 l to 100 l. In most cases, it is not the absolute signal strength of the detector which limits the detection; therefore, the injection of a larger aliquot of the analysis solution would not improve it. For some compounds, blank values are the limiting parameter (especially naphthalene and phenanthrene and, to a lesser extent, other PAHs); for this, only a larger sample volume can improve the detection limits. Many other compounds do not exhibit blank problems, if appropriate care is applied; for these, matrix noise often limits the detection. For such situations, only a better clean-up (e.g., HPLC, GPC) or a more specific detection method (GC-NCI-MS or GC-MS/MS) will improve the detection limit. Negative chemical ionization (NCI) mass spectrometric detection can be used for highly chlorinated compounds (e.g., HCB, PCBs with five or more Cl atoms, HCH) and shows extremely high sensitivity and selectivity for these compounds. More universally applicable is tandem mass spectrometry (MS/MS), which yields a similar absolute sensitivity as normal MS but much higher selectivity. Some MS/MS transitions for the detection of selected chlorinated hydrocarbons are listed in Table 1 in Appendix 2 to Annex B-13: Technical note on the determination of polycyclic aromatic hydrocarbons in biota, from the full "Guidelines".

7.2 Quantification

A multilevel calibration with at least five concentration levels is recommended. The response of the FID detector is linear. For UV and fluorescence detection, the linear range is also large. The working range should be linear and must be covered by a calibration curve.

Since the mass spectrometric detector often has no linear response curve, the use of stable deuterated isotopes is a prerequisite. Furthermore, the response of PAHs in standard solutions is often much lower than in sample extracts. Only a combination of different techniques, e.g., the use of internal standards and standard addition, might give reliable quantitative results.

The calibration curve can be checked by recalculating the standards as if they were samples and comparing these results with the nominal values. Deviations from the nominal values should not exceed 5%.

When chromatograms are processed using automated integrators, the baseline is not always set correctly, and always needs visual inspection. Because the separation of the peaks is often incomplete in HPLC analysis, the use of peak heights is recommended for quantification. In case of GC techniques, either peak heights or peak areas can be used.

Prior to running a series of samples and standards, the GC or HPLC systems should be equilibrated by injecting at least one sample extract, the data from which should be ignored. In addition, standards used for multilevel calibration should be regularly distributed over the sample series so matrix- and non-matrix-containing injections alternate. A sample series should include:

- a procedural blank,
- a laboratory reference material,
- at least five standards,
- one standard that has been treated similarly to the samples (recovery determination).

The limit of determination should depend on the purpose of the investigation. A limit of 2 ng g−1 (dry weight) or better should be attained for single compounds. The method for calculating the limit of determination should reflect QUASIMEME advice (Topping et al., 1992). The limit of determination that can be achieved depends on the blank, the sample matrix, concentrations of interfering compounds, and the volume of water taken for analysis. The typical concentration ranges of PAHs and other POPs in seawater can be found in HELCOM assessments (HELCOM, 2003a, 2003b).

8. QUALITY ASSURANCE

A number of measures should be taken to ensure a sufficient quality of the analysis. Five main areas can be identified:

1. extraction efficiency and clean-up;
2. calibrant and calibration;
3. system performance;
4. long-term stability; and
5. internal standards.

8.1 Extraction efficiency and clean-up

A check on extraction efficiency and clean-up can be performed by analysing a reference material (Annex B-7). To determine the recovery rates of the clean-up and concentration steps, it is recommended to pass a standard solution through the entire procedure. Additionally, at least one internal standard should be added to each sample before extraction, to check for recovery during the analytical procedures. If major losses have occurred, then the results should not be reported. CB29 is suggested as a recovery standard because, owing to its high volatility, losses due to evaporation are easily detected. CB29 elutes relatively late from alumina and silica columns. Small peaks that may be present in the gas chromatogram at the retention time of CB29 do not hinder the use of this CB because the recovery standard only indicates major errors in extraction or clean-up. In case of GC/MS, labelled CBs can be used as recovery standards. This allows correction for recovery, provided that each chlorination stage is represented.

8.2 Calibrant and calibration

PAH determinations should preferably be carried out using calibration solutions prepared from certified crystalline PAHs. However, the laboratory should have the appropriate equipment and expertise to handle these hazardous crystalline substances. Alternatively, certified PAH solutions, preferably from two different suppliers, can be used. Two independent stock solutions should always be prepared simultaneously to allow cross-checks to be made. Calibration solutions should be stored in ampoules in a cool, dark place. Weight loss during storage should be recorded for all standards.

CB determinations should always be carried out using calibration solutions prepared from crystalline CBs. Preferably, certified CBs should be used. Two independent stock solutions of different concentrations should always be prepared simultaneously to allow a cross-check to be made. Calibration solutions should preferably be stored in a cool, dark place. For all containers with standards, the weight loss during storage should be recorded.

After clean-up and before GC analysis, both in PAH and CB analysis, an additional internal standard is added for volume correction. Internal standards should be added in a fixed volume or weighted to all standards and samples.

8.3 System performance

The performance of the HPLC or GC system can be monitored by regularly checking the resolution of two closely eluting PAHs or CBs. A decrease in resolution indicates deteriorating HPLC or GC conditions. The signal-to-noise ratio of a low concentration standard yields information on the condition of the detector. For example, a dirty MS-source can be recognized by the presence of a higher background signal, together with a reduced signal-to-noise ratio. Additionally, the peak can be affected.

8.4 Long-term stability

One laboratory reference sample should be included in each series of samples. A quality control chart should be recorded for selected PAHs, e.g., fluoranthene (stable results), pyrene (sensitive to quenching), benzo[a]pyrene (sensitive to light), or, correspondingly, for selected CBs. If the warning limits are exceeded, the method should be checked for possible errors. When alarm limits are exceeded, the results obtained should not be reported.

A certified reference material (CRM) should be analysed at least once a year, when available, and each time the procedure is changed. Each laboratory analysing PAHs and CBs in water should participate in interlaboratory analytical performance tests on a regular basis.

8.5 Internal standards

Internal standards should be added to all standards and samples either in a fixed volume or by weight. The PAH internal standards should preferably be non-natural PAHs which are not found in water and do not co-elute with the target PAHs; several predeuterated PAHs have proved to be suitable for GC/MS as well as for HPLC analysis. For example, for GC/MS it is recommended to add four internal standards representing different ring-sizes of PAHs.

The following compounds can be used (Wise et al., 1995):

- for HPLC analysis: phenanthrene-d10, fluoranthene-d10, perylene-d12, 6-methyl-chrysene;
- for GC/MS analysis: naphthalene-d8, phenanthrene-d10, chrysene-d12, perylene-d12;
- for GC/FID analysis: 1-butylpropylene, m-tetraphenyl.

Similarly the ideal internal standard for PCBs is a compound which is not found in the samples and does not co-elute with other CBs, e.g., CBs 29, 112, 155, 198 or all 2,4,6-substituted CB congeners. Alternatively, 1,2,3,4-tetrachloronaphthalene can be used.

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ANNEX B-12 TECHNICAL NOTE ON THE DETERMINATION OF HEAVY METALS AND PERSISTENT ORGANIC COMPOUNDS IN BIOTA

- Appendix 1. Technical note on biological material sampling and sample handling for the analysis of persistent organic pollutants (PAHs, PCBs and OCPs) and metallic trace elements
- Appendix 2. Technical note on the determination of polycyclic aromatic hydrocarbons in biota
- Appendix 3. Technical note on the determination of chloronated biphenyls and organochlorine pesticides in biota
- Appendix 4. Technical note on the determination of trace metallic elements in biota
- Appendix 5. Technical note on the determination of total mercury in marine biota

ANNEX B-12, APPENDIX 1. TECHNICAL NOTE ON BIOLOGICAL MATERIAL SAMPLING AND SAMPLE HANDLING FOR THE ANALYSIS OF PERSISTENT ORGANIC POLLUTANTS (PAHS, PCBS AND OCPS) AND METALLIC TRACE ELEMENTS

1. GENERAL PRINCIPLES

Muscle tissue or liver of fish have to be dissected while they are in good condition. If biological tissue deteriorates, uncontrollable losses of determinands or cross-contamination from other deteriorating tissues and organs may occur. To avoid this, individual fish specimens must be dissected at sea if adequate conditions prevail on board, or be frozen immediately after collection and transported frozen to the laboratory, where they are dissected later.

If the option chosen is dissection on board the ship, two criteria must be met:

1. The work must be carried out by personnel capable of identifying and removing the desired organs according to the requirements of the investigations; and
2. There must be no risk of contamination from working surfaces or other equipment.

2. TOOLS AND WORKING AREA

Crushed pieces of glass or quartz knives, and scalpels made of stainless steel or titanium are suitable dissection instruments.

Colourless polyethylene tweezers are recommended as tools for holding tissues during the dissecton of biological tissue for metallic trace element analysis. Stainless steel tweezers are recommended if biological tissue is dissected for analysis of chlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) and polunuclear aromatic hydrocarbons (PAHs).

After each sample has been prepared, including the samples of different organs from the same individual, the tools should be changed and cleaned.

The following procedures are recommended for cleaning tools used for preparing samples:

1) for analysis of metallic trace elements

- a) Wash in acetone or alcohol and high purity water.
- b) Wash in HNO_3 (p.a.) diluted (1+1) with high purity water. Tweezers and haemostates in diluted (1+6) acid.
- c) Rinse with high purity water.

2) for analysis of CBs and OCPs

- a) Wash in acetone or alcohol and rinse in high purity water.

The glass plate used during dissection should be cleaned in the same manner. The tools must be stored dust-free when not in use.

The dissection room should be kept clean and the air should be free from particles. If clean benches are not available on board the ship, the dissection of fish should be carried out in the land-based laboratory under conditions of maximum protection against contamination.

3. FISH MUSCLE AND LIVER SAMPLES DISSECTION

For fish analysis, commercial catches can be used if fish transport to the laboratory does not take longer than 24 hours. The fish must be transported on ice. The dissection then takes place at the laboratory.

For analysis of **fish muscle**, the epidermis and subcutaneous tissue should be carefully removed from the fish. Samples should be taken under the red muscle layer. In order to ensure uniformity of samples, the **right** side **dorso-lateral muscle** should be taken as the sample. If possible, the entire right dorsal lateral filet should be used as a uniform sample, from which subsamples can be taken after homogenizing for replicate dry weight and contaminant determinations. If, however, the amount of material obtained by this procedure is too large to handle in practice, a specific portion of the dorsal musculature should be chosen for the sample. It is recommended that the portion of the muscle lying directly under the first dorsal fin should be utilised in this case. As both fat and water content vary significantly in the muscle tissue from the anterior to the caudal muscle of the fish (Oehlenschläger, 1994), it is important to obtain the same portion of the muscle tissue for each sample.

To sample **liver tissue**, the liver must be identified in the presence of other organs such as the digestive system or gonads (Harms and Kanisch, 2000). The appearance of the gonads will vary according to the sex of the fish and the season. After opening the body cavity with a scalpel, the connective tissue around the liver should be cut away and as much as possible of the liver is cut out in a single piece together with the gall bladder. The bile duct is then carefully clamped and the gall bladder dissected away from the liver.

When fish samples which have been frozen at sea are brought to the laboratory for analysis, they should be dissected as soon as the tissue has thawed sufficiently. The dissection of fish is easiest when the material, at least the surface layers of the muscle tissue, is half frozen. For dissection of other organs, the thawing must proceed further, but it is an advantage if, for example, the liver is still frozen. It must be noted that any loss of liquid or fat due to improper cutting or handling of the tissue makes the determinations of dry weight and fat content, and consequently the reported concentrations of determinands, less accurate.

After muscle preparations, the liver should be completely and carefully removed while still partly frozen to avoid water and fat loss. Immediately after removing it from the fish, the liver should be returned to the freezer so that it will be completely frozen prior to further handling. This is particularly important for cod liver.

4. SHELLFISH SAMPLING

The blue mussel *(Mytilus edulis)* occurs in shallow waters along almost all coasts of the Baltic Sea. It is therefore suitable for monitoring in near shore waters. No distinction is made between *M. edulis*, *M. gallopovincialis,* and *M. trossulus* because the latter species fills a similar ecological niche. A sampling size range of 20–70 mm shell length is specified to ensure availability throughout the whole maritime area.

Two alternative sampling strategies can be used: sampling to minimise natural variability and length-stratified sampling. Only details of length-stratified sampling are described in this document, as this strategy is used in monitoring programmes for temporal trends of contaminants in biota.

For shellfish, the upper limit of shell length should be chosen in such a way that at least 20 mussels in the largest length interval can easily be found. The length stratification should be determined in such a way that it can be maintained over many years for the purposes of temporal trend monitoring. The length interval shall be at least 5 mm in size. The length range should be split into at least three length intervals (small, medium, and large) which are of equal size after log transformation.

Mussels are collected by a bottom grab and selected onboard. The number of specimens selected for analysis depends on their length, e.g. 80-100 individuals are necessary to suffice material within the length range 4-5 cm.

5. STORAGE OF FISH AND MUSSEL SAMPLES

Material from single fish specimens should be packaged and stored individually.

- Samples for analysis of metallic trace elements can be stored in polyethylene, polypropylene, polystyrene or glass containers.
- Samples for analysis of CBs and OCPs should be packaged in precleaned aluminium foil or in precleaned glass containers.

Liver tissue can deteriorate rather rapidly at room temperature. Consequently, samples should be frozen as soon as possible after packaging. They can be frozen rapidly by immersion in liquid nitrogen or blast freezing, but both these techniques need care. Whatever system is used, freezing a large bulk of closely packed material must be avoided. The samples in the centre will take longer to cool and will therefore deteriorate more than those in the outer layer.

Once frozen, samples can be stored in a deep freezer at temperatures of -20°C or below.

Frozen liver tissue should not be stored longer than six months, while lean muscle tissue can be stored up to two years. Each sample should be carefully and permanently labelled. The label should contain at least the sample's identification number, the type of tissue, and the date and location of sampling.

Mussels should be shucked live and opened with minimal tissue damage by detaching the adductor muscles from the interior of at least one valve. The soft tissues should be removed and homogenised as soon as possible, and frozen in glass jars at -20 °C until analysis. Mussel tissue for trace metal determination is homogenised and decomposed in a wet state while for persistent organic pollutants determination it is homogenised and water is removed by freeze-drying. Frozen liver tissue should not be stored longer than six months, while lean muscle tissue can be stored up to two years. Each sample should be carefully and permanently labelled. The label should contain at least the sample's identification number, the type of tissue, and the date and location of sampling.

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ANNEX B-12, APPENDIX 2. TECHNICAL NOTE ON THE DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN BIOTA

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) consist of a variable number of fused aromatic rings. By definition, PAHs contain at least three fused rings, although in practice related compounds with two fused rings (such as naphthalene and its alkylated derivatives) are often determined and will be considered in these guidelines. PAHs arise from incomplete combustion processes and from both natural and anthropogenic sources, although the latter generally predominate. PAHs are also found in oil and oil products, and these include a wide range of alkylated PAHs formed as a result of diagenetic processes, whereas PAHs from combustion sources comprise mainly parent (non-alkylated) PAHs. PAHs are of concern in the marine environment for two main reasons: firstly, low-molecular weight (MW) PAHs can be directly toxic to marine animals; secondly, metabolites of some of the high-MW PAHs are potent animal and human carcinogens, benzo[a]pyrene is the prime example. Carcinogenic activity is closely related to structure, however, and benzo[e]pyrene and four benzofluoranthene isomers (all six compounds have a molecular weight of 252 Da) are much less potent. Some compounds (e.g., heterocyclic compounds containing sulphur, such as benzothiophenes and dibenzothiophenes) may also cause taint in commercially exploited fish and shellfish and render them unfit for sale.

PAHs are readily taken up by marine animals both across gill surfaces and from their diet, and may bioaccumulate, particularly in shellfish. Filter-feeding organisms such as bivalve molluscs can accumulate high concentrations of PAHs, both from chronic discharges to the sea (e.g., of sewage) and following oil spills. Fish are exposed to PAHs both via uptake across gill surfaces and from their diet, but do not generally accumulate high concentrations of PAHs as they possess an effective mixed-function oxygenase (MFO) system which allows them to metabolize PAHs and to excrete them in bile. An assessment of the exposure of fish to PAHs therefore requires also the determination of PAH-metabolite concentrations in bile samples, as turnover times can be extremely rapid. Thus, the analysis of PAHs in fish muscle tissue should normally only be undertaken for food quality assurance purposes (Law and Biscaya, 1994).

There are marked differences in the behaviour of PAHs in the aquatic environment between the low-MW compounds (such as naphthalene; 128 Da) and the high-MW compounds (such as benzo[ghi]perylene; 276 Da) as a consequence of their differing physico-chemical properties. The low-MW compounds are appreciably water soluble and can be bioaccumulated from the "dissolved" phase by transfer across gill surfaces, whereas the high-MW compounds are relatively insoluble and hydrophobic, and can attach to both organic and inorganic particulates within the water column. PAHs derived from combustion sources may actually be deposited to the sea already adsorbed to atmospheric particulates, such as soot particles. The majority of PAHs in the water column will eventually be either taken up by biota or transported to the sediments, and deep-water depositional areas may generally be regarded as sinks for PAHs, particularly when they are anoxic.

2. APPROPRIATE SPECIES FOR ANALYSIS OF PAHS

2.1 Benthic fish and shellfish

All teleost fish have the capacity for rapid metabolism of PAHs, thereby limiting their usefulness for monitoring temporal or spatial trends of PAHs. Shellfish (particularly molluscs) generally have a lesser metabolic capacity towards PAHs, and so they are preferred because PAH concentrations are generally higher in their tissues.

For the purposes of temporal trend monitoring, it is essential that long time series with either a single species or a limited number of species are obtained. Care should be taken that the sample is representative of the population and that sampling can be repeated annually. There are advantages in the use of molluscs for this purpose as they are sessile, and so reflect the degree of contamination in the local area to a greater degree than fish which are mobile. The analysis of fish tissues is often undertaken in conjunction with biomarker and disease studies, and associations have been shown between the incidence of some diseases (e.g., liver neoplasia) in flatfish and the concentrations of PAHs in the sediments over which they live and feed (Malins et al., 1988; Vethaak and ap Rheinallt, 1992). The exposure of fish to PAHs can be assessed by the analysis of PAH-metabolites in bile, and by measuring the induction of mixed-function oxygenase enzymes which affect the formation of these metabolites. At offshore locations, the collection of appropriate shellfish samples may be problematic if populations are absent, sparse or scattered, and the collection of fish samples may be simpler. Generally, the analysis of PAHs in fish muscle tissue should only be considered for the purposes of food quality assurance.

Recent monitoring studies have indicated a seasonal cycle in PAH concentrations (particularly for combustion-derived PAHs) in mussels, with maximum concentrations in the winter prior to spawning and minimum concentrations in the summer. It is particularly important, therefore, that samples selected for trend monitoring and spatial comparisons are collected at the same time of year, and preferably in the first months of the year before spawning.

2.2 Fish

Fish are not recommended for spatial or temporal trend monitoring of PAHs, but can be useful as part of biological effects studies or for food quality assurance purposes. The sampling strategy for biological effects monitoring is described in the OSPAR Joint Assessment and Monitoring Programme (JAMP).

3. TRANSPORTATION

Live mussels should be transported to the laboratory for sample preparation. They should be transported in closed containers at temperatures between 5 °C and 15 °C, preferably below 10 °C. For live animals it is important that the transport time is short and controlled (e.g., maximum of 24 hours).

Fish samples should be kept cool or frozen (at a temperature of -20 °C or lower) as soon as possible after collection. Frozen fish samples should be transported in closed containers at temperatures below -20 °C. If biomarker determinations are to be made, then it will be necessary to store tissue samples at lower temperatures, for example, in liquid nitrogen at -196 °C.

4. PRETREATMENT AND STORAGE

4.1 Contamination

Sample contamination may occur during sampling, sample handling, pretreatment and analysis, due to the environment, the containers or packing materials used, the instruments used during sample preparation, and from the solvents and reagents used during the analytical procedures. Controlled conditions are therefore required for all procedures, including the dissection of fish organs on-board a ship (see ANNEX B-13, Appendix 1). In the case of PAHs, particular care must be taken to avoid contamination at sea. On ships there are multiple sources of PAHs, such as the oils used for fuel and lubrication, and the exhaust from the ship's engines. It is important that the likely sources of contamination are identified and steps taken to preclude sample handling in areas where contamination can occur. A ship is a working vessel and there can always be procedures occurring as a result of the day-to-day operations (deck cleaning, automatic overboard bilge discharges, etc.) which could affect the sampling process. One way of minimizing the risk is to conduct dissection in a clean area, such as within a laminar-flow hood away from the deck areas of the vessel. It is also advisable to collect samples of the ship's fuel, bilge water, and oils and greases used on winches, etc., which can be used as fingerprinting samples at a later date, if there are suspicions of contamination in particular instances.

4.2 Shellfish

4.2.1 Depuration

Depending upon the situation, it may be desirable to depurate shellfish so as to void the gut contents and any associated contaminants before freezing or sample preparation. This is usually applied close to point sources, where the gut contents may contain significant quantities of PAHs associated with food and sediment particles which are not truly assimilated into the tissues of the mussels. Depuration should be undertaken under controlled conditions and in filtered sea water; depuration over a period of 24 hours is usually sufficient. The aquarium should be aerated and the temperature and salinity of the water should be similar to that from which the animals were removed.

4.2.2 Dissection and storage

When samples are processed, both at sea and onshore, the dissection must be undertaken by trained personnel on a clean bench wearing clean gloves and using clean stainless steel knives and scalpels. Stainless steel tweezers are recommended for holding tissues during dissection. After each sample has been prepared, all tools and equipment (such as homogenizers) should be cleaned.

4.3 Fish

4.3.1 Dissection and storage

The dissection of fish muscle and internal organs should be carried as soon as possible after collection. The details of fish muscle and liver dissection are given in ANNEX B-13, Appendix 1. If possible, the entire right side dorsal lateral fillet should be homogenized and sub samples taken for replicate PAH determinations. If, however, the amount of material to be homogenized would be too large, a specific portion of the dorsal musculature should be chosen. It is recommended that the portion of the muscle lying directly under the first dorsal fin is used in this case.

When dissecting the liver, care should be taken to avoid contamination from the other organs. If bile samples are to be taken for PAH-metabolite determinations, then they should be collected first. If the whole liver is not to be homogenized, a specific portion should be chosen in order to ensure comparability. Freeze-drying of tissue samples cannot be recommended for PAH determination, due to the contamination which may result from back-streaming of oil from the rotary pumps used to generate the vacuum.

If plastic bags or boxes are used, then they should be used as outer containers only, and should not come into contact with tissues. Organ samples (e.g., livers) should be stored in pre-cleaned containers made of glass, stainless steel or aluminium, or should be wrapped in pre-cleaned aluminium foil and shock-frozen quickly in liquid nitrogen or in a blast freezer. In the latter case, care should be taken that the capacity of the freezer is not exceeded (Law and de Boer, 1995). Cold air should be able to circulate between the samples in order that the minimum freezing time can be attained (maximum 12 hours). The individual samples should be clearly and indelibly labelled and stored together in a suitable container at a temperature of -20 °C until analysis. If the samples are to be transported during this period (e.g., from the ship to the laboratory), then arrangements must be made which ensure that the samples do not thaw out during transport. Sub samples for biomarker determinations should be collected immediately after death in order to minimize post-mortem changes in enzymatic and somatic activities, and stored in suitable vials in liquid nitrogen until analysis.

When samples are processed, both at sea and onshore, the dissection must be undertaken by trained personnel on a clean bench wearing clean gloves and using clean stainless steel knives and scalpels. Stainless steel tweezers are recommended for holding tissues during dissection. After each sample has been prepared, all tools and equipment (such as homogenizers) should be cleaned.

When pooling of tissues is necessary, an equivalent quantity of tissue should be taken from each fish, e.g., 10 % from each whole fillet.

5. ANALYSIS

5.1 Preparation of materials

Solvents, reagents, and adsorptive materials must be free of PAHs and other interfering compounds. If not, then they must be purified using appropriate methods. Reagents and absorptive materials should be purified by solvent extraction and/or by heating in a muffle oven, as appropriate. Glass fibre materials (e.g., Soxhlet thimbles) are preferred over filter papers and should be cleaned by solvent extraction. It should be borne in mind that clean materials can be re-contaminated by exposure to laboratory air, particularly in urban locations, and so storage after cleaning is of critical importance. Ideally, materials should be prepared immediately before use, but if they are to be stored, then the conditions should be considered critically. All containers which come into contact with the sample should be made of glass, and should be pre-cleaned before use. Appropriate cleaning methods would include washing with detergents, rinsing with water, and finally solvent-rinsing immediately before use. Heating of glassware in an oven (e.g., at 400°C for 24 hours) can also be useful in removing PAH contamination.

5.2 Lipid determination

Although PAH data are not usually expressed on a lipid basis, the determination of the lipid content of tissues can be of use in characterizing the samples. The lipid content should be determined on a separate subsample of the tissue homogenate, as some of the extraction techniques used routinely for PAH determination (e.g., alkaline saponification) destroy lipid materials. The total fat weight should be determined using the method of Smedes (1999) or an equivalent method.

5.3 Dry weight determination

Generally PAH data are expressed on a wet weight basis, but sometimes it can be desirable to consider them on a dry weight basis. Again, the dry weight determination should be conducted on a separate sub sample of the tissue homogenate, which should be air-dried to constant weight at 105 °C.

5.4 Extraction and clean-up

PAHs are lipophilic and so are concentrated in the lipids of an organism, and a number of methods have been described for PAH extraction (see, e.g., Ehrhardt *et al.,* 1991). The preferred methods generally utilize either Soxhlet extraction, or alkaline digestion followed by liquid-liquid extraction with an organic solvent. Microwave-assisted solvent extraction can be mentioned as one of the modern techniques being applied to PAH analysis (Budzinski *et al.,* 2000; During and Gaath, 2000; Vázquez Blanco *et al*., 2000; Ramil Criado *et al.,* 2002). In the case of Soxhlet extraction, the wet tissue must be dried by mixing with a chemical agent (e.g., anhydrous sodium sulphate), in which case a time period of several hours is required between mixing and extraction in order to allow complete binding of the water in the sample. Alkaline digestion is conducted on wet tissue samples, so this procedure is unnecessary. In neither case can the freeze-drying of the tissue prior to extraction be recommended, owing to the danger of contamination from oil back-streaming from the rotary pump (which provides the vacuum) into the sample. Non-polar solvents alone will not effectively extract all the PAHs from tissues when using Soxhlet extraction, and mixtures such as hexane/dichloromethane may be effective in place of solvents such as benzene and toluene, used historically for this purpose. Alkaline digestion has been extensively used in the determination of PAHs and hydrocarbons and is well documented. It is usually conducted in alcohol (methanol or ethanol), which should contain at least 10 % water, and combines disruption of the cellular matrix, lipid extraction and saponification within a single procedure, thereby reducing sample handling and treatment. For these reasons, it should be the method of choice. Solvents used for liquid-liquid extraction of the homogenate are usually non-polar, such as pentane or hexane, and they will effectively extract all PAHs.

Tissue extracts will always contain many compounds other than PAHs, and a suitable clean-up is necessary to remove those compounds which may interfere with the subsequent analysis. Different techniques may be used, both singly or in combination, and the choice will be influenced by the selectivity and sensitivity of the final measurement technique and also by the extraction method employed. If Soxhlet extraction was used, then there is a much greater quantity of residual lipid to be removed before the analytical determination can be made than in the case of alkaline digestion. An additional clean-up stage may therefore be necessary. The most commonly used clean-up methods involve the use of alumina or silica adsorption chromatography, but gel permeation chromatography and similar high performance liquid chromatography (HPLC) based methods are also employed (Nondek *et al.,* 1993; Nyman *et al.,* 1993; Perfetti *et al.,*1992). The major advantages of using HPLC-based clean-up methods are their ease of automation and reproducibility.

5.5 Pre-concentration

The sample volume should be 2 cm^3 or greater to avoid errors when transferring solvents during the clean-up stages. Evaporation of solvents using a rotary-film evaporator should be performed at low temperature (water bath temperature of 30 °C or lower) and under controlled pressure conditions, in order to prevent losses of the more volatile PAHs such as naphthalenes. For the same reasons, evaporation to dryness should be avoided. When reducing the sample to final volume, solvents can be removed by a stream of clean nitrogen gas. Suitable solvents for injection into the gas chromatograph

(GC) or GC-MS include pentane, hexane, heptane and *iso*-octane, whereas for HPLC analyses acetonitrile and methanol are commonly used.

5.6 Selection of PAHs to be determined

The choice of PAHs to be analysed is not straightforward, both because of differences in the range of PAH compounds resulting from combustion processes and from oil and oil products, and also because the aims of specific monitoring programmes can require the analysis of different representative groups of compounds. PAHs arising from combustion processes are predominantly parent (unsubstituted) compounds, whereas oil and its products contain a much wider range of alkylated compounds in addition to the parent PAHs. This has implications for the analytical determination, as both HPLC-based and GC-based techniques are adequate for the determination of a limited range of parent PAHs in samples influenced by combustion processes, whereas in areas of significant oil contamination and following oil spills only GC-MS has sufficient selectivity to determine the full range of PAHs present. The availability of pure individual PAHs for the preparation of standards is problematic and limits both the choice of determinands and, to some degree, the quantification procedures which can be used. The availability of reference materials certified for PAHs is also rather limited. A list of target parent and alkylated PAHs suitable for environmental monitoring is given in Table 1. In both cases, the list was concentrated on a subset of parent (predominantly combustion-derived) PAHs due to analytical limitations. This approach completely neglects the determination of alkylated PAHs, which allows the interpretation of PAH accumulation from multiple sources including those due to oil inputs. It will not be necessary for all of these PAH compounds and groups to be analysed in all cases, but an appropriate selection can be made from this list depending on the specific aims of the monitoring programme to be undertaken.

Table 1: Compounds of interest for environmental monitoring for which the guidelines apply

Compound	MW	Compound	MW
Naphthalene	128	C2-Phenanthrenes/Anthracenes	206
C1-Naphthalenes	142	C3-Phenanthrenes/Anthracenes	220
C2-Naphthalenes	156	Fluoranthene	202
C3-Naphthalenes	170	Pyrene	202
C4-Naphthalenes	184	C1-Fluoranthenes/Pyrenes	216

Acenaphthylene	152	C2-Fluoranthenes/Pyrenes	230
Acenaphthene	154	Benz[a]anthracene	228
Biphenyl	154	Chrysene	228
Fluorene	166	2,3-Benzanthracene	228
C1-Fluorenes	180	Benzo[a]fluoranthene	252
C2-Fluorenes	194	Benzo[b]fluoranthene	252
C3-Fluorenes	208	Benzo[j]fluoranthene	252
Dibenzothiophene	184	Benzo[k]fluoranthene	252
C1-Dibenzothiophenes	198	Benzo[e]pyrene	252
C2-Dibenzothiophenes	212	Benzo[a]pyrene	252
C3-Dibenzothiophenes	226	Perylene	252
Phenanthrene	178	Indeno[1,2,3-cd]pyrene	276
Anthracene	178	Benzo[ghi]perylene	276
C1-Phenanthrenes/ Anthracenes	192	Dibenz[ah]anthracene	278

5.7 Instrumental determination of PAHs

Unlike the situation for chlorobiphenyls (CBs), where GC techniques (particularly GC-ECD) are used exclusively, two major approaches based on GC and HPLC are followed to an equal extent in the analysis of PAHs. The greatest sensitivity and selectivity in routine analyses are achieved by combining

HPLC with fluorescence detection (HPLC-UVF) and capillary gas chromatography with mass spectrometry (GC-MS). In terms of flexibility, GC-MS is the most capable technique, as in principle it does not limit the selection of determinands in any way, while HPLC is suited only to the analysis of parent PAHs. In the past, analyses have also been conducted using HPLC with UV-absorption detection and GC with flame-ionization detection, but neither can be recommended because of their relatively poor selectivity.

Intercomparison exercises have demonstrated a serious lack of comparability between specific hydrocarbon concentrations measured in different laboratories and using both analytical approaches described above (Farrington *et al.,* 1986). An interlaboratory performance study has been carried out within the QUASIMEME laboratory testing scheme in order to assess the level of comparability among laboratories conducting PAH analyses and to identify improvements in methodology (Law and Klungsøyr, 1996; Law *et al.,* 1998, QUASIMEME).

Limits of determination within the range of 0.2 to 10 µg kg-1 wet weight for individual PAH compounds should be achievable by both GC-MS and HPLC-UVF techniques.

5.8 HPLC

Reversed-phase columns (e.g., octadecylsilane (RP-18)) 15–30 cm in length are used almost exclusively, in conjunction with gradient elution using mixtures of acetonitrile/water or methanol/water. A typical gradient may start as a 50 % mixture, changing to 100 % acetonitrile or methanol in 40 minutes. This flow is maintained for 20 minutes, followed by a return to the original conditions in 5 minutes and 5–10 minutes' equilibration before the next injection. The use of an automatic injector is strongly recommended. Also, the column should be maintained in a column oven heated to 10–30°C. The systems yielding the best sensitivity and selectivity utilize fluorescence detection. As different PAH compounds yield their maximum fluorescence at different wavelengths, for optimum detection of PAHs the wavelengths of the detector should be programmed so that the excitation/emission wavelengths detected are changed at pre-set times during the analytical determination. For closely eluting peaks, it may be necessary to use two detectors in series utilizing different wavelength pairs, or to affect a compromise in the selected wavelengths if a single detector is used. As the fluorescence signals of some PAHs (e.g., pyrene) are quenched by oxygen, the eluents must be degassed thoroughly. This is usually achieved by continuously bubbling a gentle stream of helium through the eluent reservoirs, but a vacuum degasser can also be used. Polytetrafluorethylene (PTFE) tubing must not then be used downstream of the reservoirs as this material is permeable to oxygen; stainless steel or polyether-etherketone (PEEK) tubing is preferred.

5.9 GC-MS

The two injection modes commonly used are splitless and on-column injection. Automatic sample injection should be used wherever possible to improve the reproducibility of injection and the precision of the overall method. If splitless injection is used, the liner should be of sufficient capacity to contain the injected solvent volume after evaporation. For PAH analysis, the cleanliness of the liner is also very important if adsorption effects and discrimination are to be avoided, and the analytical column should not contain active sites to which PAHs can be adsorbed. Helium is the preferred carrier gas, and only capillary columns should be used. Because of the wide boiling range of the PAHs to be determined and the surface-active properties of the higher PAHs, the preferred column length is 25–30 m, with an internal diameter of 0.15 mm to 0.3 mm. Film thicknesses of 0.3 µm to 1 µm are generally used; this choice has little impact on critical resolution, but thicker films are often used when one-ring aromatic compounds are to be determined alongside PAHs, or where a high sample loading is needed. No stationary phase has been found on which all PAH isomers can be resolved; the most commonly used stationary phase for PAH analysis is 5 % phenyl methylsilicone (DB-5 or equivalent). This will not, however, resolve critical isomers such as benzo[*b*], [*j*] and [*k*]fluoranthenes, or chrysene from triphenylene. These separations can be made on other columns, if necessary. For PAHs there is no sensitivity gain from the use of chemical ionization (either positive or negative ion), so analyses are usually conducted in electron-impact mode at 70 eV. The choice of full-scan or multiple-ion detection is usually made in terms of sensitivity. Some instruments such as ion-trap mass spectrometers exhibit the same sensitivity in both modes, so full-scan spectra are collected, whereas for quadrupole instruments greater sensitivity is obtained if the number of ions scanned is limited. In that case, the masses to be detected are programmed to change during the analysis as different PAHs elute from the capillary column.

6. CALIBRATION AND QUANTIFICATION

6.1 Standards

A range of fully deuterated parent PAHs is available for use as standards in PAH analysis. The availability of pure PAH compounds is limited (Annex B-7). Although most of the parent compounds can be purchased as pure compounds, the range of possible alkyl-substituted PAHs is vast and only a limited selection of them can be obtained. In HPLC, where the resolving power of the columns is limited and the selectivity less than that which can be obtained using MS detection, only a single internal standard is normally used (e.g., phenanthrene-d10), although fluoranthene-d10 and 6-methyl chrysene, among others, have also been used. If GC-MS is used, then a wider range of deuterated PAHs can be utilized, both because of the wide boiling range of PAHs present and because that allows the use of both recovery and quantification standards. Suitable standards could range from naphthalene-d8 to perylene-d10. It is always recommended to use at least two and preferably three internal standards of hydrocarbons of small, medium, and high molecular weight (e.g., naphthalene-d8, phenanthrene-d10, perylene-d12. Crystalline PAHs of known purity should be used for the preparation of calibration standards. If the quality of the standard materials is not guaranteed by the producer or supplier (as for certified reference materials), then it should be checked by GC-MS analysis. Solid standards should be weighed to a precision of 10-5 grams. Calibration standards should be stored in the dark because some PAHs are photosensitive, and ideally solutions to be stored should be sealed in amber glass ampoules. Otherwise, they can be stored in a refrigerator in stoppered measuring cylinders or flasks that are gas tight to avoid evaporation of the solvent during storage.

6.2 Calibration

Multilevel calibration with at least five calibration levels is preferred to adequately define the calibration curve. In general, GC-MS calibration is linear over a considerable concentration range but exhibits non-linear behaviour when the mass of a compound injected is low due to adsorption. Quantification should be conducted in the linear region of the calibration curve, or the non-linear region must be well characterized during the calibration procedure. For HPLC-UVF, the linear range of the detection system should be large, and quantification should be made within the linear range. External standardization is often used with HPLC due to the relatively limited resolution obtainable with this technique as generally employed.

6.3 Recovery

The recovery of analytes should be checked and reported. Given the wide boiling range of the PAHs to be determined, the recovery may vary with compound group, from the volatile PAHs of low molecular weight to the larger compounds. For GC-MS analysis, deuterated standards can be added in two groups: those to be used for quantification are added at the start of the analytical procedure, whilst those from which the absolute recovery will be assessed are added prior to GC-MS injection. This ensures that the calculated PAH concentrations are corrected for the recovery obtained in each case. In the case of HPLC, where only a single deuterated PAH standard is used, it is more common to assess recovery periodically by carrying a standard solution through the whole analytical procedure, then assessing recovery by reference to an external standard. This technique does not, however, correct for matrix effects, and so may be used in conjunction with the spiking of real samples.

7. ANALYTICAL QUALITY CONTROL

Planners of monitoring programmes must decide on the accuracy, precision, repeatability, and limits of detection and determination which they consider acceptable. Achievable limits of determination for each individual component are as follows:

- for GC-MS measurements: 0.2 µg kg-1 ww;
- for HPLC measurements: 0.5–10 µg kg-1 ww.

Further information on analytical quality control procedures for PAHs can be found elsewhere (Law and de Boer, 1995). A procedural blank should be measured with each sample batch, and should be prepared simultaneously using the same chemical reagents and solvents as for the samples. Its purpose is to indicate sample contamination by interfering compounds, which will result in errors in quantification. The procedural blank is also very important in the calculation of limits of detection and limits of quantification for the analytical method. In addition, a laboratory reference material (LRM) should be analysed within each sample batch. Test materials from the former runs of QUASIMEME Laboratory Proficiency Testing can be used as Laboratory Reference Material. The LRM must be homogeneous and well characterized for the determinands of interest within the analytical laboratory. Ideally, stability tests should have been undertaken to show that the LRM yields consistent results over time. The LRM should be of the same matrix type (e.g., liver, muscle, mussel tissue) as the samples, and the determinand concentrations should be in the same range as those in the samples. Realistically, and given the wide range of PAH concentrations encountered, particularly in oil spill investigations, this is bound to involve some compromise. The data produced for the LRM in successive sample batches should be used to prepare control charts. It is also useful to analyse the LRM in duplicate from time to time to check within-batch analytical variability. The analysis of an LRM is primarily intended as a check that the analytical method is under control and yields acceptable precision, but a certified reference material (CRM) of a similar matrix should be analysed periodically in order to check the method bias. The availability of biota CRMs certified for PAHs is very limited (Annex B-7; QUASIMEME), and in all cases the number of PAHs for which certified values are provided is small. At regular intervals, the laboratory should participate in an intercomparison or proficiency exercise in order to provide an independent check on the performance.

8. DATA REPORTING

The calculation of results and the reporting of data can represent major sources of error, as has been shown in intercomparison studies for PAHs. Control procedures should be established in order to ensure that data are correct and to obviate transcription errors. Data stored in databases should be checked and validated, and checks are also necessary when data are transferred between databases. Data should be reported in accordance with the latest ICES reporting formats.

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ANNEX B-12, APPENDIX 3. TECHNICAL NOTE ON THE DETERMINATION OF CHLORINATED BIPHENYLS AND ORGANOCHLORINE PESTICIDES IN BIOTA

1. INTRODUCTION

The analysis of chlorinated biphenyls (CBs) and organochlorine pesticides (OCPs) in fish samples generally involves extraction from the respective matrix with organic solvents, followed by clean-up and gas chromatographic separation with electron capture (GC-ECD) or mass spectrometric (GC-MS) detection.

The analytical procedure is liable to systematic errors due to insufficiently optimized gas chromatographic conditions, determinant losses (evaporation, unsatisfactory extraction yield), and/or contamination from laboratory ware, reagents and the laboratory environment. It is therefore essential that the sources of systematic errors are identified and eliminated as far as possible.

In the following paragraphs, the guidelines drafted by the OSPAR Ad Hoc Working Group on Monitoring (OSPAR, 1996) have been taken into consideration.

2. PRE-TREATMENT OF LABORATORY WARE AND REAGENTS; CONTAMINATION CONTROL

Glassware, reagents, solvents, column adsorption materials and other laboratory equipment that come into contact with the sample material to be analysed should be free of impurities that interfere with the quantitative determination of CBs and OCPs.

For cleaning purposes, the following procedures should be followed:

1. Glassware should be thoroughly washed with detergents, dried with acetone and rinsed with a non-polar solvent such as *n*-pentane, and heated to > 100 ^{o}C prior to use.
2. Glass fibre Soxhlet thimbles should be pre-extracted with an organic solvent. The use of paper Soxhlet thimbles should be avoided. Alternatively, glass fibre thimbles or full glass Soxhlet thimbles, with a G1 glass filter at the bottom, are recommended.
3. Solvents should be checked for impurities using GC after concentrating the volume normally used in the procedure to 10 % of the final volume. If necessary, solvents can be purified by controlled re-distillation and rectification over KOH in an all-glass distillation column.
4. Reagents and column adsorption materials should be checked for contamination before use by extraction with an organic solvent (e.g., *n*-pentane) and analysis by GC, using the detector which will also be used for the final determination (ECD or MS).
5. Laboratory air can also be contaminated with CBs, OCPs or compounds interfering with the CB/OCP analysis. A good estimation of the contamination of the air can be found by placing a petri dish with 2 grams of C18-bonded silica for two weeks in the laboratory. After this period, the material is transferred to a glass column and eluted with 10 ml of 10% diethylether in hexane. After concentrating the eluate, the CB concentrations can be measured. Absolute

amounts of <1 ng show that the contamination of the air is at an acceptably low level in that laboratory (Smedes and de Boer, 1994).

3. SAMPLE PRETREATMENT

To ensure complete extraction of the lipophilic CBs and OCPs from biological sample matrices, it is essential to dry the material and disrupt the cell walls of the biological matrix to be analysed. This can be achieved by Ultra Turrax mixing or grinding of the sample with a dehydrating reagent, such as Na_2SO_4, followed by multiple solid/liquid extraction with a mixture of polar and non-polar solvents (e.g., acetone/hexane or methanol/dichloromethane). It is essential to allow complete binding of the water present in the sample with the dehydrating reagent (this requires at least several hours) prior to starting the extraction step. The extraction efficiency must be checked for different types and amounts of biological matrices to be investigated (see 'recovery section’).

4. CLEAN-UP

The crude extract obtained from sample pretreatment requires a clean-up in order to remove co-extracted lipophilic compounds that interfere with the gas chromatographic determination of CBs and OCPs. Normal-phase solid/liquid chromatography, using deactivated Al2O3 or deactivated silica as adsorbents and hexane or iso-octane as solvents, is an appropriate technique for the separation of the determinands from lipids or other interfering compounds.

Effective removal of high molecular weight compounds can be achieved by gel permeation chromatography (GPC). However, GPC does not separate CBs from other compounds in the same molecular range, such as organochlorine pesticides (OCPs). Therefore, additional clean-up may be required. Treatment of the OCP fraction with concentrated H_2SO_4 can improve the quality of the subsequent gas chromatogram. However, this treatment is not recommended if determinands of the dieldrin type or heptachloroepoxides, which are easily broken down by H2SO4, are to be determined.

5. DETERMINATION BY GAS CHROMATOGRAPHY

Because of the large number of organochlorine compounds to be determined, high resolution gas chromatography (GC) using, preferably narrow bore, fused silica wall-coated open-tubular (capillary) columns is necessary.

Carrier gas

Hydrogen is the preferred carrier gas and is indispensable for columns with very small inner diameters. For safety reasons, hydrogen should not be used without a safety module which is able to check for small hydrogen concentrations inside the GC oven coming from possible leakages. As a compromise to safety aspects, helium is also acceptable.

Columns

In order to achieve sufficient separation, capillary columns should have a length of >60 m, an internal diameter of < 0.25 mm (for diameters below 0.18 mm the elevated pressure of the carrier gas needs special instrumentation) and a film thickness of the stationary phase of < 0.25 µm. For routine work, the SE 54 (Ultra 2, DB 5, RTx 5, CP-Sil 8) phase (94 % dimethyl-, 5 % phenyl-, 1 % vinyl-polysiloxane) or medium polar columns (CP-Sil 19, OV-17, OV 1701, DB 17) have been shown to give satisfactory chromatograms. A second column with a stationary phase different, from that used in the first column, may be used for confirmation of the peak identification.

Injection

Splitless and on-column injection techniques may both be used. Split injection is not recommended because strong discrimination effects may occur. Other techniques such as temperature-programmed or pressure-programmed injection may have additional advantages, but should be thoroughly optimized before use.

In splitless injection, the volume of the liner should be large enough to contain the gas volume of the evaporated injected solvent. If the liner is too small, memory effects can occur due to contamination of the gas tubing attached to the injector. Very large liner volumes, in contrast, can cause a poor transfer of early eluting components.

A 1 µl injection normally requires a ca. 1 ml liner. The occurrence of memory effects should be tested by injection of iso-octane after analysis of a CB or OCP standard. The use of a light packing of silylated glass wool in the liner improves the response and reproducibility of the injection. However, some organochlorine pesticides such as DDT may disintegrate when this technique is used. In splitless injection, discrimination effects can occur.

The splitless injection time should therefore be optimized to avoid discrimination. This can be done by injecting a solution containing an early-eluting and a late-eluting CB, e.g., CB28 and CB180. Starting with a splitless injection time of 0.5 minutes, the peak height of the late-eluting compound will presumably increase relative to that of the first compound. The optimum is found at the time when the increase does not continue any further. The split ratio is normally set at 1:25 and is not really critical. The septum purge, normally approximately 2 ml min-1, should be stopped during injection. This option is not standard in all GCs.

Due to the variety of on-column injectors, a detailed optimization procedure cannot be given. More information on the optimization of on-column parameters may be obtained from Snell *et al*. (1987).

The reproducibility of injection is controlled by the use of an internal standard not present in the sample.

Detector

Quantitative analysis is performed by comparing the detector signal produced by the sample with that of defined standards. The use of an electron capture detector (ECD) sensitive to chlorinated compounds or - more generally applicable - a mass selective detector (MSD) or (even) a mass spectrometer (MS) is essential.

Due to incomplete separation, several co-eluting compounds can be present under a single detector signal. Therefore, the shape and size of the signal have to be critically examined. With a MSD or MS used as detector, either the molecular mass or characteristic mass fragments should be recorded for that purpose. If only an ECD is available, the relative retention time and the signal size should be confirmed on columns with different polarity of their stationary phases, or by the use of multi-dimensional GC techniques (de Boer *et al*., 1995; de Geus *et al*., 1996).

Calibration

Stock solutions of individual organohalogen compounds should be prepared using iso-octane as the solvent and weighed solid individual standard compounds of high purity (> 99 %). Stock solutions can be stored in measuring flasks in a refrigerator or in a dessicator with a saturated atmosphere of iso-octane, but losses can easily occur, particularly when storing in refrigerators (Law and de Boer, 1995). Loss of solvents in stock solutions can be controlled by recording the weight and filling up the missing amount before a new aliquot is taken. However, aliquots stored in sealed glass ampoules are much more appropriate and can normally be stored for several years. Fresh stock standard solutions should be prepared in duplicate and compared with the old standard solutions. Working standards should be prepared gravimetrically from stock solutions for each sample series. All manipulations with solvents, including pipetting, diluting and concentrating, should preferably be checked by weighing. Due to day-to-day and season-to-season temperature differences in laboratories and due to the heating of glassware after cleaning, considerable errors can be made when using volumetric glassware as a basis for all calculations.

The GC should be calibrated before each batch of measurements. Since the ECD has a non-linear response curve, a multilevel calibration is strongly advised. Megginson *et al*. (1994) recommend a set of six standard solutions for CB determination or five standard solutions for OCP determination. Standards used for multilevel calibration should be regularly distributed over the sample series, so that matrix and non-matrix containing injections alternate.

When concentrations of compounds in the sample fall outside either side of the calibration curve, a new dilution or concentrate should be made and the measurement repeated. Considerable errors can be made when measuring concentrations which fall outside the calibration curve.

For MS detection, a multi-level calibration is also recommended.

Recovery

For the purpose of determining recovery rates, an appropriate internal standard should be added to each sample at the beginning of the analytical procedure. The ideal internal standard is a CB which is not present in the sample and which does not interfere with other CBs. All 2,4,6-substituted CB congeners are, in principle, suitable. Alternatively, 1,2,3,4-tetrachloronaphthalene or the homologues of dichloroalkylbenzylether can be used. For GC with mass selective detection (GC-MSD), 13C-labelled CBs must be used as internal standards. With GC/MS, 13C-labelled CBs should preferably be used as internal standards.

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ANNEX B-12, APPENDIX 4: TECHNICAL NOTE ON THE DETERMINATION OF TRACE METALLIC ELEMENTS IN BIOTA

Attachment 1. Technical note on the determination of total mercury in marine biota by Cold Vapour Atomic Absorption Spectroscopy

1. INTRODUCTION

Metallic elements appear in different marine biological matrices in trace concentrations, ranging from the mg/kg through the ƒÊg/kg to the ng/kg level. Stoeppler (1991) provided a comprehensive review of the most frequently used techniques for quantitative analysis of metallic trace elements, such as optical atomic absorption, fluorescence or emission spectrometry, anodic, cathodic or adsorptive stripping voltammetry, isotope dilution mass spectrometry and total reflection X-ray fluorescence, respectively. In spite of the powerful instrumental techniques presently in use, various analytical error sources have to be taken into consideration that may significantly influence the accuracy of the analytical data.

2. WORKING CONDITIONS

For each step of the analytical procedure, contamination of the sample may occur from the environment (laboratory air dust particles and the analyst), from sample containers or packing materials, from instruments used during sample pre-treatment and sample preparation, and from the chemical reagents used for analysis.
The predominant purpose of the analytical clean laboratory is to eliminate contamination, which may be airborne or laboratory-induced, as far as possible and to control the total analytical blank. Contamination by particles from the laboratory air may be controlled by a high-efficiency particulate filter. (A clean room is designed to maintain air with 100 particles per ft3 or 3.6.103 per m3 of 0.5 ƒÊm particles (class 100 of U.S. Federal Standards 209), or better, preferably with a minimum of activity in the room.) U.S. Federal Standards 209 describes designs for complete laminar flow rooms, clean benches, and fume hoods, and contains information on design, testing, and maintenance of clean rooms, and should be considered an essential reference for those interested in a clean laboratory.

To control the analytical blank for analysis of metallic trace elements, one must not only maintain good laboratory air quality, but also select the appropriate composition and type of construction materials used to build the laboratory.
Principally, contaminants must be effectively removed at the source to minimize their uncontrolled distribution in the analytical clean laboratory. Accordingly, the laboratory's walls should be cleaned easily and therefore painted with special metal-free wipe-resistant paints. Surfaces of working areas should be protected with, for example, disposable plastic (polyethylene, PTFE) foils. The floors should, for example, be covered with adhesive plastic mats. Details of the design that are essential for obtaining a working laboratory with low trace element blanks are described by Moody (1982), Mitchell (1982a), Boutron (1990), and Schmidt and Gerwinski (1994).

3. Pretreatment of laboratory ware and reagents; contamination control'

Chemically resistant materials, used in the production of high-quality laboratory ware appropriate for metallic trace element analysis, include low- and high-density polyethylene (LDPE and HDPE),

polypropylene (PP), polytetrafluorethylene (PTFE), perfluoralkoxy (PFA), ethylenetetrafluorethylene (ETFE), tetrafluorethyleneper- fluorpropylene (FEP), borosilicate and quartz glass, respectively. With appropriate pretreatment and handling, these materials meet the requirements of purity necessary for the required analytical investigations. Cleaning procedures for plastic and glass laboratory ware were comprehensively dealt with by Moody and Lindstrom (1977), Tschopel et al. (1980), Kosta (1982) and Boutron (1990). Generally, immersion in diluted (10-25 % v/v) high-purity nitric acid at room temperature for a period of one to three days, followed by repeated rinsing with high-purity water, is recommended.
Steaming in high-purity acids (predominantly nitric acid) is also very effective to remove impurities from container surfaces and condition them for subsequent analysis.

The materials mentioned above for the production of laboratory ware exhibit some adsorptive or exchange properties. Boundary-surface interactions can be important, particularly when very dilute analytical solutions are being handled, since uncontrollable losses through sorption of element ions can occur (Tschopel et al., 1980; Harms, 1985). Based on this information, it is imperative that volumetric flasks, reagent vessels, pipette tips, etc., for handling samples, sample solutions and low-level reference or analyte solutions must never be used for transferring or processing stock calibration solutions, analytes solutions or concentrated reagents. Considerable quantities of analytes may be adsorbed from such solutions by the respective container surfaces, residuals of which may be leached later when dilute sample or analyte solutions are handled.

The availability of high-purity reagents is a key condition for reliable investigations of metallic trace element concentrations. For many analytical problems, the level of a specific contaminant can adequately be controlled only by applying specific purification methods.

The first order of priority in regard to high-purity reagents is a sufficient supply of high-purity water. Ion-exchange units are universally accepted as an effective means of removing dissolved ionic species from water. Since high-purity water is frequently used in metallic trace element analysis, equipment for sustainable production of high-purity water by high-purity mixed-bed ion exchange resins should be available.
The next most important group of reagents are mineral acids. Contamination of the sample by residual concentrations of metallic trace elements in the acids used for dissolution or decomposition represents a major problem. Purification of the acids is essential to ensure acceptable blanks.

Isothermal (isopiestic) distillation can produce volatile acids (and ammonia) of medium concentration in high-purity form. For example, pure hydrochloric acid (and ammonia) can be generated by placing an open container of concentrated reagent-grade acid adjacent to a container of high-purity water, within a closed system (such as a desiccator) at room temperature. Acid vapours are continuously transferred into the water until equilibrium is obtained. Purification by sub-boiling distillation is based on motionless evaporation of the liquid by infrared heating at the surface to prevent violent boiling. Different purification systems are described in detail by Matthinson (1972), Kuehner et al. (1972), Dabeka et al. (1976), Tschopel et al. (1980), Mitchell (1982b), Moody and Beary (1982), Moody et al. (1989), and Paulsen et al. (1989). Acids of extremely high purity are produced by multiple batchwise distillation of reagent-grade acids in a silica apparatus, which is placed in a laminar-flow hood.

4. SAMPLE PRETREATMENT

If the determinands are heterogeneously distributed in the sample material, it may be preferable to homogenize prior to taking subsamples for analysis. However, this procedural step is problematic, since uncontrollable contamination through the homogenizing tool may occur. Cryogenic homogenization at liquid nitrogen temperature and application of high-purity material such as quartz, PTFE, titanium or stainless steel for the construction of homogenizing devices may help to minimize contamination (Iyengar, 1976; Iyengar and Kasperek, 1977; Klussmann et al., 1985).

5. SAMPLE DECOMPOSITION

For accurate direct measurements of metallic trace element contents in biological matrices, appropriate calibration (reference) standards are lacking in most instances. Therefore, multi-stage, easy to calibrate methods are still necessary, which include decomposition procedures and transformation of biological material into solution.

As a general rule wet sample is to be subject to decomposition procedures to avoid contamination or loss of determinands. A general sample decomposition procedure cannot be recommended due to the diverse composition of materials to be analysed, as well as to the different elements to be determined, and also because of the variety of possible analytical methods applied. However, the following minimum requirements should be met:

- complete destruction of all organic material of the sample,
- avoidance of determinand losses,
- avoidance of contamination.

Complete decomposition of the organic matrix is a prerequisite for a variety of the subsequently used instrumental determination techniques. Residual dissolved organic carbon from biological materials incompletely disintegrated after decomposition with nitric acid causes problems particularly in voltammetric and polarographic determinations. Both are sensitive to interference from chelating and electroactive organic components coexisting in incompletely decomposed samples during analysis (Pratt et al., 1988; Wurfels et al., 1987, 1989). Residual dissolved organic carbon compounds even of low molecular weight can change the equilibria in the spray chambers for sample introduction in atomic emission spectrometry (AES), optical emission spectrometry (OES), and atomic absorption spectrophotometry (AAS) by changing the viscosity of the sample solution. In such cases, comparison with pure aquatic calibration standard solutions can lead to erroneous results. In graphite furnace atomic absorption spectrophotometry (GFAAS), residual organic carbon may undergo complicated secondary reactions with the analyte prior to or during the atomization process. Such 'matrix interferences' alter the rate at which atoms enter the optical path relative to that obtained for an undisturbed element standard (Harms, 1985; and other references cited there).

The comparatively simple dry ashing method using a muffle furnace is problematic, since both uncontrollable losses of the determinands and contamination through contact with the furnace material may occur.

Both, application of a carefully developed and controlled temperature programme and modifying the matrix prior to the ashing procedure (addition of ashing aids agents) may be suitable to prevent losses of volatile elements (special analytical problems concerning mercury determination are described in

Attachment 1). The use of special materials (quartz, titanium, stainless steel) for the construction of sample containers may be helpful to minimise contamination.

In the widely applied wet ashing procedure in open systems, the sample is treated with acids, mainly nitric, sulphuric and perchloric acids, in different ratios and under different conditions. Usually large quantities of reagents and voluminous apparatus with large surfaces are needed for complete destruction of the organic material. Serious contamination problems (too high blank values) may arise, if insufficiently purified acids are used.

The rate of reaction and efficiency of acid decomposition increase substantially with elevated temperatures. Accordingly, closed-vessel techniques, using conventional heating or microwave energy, have an advantage over open systems. As a result of the closed systems with vessels manufactured of dense and very pure material (PTFE, PFA, quartz), loss of elements through volatilisation and contamination by desorption of impurities from the vessel surface are significantly reduced. In addition, since only small quantities of high-purity acid (usually nitric acid) need to be used, extremely low analytical blanks can be obtained.

Kingston and Jassie (1986, 1988) comprehensively considered the fundamental parameters governing closed vessel acid decomposition at elevated temperatures using a microwave radiation field. Microwave systems enable a very fast energy transfer to the sample and a very rapid build up of high internal vessel temperature and pressure, with the advantage of an enormous reduction in digestion time occurs. Furthermore, a reduction of acid volume (McCarthy and Ellis, 1991) and contamination reduction during the decomposition process were found (Dunemann, 1994; Sheppard et al., 1994).

The application of microwave energy must be carefully controlled to avoid explosions; a pressure-relief system is recommended for safe operation (Gilman and Grooms, 1988). At this stage of development, it can be concluded that advances in pressure and temperature feedback control features have contributed to the acceptance of microwave sample decomposition in analytical chemistry.

6. CALIBRATION

For calibration purposes, single element standard stock solutions at a concentration of 1000 mg/l, purchased from a qualified manufacturer, should be available. The actual concentration of the named element should be stated on the label together with the date of the preparation of the standard solution.

Fresh stock standard solutions should be compared with the old standard solutions. Traceability can be ensured by the use of CRM(s) or participation in intercomparison exercises (EURACHEM, 2003).

Single or mixed element working standard solutions for calibration purposes are prepared by dilution of the standard stock solutions using dilute acid, as required.

Both stock standard and working standard solutions are stored in polyethylene, borosilicate or quartz volumetric flasks. Working standard solutions at concentrations less than 100 ƒÊg/l should be freshly prepared for every batch of samples and kept no longer than two weeks.

The calibration procedure must meet some basic criteria in order to give the best estimate of the true (but unknown) element concentration of the sample analysed. These criteria are as follows:

- The amounts or concentrations of standards for the establishment of the calibration function must cover the range as related to practical conditions. The mean of the range should be roughly equal to the expected analyte concentration in the sample.
- The required analytical precision must be achievable and known throughout the entire range.
- The measured value (response) at the lower end of the range must be significantly different from the procedural analytical blank.
- The chemical and physical properties of the calibration standards must closely resemble those of the sample under investigation.
- The calibration standards must be processed through the entire analytical procedure in the same manner as the sample.
- The standard addition technique should be used only under very special circumstances (Cardone, 1986a, 1986b).

7. DETERMINATION

In an analytical series, especially with the number of samples >10, the control of calibration settings should be carried out with 2-3 calibration solution between environmental 10 samples. The analytical series should contain also a control sample of LRM or CRM.

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ATTACHMENT 1. TECHNICAL NOTE ON THE DETERMINATION OF TOTAL MERCURY IN MARINE BIOTA BY COLD VAPOUR ATOMIC ABSORPTION SPECTROSCOPY

1. POSSIBILITIES OF USING COLD VAPOUR ATOMIC ABSORPTION SPECTROMETRY IN TOTAL MERCURY ANALYSIS

The most widely used method for the determination of total mercury in biological tissues is cold vapour atomic absorption spectrometry (CV-AAS), based on a technique elaborated in detail by Hatch and Ott (1968). In this method, (divalent) ionic mercury is reduced to its metallic form (Hg^o) in acidic solution using a powerful reducing agent. Subsequently, the elemental mercury is volatilized (purged) by a carrier gas and transported into an absorption cell, where the 253.65 nm wavelength absorbance of mercury atoms is measured.

CV-AAS analysis can be performed manually using batch CV-AAS or automatically using flow injection (FI) techniques. FI is a very efficient approach for introducing and processing liquid samples in atomic absorption spectrometry. The FI technique, combined with a built-in atomic absorption spectrometer optimised for mercury determination, reduces sample and reagent consumption, has a higher tolerance of interferences, lower determination limits and improved precision compared with conventional cold vapour techniques.

The efficiency of various flow injection mercury systems has been reported by several groups (Tsalev *et al*., 1992a, 1992b; Welz *et al*., 1992; Guo and Baasner, 1993; Hanna and McIntosh, 1995; Kingston and McIntosh, 1995; Lippo *et al*., 1997).

Better sensitivities of both conventional CV-AAS and FI-CV-AAS can be obtained by collecting mercury vapour released from the sample solution on a gold adsorber (Welz and Melcher, 1984). This so-called amalgamation technique eliminates kinetic interferences due to a different vaporization rate or a different distribution function of the elemental mercury between the liquid and the gaseous phases. The amalgamation ability of the gold adsorber must be carefully and regularly checked. Volatile compounds (in particular sulfur-containing compounds) evaporating together with the elemental mercury from the sample solution may deactivate the adsorber surface. This means an increased risk of underestimation, as unknown quantities of mercury are not collected by the adsorber.

2. SAMPLE PRETREATMENT

It is generally agreed that oxidative conversion of all forms of mercury in the sample to ionic Hg(II) is necessary prior to reduction to elemental Hg and its subsequent measurement by CV-AAS. Therefore, the initial procedural step in mercury analysis is a sample pretreatment, which is aimed at liberating the analyte element from its chemical bonding to the organic matrix and thus transforming all of the analyte species into a well-defined oxidation state. For this purpose, a wide variety of combinations of strong acids (HCl, H_2SO_4, HNO_3) and oxidants (H_2O_2, $KMnO_4$, $K_2Cr_2O_7$, $K_2S_2O_8$) have been tested and recommended (Kaiser *et al*., 1978; Harms, 1988; Vermeir*et al*., 1989; Ping and Dasgupta, 1989; Baxter and Frech, 1990; Landi *et al*., 1990; Navarro *et al*., 1992; Lippo *et al*., 1997).

A suitable sample pretreatment, which implies the complete transformation of all organomercury species into inorganic mercury ions, requires the following:

- oxidation mixtures with a high oxidation potential;
- rapid oxidation (usually promoted by high reaction temperatures), preferably in closed systems;
- compatibility with CV-AAS techniques;
- stability of sample solutions during storage (at least short term);
- no formation of solid reaction products.

On-line sample pretreatment is of particular interest in total mercury determinations because it allows reduction of the well-known problems associated with the inherent risk of contamination, and volatilization and adsorption losses. At present, suitable procedures for on-line pretreatment of solid biological samples are lacking. However, several authors (Tsalev *et al*., 1992a 1992b; Welz *et. al*., 1992; Guo and Baasner, 1993) have demonstrated that microwave digestion coupled with FI-CV-AAS can successfully be applied to the analysis of liquid samples.

3. CONTROL OF CONTAMINATION AND ANALYTE LOSSES

Major difficulties arise due to the mobility and reactivity of mercury and its compounds, respectively, during sample preparation, sample pretreatment, and analysis. Therefore, the stability of samples and standard solutions is of prime importance, and it is advisable to test the stability of typical standard and sample solutions under typical laboratory conditions.

Mercury can disappear from solution due to several mechanisms, including volatilization of mercury compounds, reduction of such compounds followed by volatilization of elemental (metallic) mercury, adsorption on container walls, adsorption onto colloids or particles, incorporation into stable chemical complexes, or incorporation, upon reduction, into stable amalgams.

Thermodynamic considerations of Toribara *et al*. (1970) showed that loss of mercury from a solution containing the element in the monovalent form may occur readily through disproportion and subsequent loss of metallic mercury. Because of the high oxidation potential of the mercury(II)-mercury(I) system, almost any reducing substance could convert some divalent mercury ions into monovalent mercury ions, which then spontaneously disproportion into mercury(II) and mercury(o). The latter escape as metallic vapour from the solution into the gas phase. Because of the almost impossibility of preventing the introduction of small amounts of reducing substances by reagents or solvents, the more dilute mercury(II) solutions would be less stable and lose mercury more readily. The only practical method for stabilizing such solutions is to add a small excess of an oxidising substance (such as permanganate), which has a higher oxidation potential than the mercury(II)-mercury(I) system.

Similarly, Feldman (1974) concluded from his experiments that solutions with 0.1 µg divalent Hg dm-3 in distilled water could be stored in glass vials for as long as five months without deteriorating if the solutions contained 5 % (v/v) HNO_3 and 0.01 % $Cr_2O_7^{2-}$. Storage of such solutions was safe in polyethylene vials for at least 10 days if the solutions contained 5 % (v/v) HNO_3 and 0.05 % $Cr_2O_7^{2-}$. The efficiency of this mixture was probably due to its ability to prevent the hydrolysis of dissolved mercury and prevent its reduction to valencies lower than +2.

4. REDUCING REAGENTS

Tin(II) chloride and sodium tetrahydroborate are predominantly used as reducing reagents for the determination of total mercury by CV-AAS. Sodium tetrahydroborate has been found advantageous for several applications owing to its higher reducing power and faster reaction (Toffaletti and Savory, 1975). In addition, this reductant has been successfully used even in the presence of interfering agents such as iodide and selenium (Kaiser *et al*., 1978). However, potential interferences can occur from metal ions (e.g., Ag(I), Cu(II), Ni(II)), which are themselves reduced to the metallic state and so may occlude mercury through amalgamation.

Welz and Melcher (1984) showed that sodium tetrahydroborate could more readily attack those organic mercury compounds which were not reduced to metallic mercury by tin(II) chloride. However, they stated that sodium tetrahydroborate could not be recommended as the reducing reagent for the amalgamation technique. They found that, due to the rather violent reaction with sodium tetrahydroborate, fine droplets of the sample solution were carried by the gas stream and contaminated or deactivated the adsorber surface. Further, they considered even more important the fact that not only mercury but all gaseous hydride-forming elements (e.g., arsenic, antimony, selenium) were volatilized when sodium tetrahydroborate was used as reductant. These hydrides reacted with the adsorber material and deactivated its surface, thus no longer permitting a sensitive and reproducible determination of mercury.

5. INTERFERENCES

Interferences by volatile nitrogen oxides in the determination of mercury by FI-CV-AAS were studied by Rokkjaer et al. (1993). The main symptom of the interference effects was a suppression, broadening or even splitting of the mercury signal. The authors postulated that volatile nitrogen oxides formed as reaction products of nitric acid during sample decomposition scavenged the reducing agent and concomitantly inhibited the reduction of mercury(II). The rate of the reaction of nitrogen oxides with the reducing agent was considered to be so fast that it was consumed before the reduction of mercury was complete. Rokkjaer et al. (1993) demonstrated that the interference could easily be remedied by purging the sample solution with an inert gas prior to the introduction of the reducing agent. Lippo et al. (1997) concluded from their experiments that nitrogen mono- and dioxide, having molecular absorption bands at 253.63 nm and 253.85 nm, respectively, might cause unspecific absorption at the specific mercury wavelength of 253.65 nm, leading to enhanced and broadened mercury signals if not properly compensated for by adequate instrumental background correction.

6. INTERNAL (ROUTINE) QUALITY CONTROL

In order to demonstrate that the analytical method applied is fit for the purpose of the investigations to be carried out, control materials should be regularly analysed alongside the test materials (cf. Chapter B.5 of the Manual).

The control materials - preferably certified reference materials (CRM) - should be typical of the test materials under investigation in terms of chemical composition, physical properties and analyte concentration. Fitness for purpose is achieved if the results obtained from the analysis of the control materials are within the defined limits of permissible tolerances in analytical error (see Chapters B.3.5, B.4.2.5 and B.4.2.5.2b of the Manual).

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ANNEX B-12 APPENDIX 5. TECHNICAL NOTE ON THE DETERMINATION OF TOTAL MERCURY IN MARINE BIOTA

1. GENERAL REMARKS TO THE AVAILABLE ANALYTICAL METHODS

The most widely used method for the determination of total mercury in biological tissues and sediments is cold vapour atomic absorption spectrometry (CV-AAS), based on a technique elaborated in detail by Hatch and Ott (1968). In this method, (divalent) ionic mercury is reduced to its metallic form (Hg^o) in acidic solution using a powerful reducing agent. Subsequently, the elemental mercury is volatilized (purged) by a carrier gas and transported into an absorption cell, where the 253.65 nm wavelength absorbance of mercury atoms is measured.

More recent atomic fluorescence (AFS) was introduced as an alternative detection method. It is part of the US EPA Method 1631 Revision E (US EPA 2002), which describes the measurement of trace concentrations of mercury in water samples. Comparing both methods the CV-AAS is may be more appropriate for samples containing higher levels of mercury and the CV-AFS method more for lower level samples.

Analysis can be performed manually using batch operation or automatically using flow injection (FI) techniques. FI is a very efficient approach for processing and introducing liquid samples into the detector. The FI technique, combined with a built-in detector optimised for mercury determination, reduces sample and reagent consumption, has a higher tolerance of interference's, lower determination limits and improved precision compared with conventional cold vapour techniques.

The efficiency of various flow injection mercury systems has been reported by several groups (Tsalev *et al*., 1992a, 1992b; Welz *et al*., 1992; Guo and Baasner, 1993; Hanna and McIntosh, 1995; Kingston and McIntosh, 1995; Lippo *et al*., 1997).

Further improvement of sensitivity could be reached by the insertion of an amalgamation unit between reduction unit and detector. In this case the mercury vapour, released from the sample solution, will be pre concentrated on a gold adsorber (Welz and Melcher, 1984). This technique eliminates kinetic interferences due to a different vaporization rate or a different distribution function of the elemental mercury between the liquid and the gaseous phases. The amalgamation ability of the gold adsorber must be carefully and regularly checked, because volatile compounds, sulfur-containing in particular,evaporating together with the elemental mercury from the sample solution and may deactivate the adsorber surface. This means an increased risk of underestimation, as unknown quantities of mercury are not collected by the adsorber.

2. SAMPLE PRE-TREATMENT

It is generally agreed that oxidative conversion of all forms of mercury in the sample to ionic Hg (II) is necessary prior to reduction to elemental Hg and its subsequent measurement by CV-techniques. Therefore, the initial procedural step in mercury analysis is a sample pre-treatment, which is aimed at liberating the analyte element from its chemical bonding to the organic matrix and thus transforming all of the analyte species into a well-defined oxidation state. For this purpose, a wide variety of combinations of strong acids (HCl, H_2SO_4, and HNO_3) and combustion with oxidants (H_2O_2, $KMnO_4$, $K_2Cr_2O_7$, $K_2S_2O_8$) have been tested and recommended (Kaiser *et al*., 1978; Harms, 1988; Vermeir *et al*., 1989; Ping and Dasgupta, 1989; Baxter and Frech, 1990; Landi *et al*., 1990; Navarro *et al*., 1992; Lippo*et al*., 1997).

A suitable sample pre-treatment, which implies the complete transformation of all organomercury species into inorganic mercury ions, requires the following:

- oxidation mixtures with a high oxidation potential;
- rapid oxidation (usually promoted by high reaction temperatures), preferably in closed systems;
- compatibility with CV techniques;
- stability of sample solutions during storage (at least short term);
- no formation of solid reaction products

Several authors (Tsalev *et al*., 1992a 1992b; Welz *et. al*., 1992; Guo and Baasner, 1993) have demonstrated that microwave digestion can be successfully applied to the analysis of liquid samples.

3. CONTROL OF CONTAMINATION AND ANALYTE LOSSES

Major difficulties arise due to the mobility and reactivity of mercury and its compounds, respectively, during sample preparation, sample pre-treatment, and analysis. Therefore, the stability of samples and standard solutions is of prime importance, and it is advisable to test the stability of typical standard and sample solutions under typical laboratory conditions.

Mercury can disappear from solution due to several mechanisms, including volatilization of mercury compounds, reduction of such compounds followed by volatilization of elemental (metallic) mercury, adsorption on container walls, adsorption onto colloids or particles, incorporation into stable chemical complexes, or incorporation, upon reduction, into stable amalgams.

Thermodynamic considerations of Toribara *et al*. (1970) showed that loss of mercury from a solution containing the element in the monovalent form may occur readily through disproportion and subsequent loss of metallic mercury. Because of the high oxidation potential of the mercury(II)-mercury(I) system, almost any reducing substance could convert some divalent mercury ions into monovalent mercury ions, which then spontaneously disproportion into mercury(II) and mercury(o). The latter escapes as metallic vapour from the solution into the gas phase. Because of the almost impossibility of preventing the introduction of small amounts of reducing substances by reagents or solvents, the more dilute mercury(II) solutions would be less stable and lose mercury more readily. The only practical method for stabilizing such solutions is to add a small excess of an oxidizing substance (such as permanganate), which has a higher oxidation potential than the mercury(II)-mercury(I) system.

Similarly, Feldman (1974) concluded from his experiments that solutions with 0.1 µg divalent Hg dm^{-3} in distilled water could be stored in glass vials for as long as five months without deteriorating if the solutions contained 5 % (v/v) HNO_3 and 0.01 % $Cr_2O_7^{2-}$. Storage of such solutions was safe in polyethylene vials for at least 10 days if the solutions contained 5 % (v/v) HNO_3 and 0.05 % $Cr_2O_7^{2-}$. The efficiency of this mixture was probably due to its ability to prevent the hydrolysis of dissolved mercury and prevents its reduction to valances lower than +2.

4. REDUCING REAGENTS

Tin(II) chloride and sodium tetrahydroborate are predominantly used as reducing reagents for the determination of total mercury by CV-Methods. Sodium tetrahydroborate has been found advantageous for several applications owing to its higher reducing power and faster reaction (Toffaletti and Savory, 1975). In addition, this reductant has been successfully used even in the presence of interfering agents such as iodide and selenium (Kaiser *et al*., 1978). However, potential interferences can occur from metal ions (e.g., Ag(I), Cu(II), Ni(II)), which are themselves reduced to the metallic state and so may occlude mercury through amalgamation.

Welz and Melcher (1984) showed that sodium tetrahydroborate could more readily attack those organic mercury compounds which were not reduced to metallic mercury by tin(II) chloride. However, they stated that sodium tetrahydroborate could not be recommended as the reducing reagent for the amalgamation technique. They found that, due to the rather violent reaction with sodium tetrahydroborate, fine droplets of the sample solution were carried by the gas stream and contaminated or deactivated the adsorber surface. Further, they considered even more important the fact that not only mercury but all gaseous hydride-forming elements (e.g., arsenic, antimony, and selenium) were volatilized when sodium tetrahydroborate was used as reductant. These hydrides reacted with the adsorber material and deactivated its surface, thus no longer permitting a sensitive and reproducible determination of mercury.

5. INTERFERENCES

Interferences by volatile nitrogen oxides in the determination of mercury by FI-CV-AAS were studied by Rokkjaer *et al*. (1993). The main symptom of the interference effects was suppression, broadening or even splitting of the mercury signal. The authors postulated that volatile nitrogen oxides formed as reaction products of nitric acid during sample decomposition scavenged the reducing agent and concomitantly inhibited the reduction of mercury(II). The rate of the reaction of nitrogen oxides with the reducing agent was considered to be so fast that it was consumed before the reduction of mercury was complete. Rokkjaer *et al*. (1993) demonstrated that the interference could easily be remedied by purging the sample solution with an inert gas prior to the introduction of the reducing agent.

Lippo *et al*. (1997) concluded from their experiments that nitrogen mono- and dioxide, having molecular absorption bands at 253.63 nm and 253.85 nm, respectively, might cause unspecific absorption at the specific mercury wavelength of 253.65 nm, leading to enhanced and broadened mercury signals if not properly compensated for by adequate instrumental background correction.

The presence of water vapour in the fluorescence detector may produce scattering effects, positive interferences and degradation of the analytical signal. The use of a dryer tube is required to remove any water vapour from the flow before reaching the detector.

6. INTERNAL (ROUTINE) QUALITY CONTROL

In order to demonstrate that the analytical method applied is fit for the purpose of the investigations to be carried out, control materials should be regularly analysed alongside the test materials (cf. Chapter 5, 6).

The control materials--preferably certified reference materials--should be typical of the test materials under investigation in terms of chemical composition, physical properties and analyte concentration.

Fitness for purpose is achieved if the results obtained from the analysis of the control materials are within the defined limits of permissible tolerances in analytical error (see Chapters 5).

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ANNEX B-13 TECHNICAL NOTE ON THE DETERMINATION OF HEAVY METALS AND PERSISTENT ORGANIC COMPOUNDS IN MARINE SEDIMENTS

- Appendix 1. Technical note on the determination of polycyclic aromatic hydrocarbons in marine sediment
- Appendix 2. Technical note on the determination of chlorinated biphenyls in marine sediment
- Appendix 3. Technical note on the determination of heavy metals in marine sediment

ANNEX B-13 APPENDIX 1. TECHNICAL NOTE ON THE DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHS) IN SEDIMENT

1. INTRODUCTION

This Technical Note provides advice on PAH analysis in total sediment, sieved fractions, and suspended particulate matter. The analysis of polycyclic aromatic hydrocarbons (PAHs) in sediments generally includes extraction with organic solvents, clean-up, and high performance liquid chromatography (HPLC) with ultraviolet or fluorescence detection or gas chromatographic separation (GC) with flame ionization (FID) or mass spectrometric (MS) detection (Fetzer and Vo-Dinh, 1989; Wise *et al.,* 1995). All steps in the procedure are susceptible to insufficient recovery and/or contamination. Quality control procedures are recommended in order to check the performance of the method. These guidelines are intended to encourage and assist analytical chemists to critically reconsider their methods and to improve their procedures and/or the associated quality control measures, where necessary.

These guidelines are not intended as a complete laboratory manual. If necessary, guidance should be sought from highly specialized research laboratories. Whichever procedure is adopted, each laboratory must demonstrate the validity of each step of its procedure. In addition, the use of a second (and different) method, carried out concurrently to the routine procedure, is recommended for validation. The analyses should be carried out by experienced staff.

2. SAMPLING AND STORAGE

The major criterion for successful sediment sampling is to guarantee a fairly undisturbed sample stratification. (For further details about sampling, see “Technical note on the determination of heavy metals in marine sediments”.) Plastic materials must not be used for sampling or storage owing to possible adsorption of the PAHs onto the container material. Samples should be transported in closed containers and at temperatures between 5 °C and 15 °C, preferably below 10 °C. If the samples are not analysed within 48 hours after collection, they must be stored at 4 °C (short-term storage). Storage over several months is only possible for frozen (i.e., below -20 °C) and/or dried samples (Law and de Boer, 1995).

As PAHs are sensitive to photo-degradation, exposure to direct sunlight or other strong light must be avoided during storage of the samples as well as during all steps of sample preparation, including extraction and storage of the extracts (Law and Biscaya, 1994). The use of amber glassware is strongly recommended.

3. BLANKS AND CONTAMINATION

The procedural detection limit is determined by the blank value. In order to keep the blank value as low as possible, PAHs or other interfering compounds should be removed from all glassware, solvents, chemicals, adsorption materials, etc., that are used in the analysis. The following procedures should be used:

- Glassware should be thoroughly washed with detergents and rinsed with an organic solvent prior to use. Further cleaning of the glassware, other than calibrated instruments, can be carried out by heating at temperatures > 250 °C.
- All solvents should be checked for impurities by concentrating the amount normally used to 10 % of the normal end volume. This concentrate can then be analysed by HPLC or GC and should not contain significant amounts of PAHs or other interfering compounds.
- All chemicals and adsorption materials should be checked for impurities and purified (e.g., by heating or extraction), if necessary. Soxhlet thimbles should be pre-extracted. Glass fiber thimbles are preferred over paper thimbles. Alternatively, full glass Soxhlet thimbles, with a G1 glass filter at the bottom, can be used. The storage of these supercleaned materials for a long period is not recommended, as laboratory air can contain PAHs that will be adsorbed by these materials. Blank values occurring despite all the above-mentioned precautions may be due to contamination from the air. The most volatile compounds will usually show the highest blanks (Gremm and Frimmel, 1990).

4. PRETREATMENT

Before taking a subsample for analysis, the samples should be sufficiently homogenized. The intake mass is dependent on the expected concentrations. For the marine environment, as a rule of thumb, the mass of sample taken for analysis can be equal to an amount representing 50–100 mg organic carbon.

PAHs can be extracted from wet or dried samples. However, storage, homogenization, and extraction are much easier when the samples are dry.

Drying the samples at ambient or elevated temperatures as well as freeze-drying may alter the concentrations, e.g., by contamination or by loss of compounds through evaporation (Law *et al.,* 1994). Possible losses and contamination have to be checked. Contamination can be checked by exposing 1–2 g CIS-bonded silica to drying conditions and analysing it as a sample (clean-up can be omitted) (Smedes and de Boer, 1998). Contamination during freeze-drying is reduced by placing a lid, with a hole about 3 mm in diameter, on the sample container, while evaporation of the water is not hindered.

Chemical drying of samples can be performed by grinding with Na2SO4, or MgSO4 until the sample reaches a sandy consistency. It is essential that at least several hours elapse between grinding and extraction to allow for complete dehydration of the sample. Residual water will decrease extraction efficiency.

5. EXTRACTION AND CLEAN-UP

Exposure to light must be kept to a minimum during extraction and further handling of the extracts (Law and Biscaya, 1994). Since photo-degradation occurs more rapidly in the absence of a sample matrix, first of all the standard solution used for checking the recovery of the procedure will be affected, allowing a proper detection of the influence of light. The most photo-sensitive PAH is benzo[*a*]pyrene, followed by anthracene.

5.1 EXTRACTION OF WET SEDIMENTS

A commonly used and very efficient method for PAH extraction from sediments is alkaline saponification; apart from having a short extraction time (approximately 1.5 hrs under the reflux), it also eliminates organic sulphur and other interfering compounds such as lipids and yields an extract that is relatively easy to clean up.

Wet sediments could also be extracted using a stepwise procedure by mixing with organic solvents. Extraction is enhanced by shaking, Ultra Turrax mixing, ball mill tumbling or ultrasonic treatment. Water-miscible solvents, such as acetone, methanol, or acetonitrile, are used in the first step. The extraction efficiency of the first step will be low as there is a considerable amount of water in the liquid phase. For sufficient extraction, at least three subsequent extractions are needed. The contact time with the solvent should be sufficient to complete the desorption of the PAHs out of the sediment pores. The contact time for the desorption of PAHs from sediments may vary with sediment type up to 24 hrs. If there is any doubt, a second extraction step should be performed and quantities of PAHs in the two extracts combined.

The contact time of the sediment with the solvent can be reduced by using microwave heating or a Soxhlet apparatus. When utilizing Soxhlet, the extraction of wet sediments should be conducted in two steps. First, a polar solvent, such as acetone, is used to extract the water from the sediment, then the flask is replaced and the extraction continued with a less polar solvent or solvent mixture (e.g., acetone/hexane). Thereafter, the extracts must be combined.

For both batch and Soxhlet extraction, water must be added to the combined extracts and the PAHs must be extracted to a non-polar solvent.

5.2 EXTRACTION OF DRY SEDIMENTS

Although all the methods mentioned above can also be used for dried sediments, Soxhlet extraction is the most frequently applied technique to extract PAHs from dried sediments. Medium-polar solvents such as dichloromethane or toluene, or mixtures of polar and non-polar solvents can be used. When using dichloromethane, losses of PAHs have occasionally been observed. Although toluene is not favoured because of its high boiling point, it should be chosen as solvent when it is expected that sediment samples contain soot particles. For routine marine samples, the use of a mixture of a polar and a non-polar solvent (e.g., acetone/hexane (1/3, v/v)) is recommended.

The extraction can be carried out with a regular or a hot Soxhlet (Smedes and de Boer, 1998). A sufficient number of extraction cycles must be performed (approximately 8 hours for the hot Soxhlet and 12 to 24 hours for the normal Soxhlet). The extraction efficiency has to be checked for different types of sediments by a second extraction step. These extracts should be analysed separately. A recovery during the first extraction step of over 90 % is considered adequate.

All the methods described, both for wet and dry samples, are in principle suitable for the extraction of PAHs from sediments. However, Soxhlet extraction is recommended over mixing methods, especially for dry samples. For naphthalene, which can easily be lost in several steps of the sample preparation, headspace or purge and trap analysis might provide a suitable alternative to extraction methods.

5.3 CLEAN-UP

The crude extract requires a clean-up to remove the many other compounds that are co-extracted (Wise *et al.,* 1995). Due to chlorophyll-like compounds extracted from the sediment, the raw extract will be coloured and will also contain sulphur and sulphur-containing compounds, oil, and many other natural and anthropogenic compounds. Selection of the appropriate clean-up method depends on the subsequent instrumental method to be used for analysis. Prior to the clean-up, the sample must be concentrated and polar solvents used in the extraction step should be removed. The recommended acetone/hexane mixture will end in hexane when evaporated because of the formation of an azeotrope. Evaporation can be done using either a Kuderna-Danish or a rotary evaporator. Especially for the latter, care should be taken to stop the evaporation in time at about 5 cm3. For further reducing the volume, a gentle stream of nitrogen should be applied. The extract should never be evaporated to dryness.

For removing more polar interferences from the extract, deactivated aluminium oxide (10 % water), eluted with hexane, as well as silica or modified silica columns, e.g., aminopropylsilane, eluted with toluene or a semipolar solvent mixture such as hexane/acetone (95/5, v/v) or hexane/dichloromethane (98/2, v/v) can be used. Gel permeation chromatography (GPC) can be used to remove high molecular weight material and sulphur from the extracts.

When using HPLC/fluorescence detection, for the majority of samples polar interferences can be removed from the extract using an aluminium oxide (deactivated with 10 % water) column that is eluted with hexane. If interferences appear to be present in the chromatogram, a clean-up combination of silica and a cyanopropyl phase, eluted with, e.g., hexane/acetone, is suitable. For GC/MS analysis, sulphur should be removed from the extracts, in order to protect the detector. This can be achieved by the addition of copper powder, wire or gauze during or after Soxhlet extraction. Ultrasonic treatment might improve the removal of sulphur. As an alternative to copper, other methods can be used (Smedes and de Boer, 1998).

Analysis by GC or HPLC/UV requires a more elaborate clean-up. Aliphatic hydrocarbons originating from mineral oil interfere with the flame ionization detection. They can be removed from the extract by fractionation over columns filled with activated aluminium oxide or silica. The first fraction eluting with hexane is rejected. The PAHs elute in a second fraction with a more polar solvent, e.g., diethylether or acetone/hexane. When applying fractionation, the elution pattern has to be checked frequently. This should be carried out in the presence of sample matrix, as that can partially deactivate the clean-up column, resulting in earlier elution of the PAHs than in the standard solution.

Alkylated PAHs are difficult to remove from extracts by column clean-up. When excessive amounts of these compounds are present, they may interfere with HPLC analysis and such samples are better analysed by GC/MS. An alternative could be preparative HPLC fractionation using a normal phase silica, cyanopropyl or aminopropyl column. After clean-up, the eluate or fractions must be concentrated to 1–2 cm3. Concentrating the extract by evaporation, e.g., in a rotary evaporator, can easily result in losses of PAHs. Care should be taken that the extracts are never evaporated to dryness and the water bath temperature should be carefully controlled (< 30 ºC).

HPLC and GC require different solvents for injection of the extract. The methods suggested above all yield an extract in which non-polar solvents are dominant. In HPLC, even small amounts of non-polar solvents result in a shift of retention time and broadening of the peaks (Reupert and Brausen, 1994). As for solvent exchange, evaporation to dryness must be avoided; hexane should be removed by the addition of 5 cm3 acetonitrile for each cm3 of extract and subsequent evaporation to 1–2 cm3. Azeotropic evaporation leaves only acetonitrile. Although this also works with methanol, acetonitrile is preferred because PAHs show a better stability when dissolved in acetonitrile. Azeotropic exchange can also be applied the other way round. In that case, 5 cm3 hexane must be added for each cm3 of acetonitrile. For GC methods, iso-octane or toluene are suitable solvents for injection and can already be added, before evaporation to the required volume, as a keeper.

5.4 EXTRACTION EFFICIENCY AND CLEAN-UP

A check on extraction efficiency and clean-up can be performed by analysing a reference material. To determine the recovery rates of the clean-up and concentration steps of each sample series, a standard solution should be put through the entire procedure. It is recommended to always use two, and preferably three, internal standards: hydrocarbons of small, medium, and high molecular weight, e.g., naphthalene-d8, phenanthrene-d10, perylene-d12, to check for recovery during the analytical procedures. If major losses have occurred, then the results obtained should not be reported.

6. CHROMATOGRAPHIC DETERMINATION

The separation of PAHs should be optimized for at least the compounds listed in Annex B-13 (Appendix 1, Table 1) (Keith and Telliard, 1979). Separation should not only be optimized for a standard solution but also for a sample, as samples often contain several non-target PAHs that should be separated from the target compounds, if possible.

In the guidelines, both the HPLC-fluorescence and GC/MS methods are considered to be equally valid approaches. Although this may be the case for the parent PAHs, it is certainly not the case for alkylated species, as this range of compounds cannot be satisfactorily analysed using HPLC. This is particularly relevant for the future as additional PAHs, including both additional parent compounds of 5- and 6-rings, and the alkylated PAHs gain increasing interest.

6.1 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

For adequate HPLC analysis of PAHs, the equipment should meet some minimum requirements. At a minimum, a binary gradient is necessary to achieve proper separation. Using HPLC and measuring concentrations with the peak height, a 50 % valley should be considered as adequate separation. Solvents must be degassed in order to allow proper operation of the high pressure pump. Sample injection should be carried out with an autosampler.

6.1.1 COLUMNS

The column specifications are:

- stationary phases: e.g., octadecylsilane (RP-18), or special PAH column material;
- length: 15–25 cm;
- inner diameter: 4.6 mm or less;
- particle size: 5 µm or less.

Columns with diameters smaller than 4.6 mm can be chosen in order to reduce the flow of the eluent and thus save solvents, if the dimensions of the detector cell and the tubings are appropriate. When using a smaller diameter column, the amount injected should also be reduced (e.g., 25–50 µl for a 4.6 mm column, 10 to 20 µl for a 3 mm column).

6.1.2 ELUTION

At a minimum, a binary gradient is necessary to allow for a proper separation. For elution, e.g., methanol/water or acetonitrile/water can be applied. Acetonitrile allows more rapid flow, but presents a greater health risk than methanol. A typical gradient (1–1.5 ml min-1 for a 4.6 mm column) starts at 50 % methanol/water or acetonitrile/water and runs to 100 % methanol or acetonitrile in 40 minutes, where it remains for 20 minutes and then returns to the initial conditions again for about 5 minutes. Prior to the next injection, the equilibrium time should be about 5–10 minutes (3–5 times the dead volume).

100 % methanol or acetonitrile may not be sufficient to elute all non-target compounds from the column, resulting in peaks that disturb the baseline in the subsequent chromatogram. To avoid this, a further elution step using acetone/methanol (1/1) or acetonitrile/acetone (1/1) can be applied. A ternary gradient is then necessary.

In order to obtain reproducible retention times, the equilibrium time after each run should be constant. Therefore, automatic injection is strongly recommended. In addition, a thermostated column compartment (10–30 °C) should be used. Not only retention times but also the resolution between some PAHs can be affected by varying the temperature.

6.1.3 DETECTION

For the detection of PAHs, the more sensitive and selective fluorescence detector is preferred to a UV detector. The excitation and emission wavelengths should be programmable to allow the detection of PAHs at their optimum wavelength (Reupert and Brausen, 1994; ISO, 1995). However, when PAHs elute close to each other, wavelength switching cannot be carried out between these peaks and a wavelength pair appropriate for the respective compounds has to be chosen. The use of two detectors in series, or running the analysis twice with different wavelength programmes, can minimize the need for such compromises.

As the fluorescence signals of some PAHs can decrease by up to a factor of ten in the presence of oxygen, the eluents must be degassed thoroughly. This can be done either by continuously passing a gentle stream of helium through the eluents or using a commercially available vacuum degasser. In addition, after degassing the eluents, they should not pass PTFE tubings, as this material is permeable to oxygen and allows oxygen to enter the system again. The use of stainless steel or PEEK (polyetheretherketone) tubing is recommended.

Acenaphthylene is not detectable with fluorescence. A UV or diode-array detector can be used for detection.

6.2 GAS CHROMATOGRAPHY

6.2.1 COLUMNS

Column dimensions for the determination of PAHs should be the following:

- length: minimum 25 m;
- inner diameter: maximum 0.25 mm;
- film thickness: between 0.2 µm and 0.4 µm;
- stationary phases: A wide range of non-polar or slightly polar stationary phases can be used for the separation of PAHs, e.g., a 5 % phenyl-substituted methyl polysiloxane phase.

Better resolution can be obtained by increasing the length and reducing the inner diameter to 0.20 mm or less. Below a diameter of 0.15 mm, the carrier gas pressure rises to values greater than 500 kPa, which are not compatible with normal GC equipment. Also, the risk of leakages increases.

6.2.2 CARRIER GAS

Preferably helium should be used as the carrier gas for GC/MS. When using columns with very small inner diameters, the use of hydrogen is essential. The linear gas velocity should be optimized. Appropriate settings for 0.25 mm i.d. columns range from 20–40 cm s-1 and for 0.15 mm i.d. columns from 30–50 cm s-1.

6.2.3 INJECTION TECHNIQUES

An autosampler should be used for injection. The two systems commonly used are splitless and on-column injection. Other techniques such as temperature-programmed or pressure-programmed injection may have additional advantages, but should be thoroughly optimized before use. Due to their high boiling points, for PAHs on-column injection is recommended.

6.2.4 TEMPERATURE PROGRAMMING

The temperature program must be optimized for a sufficient separation of the PAH compounds. For GC/MS analysis peak area is generally used, and a 10 % valley would represent a good separation. Less resolved peaks may also be quantified (for instance, by dropping perpendiculars to the baseline), but increasing errors may result. In addition to a reproducible temperature program, a fixed equilibration time is important for a correct analysis and constant retention times.

6.2.5 DETECTION

A frequently used detector for PAH analysis is a mass spectrometric detector, used in the Selected Ion Monitoring (SIM) mode. Electron impact ionization (El) may be used as the ionization method. The selectivity of a mass spectrometric detector is excellent and the chromatographic noise of a standard is similar to that of a sample. However, major drawbacks are the matrix-dependent response and the convex calibration curves that both often occur and make quantification difficult. As another technique of PAH identification, the full-scan MS using an ion trap can be mentioned; it operates with the same sensitivity as SIM but is a much more powerful analytical tool. The use of a flame ionization detector (FID) is also possible, but since the selectivity of the FID is low, it is not recommended.

6.3 IDENTIFICATION

The individual PAHs are identified by comparing the retention time of the substance in a sample with that of the respective compound in a standard solution analysed under the same conditions. In case of doubt, it is recommended to confirm the results by using a different wavelength for UV-absorption or a different combination of wavelengths for fluorescence detection. Using a GC/MS system, the molecular mass or characteristic mass fragments are a suitable way to prove the identification of the PAH compound. Using GC/MS on a modern instrument, the retention times should be reproducible to within ±0.05 minutes, and additionally there are deuterated analogues of many of the parent compounds present for comparative purposes. For HPLC, reproducibility of retention times may be less good, but should certainly be within ±1 minute.

6.4 QUANTIFICATION

PAH determinations should preferably be carried out using calibration solutions prepared from certified, crystalline PAHs. However, the laboratory should have the appropriate equipment and the expertise to handle these hazardous crystalline substances. Alternatively, certified PAH solutions, preferably from two different suppliers, can be used. Two independent stock solutions should always be prepared simultaneously to allow a cross-check to be made. Calibration solutions should be stored in ampoules in a cool, dark place. Weight loss during storage should be recorded for all standards.

Internal standards should be added to all standards and samples either in a fixed volume or by weight. The internal standards should preferably be non-natural PAHs which are not found in sediment samples and do not co-elute with the target PAHs. Several perdeuterated PAHs have proved to be suitable for GC/MS as well as for HPLC analysis. The use of several deuterated PAHs spanning the entire molecular weight range as internal standards is encouraged. For example, for GC/MS it is recommended to add four internal standards representing different ring-sizes of PAHs.

The following compounds can be used (Wise *et al.,* 1995):

- for HPLC analysis: phenanthrene-d10, fluoranthene-d10, perylene-d12, 6-methyl-chrysene;
- for GC/MS analysis: naphthalene-d8, phenanthrene-d10, chrysene-dl2, perylene-dl2.

After clean-up and before GC analysis, an additional internal standard is added for volume correction.

A multilevel calibration with at least five concentration levels is recommended. For UV and fluorescence detection, the linear range is large. The calibration curve should be linear and should cover the working range.

Since the mass spectrometric detector often has no linear response curve, the use of stable, deuterated isotopes is a prerequisite. Furthermore, the response of PAHs in standard solutions is often much lower than in sample extracts. Only a combination of different techniques, e.g., the use of internal standard and standard addition, might give reliable quantitative results.

The calibration curve can be checked by recalculating the standards as if they were samples and comparing these results with the nominal values. Deviations from the nominal values should not exceed 5 %.

When chromatograms are processed using automated integrators, the baseline is not always set correctly, and always needs visual inspection. Because in HPLC analysis the separation of the peaks is often incomplete, the use of peak heights is recommended for quantification. Using GC techniques, either peak heights or peak areas can be used.

Prior to running a series of samples and standards, the GC or HPLC systems should be equilibrated by injecting at least one sample extract, the data of which should be ignored. In addition, standards used for multilevel calibration should be regularly distributed over the sample series so matrix-and non-matrix-containing injections alternate. A sample series should include:

- a procedural blank;
- a laboratory reference material;
- at least five standards;
- one standard that has been treated similarly to the samples (recovery determination).

The limit of determination should depend on the purpose of the investigation. A limit of 2 ng g-1 (dry weight) or better should be attained for single compounds. The method for calculating the limit of determination should reflect the advice in Part B-4.2.3 (COMBINE manual). The limit of determination that can be achieved depends on the blank, the sample matrix, concentrations of interfering compounds, and the mass of sediment taken for analysis.

6.5 SYSTEM PERFORMANCE

The performance of the HPLC or GC system can be monitored by regularly checking the resolution of two closely eluting PAHs. A decrease in resolution indicates deteriorating HPLC or GC conditions. The signal-to-noise ratio yields information on the condition of the mass spectrometric (MS) detector. A dirty MS-source can be recognized by the presence of a higher background signal, together with a reduced signal-to-noise ratio. Additionally, the peak shape can be affected.

6.6 LONG-TERM STABILITY

One laboratory reference sample should be included in each series of samples. A quality control chart should be recorded for selected PAHs, e.g., fluoranthene (stable results), pyrene (sensitive to quenching), benzo[*a*]pyrene (sensitive to light). If the warning limits are exceeded, the method including calibration solutions should be checked for possible errors. When alarm limits are exceeded, the results should not be reported. A Certified Reference Material should be analysed at least twice a year and each time the procedure is changed. Each laboratory analysing sediments should also participate in interlaboratory studies on the determination of PAHs in sediments on a regular basis.

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ANNEX B-13 APPENDIX 2. TECHNICAL NOTE ON THE DETERMINATION OF CHLORINATED BIPHENYLS IN SEDIMENT

1. INTRODUCTION

These guidelines are based on the review papers by Smedes and de Boer (1994, 1998). The analysis of chlorinated biphenyls in sediments generally involves extraction with organic solvents, clean-up, removal of sulphur, column fractionation and gas chromatographic separation, mostly with electron capture or mass-spectrometric detection. All of the steps in the procedure are susceptible to insufficient recovery and/or contamination. Hence, quality control procedures are recommended in order to check the method performance. In addition, the quality control aspects relating to calibrants, extraction, clean-up, etc., are considered important. These guidelines are intended to encourage and assist analytical chemists to (re)consider their methods critically and to improve their procedures and/or the associated quality control measures, where necessary. It should be noted that these guidelines do not cover the determination of non-*ortho* substituted CBs. Due to the low concentrations of non-*ortho* CBs in sediments compared to those of other CBs, their determination requires an additional separation and concentration step.

These guidelines can also be applied for the determination of several other types of organochlorine compounds, e.g., chlorobenzenes, DDT and its metabolites, and hexachlorocyclohexanes. The recovery in the clean-up procedures must be checked carefully. In particular, treatment with H_2SO_4 results in loss of, e.g., dieldrin and endosulfanes. Also, the clean-up procedure with silver ions can result in low recoveries for certain pesticides.

It is neither possible nor desirable to provide fully detailed guidelines for the analysis of sediments. If necessary, guidance should be sought from highly specialized research laboratories. Whichever procedure is adopted, each laboratory must demonstrate the validity of each step of its procedure. The use of a second, different method, in addition to the routine procedure, is recommended as a validation. The analyses have to be carried out by experienced staff.

2. SAMPLING AND STORAGE

The major criteria for successful sediment sampling is to guarantee a fairly undisturbed sample stratification. (For further details about sampling, see the “Technical note on the determination of heavy metals in marine sediments”.) Plastic materials (except polyethylene or polytetra-fluorethene) must not be used for sampling due to adsorption of determinands to the container material.

The samples should be transported in closed containers; a temperature of 25 °C should not be exceeded. If the samples are not to be analysed within 48 hours after sampling, the sample has to be stored at 4 °C (short-term storage). Storage over several months is only possible for frozen (below -20 °C) and dried samples.

3. BLANKS AND CONTAMINATION

The procedural detection limit is determined by the blank value. In order to keep the blank value as low as possible, all glassware, solvents, chemicals, adsorption materials, etc., should be free of CBs or other interfering compounds.

Glassware should be washed thoroughly with detergents, heated to > 250 °C, and rinsed with an organic solvent prior to use.

All solvents should be checked for impurities by concentrating the volume normally used in the procedure to 10 % of the normal end volume. The presence of CBs and other compounds in the solvents can then be checked by gas chromatographic (GC) analysis.

All chemicals and adsorption materials should be checked for impurities and purified (e.g., by heating or extraction), if necessary. Glassfiber Soxhlet thimbles should be pre-extracted. The use of paper thimbles should be avoided. Alternatively, full glass Soxhlet thimbles with a G1 glass filter at the bottom can be used. Storage of these super-cleaned materials for a long period of time is not recommended, as laboratory air can contain CBs that will be adsorbed by these materials. The occurrence of blank values despite having taken all the above-mentioned precautions may be due to contamination from the air.

4. PRETREATMENT

CBs can be extracted from wet or dried samples. Storage, homogenization, and extraction are much easier when the samples are dry. However, drying the samples may alter the concentrations, e.g., by loss of compounds through evaporation or by contamination.

Before taking a subsample for analysis, the sample should be sufficiently homogenized.

Chemical drying of samples can be performed by grinding with Na_2SO_4 or $MgSO_4$ until the samples reach a sandy consistency. It is essential that the operations of grinding and extraction are separated by at least several hours to allow proper binding of the water and avoid insufficient extraction.

Freeze-drying is becoming a more popular technique. Losses through evaporation are diminished by keeping the temperature in the evaporation chamber below 0 °C. Possible losses or contamination must be checked. Contamination during freeze-drying is reduced by putting a lid, with a hole of about 3 mm in diameter, on the sample container.

5. EXTRACTION AND CLEAN-UP

5.1. EXTRACTION

The target compounds must be extracted from the sediment with an organic solvent prior to further analysis.

Wet sediments are extracted by mixing with organic solvents. Extraction is enhanced by shaking, ultra-turrax mixing, ball mill tumbler, or ultrasonic treatment. Water-miscible solvents, such as methanol, acetone, and acetonitrile, are used, especially in the first step. The extraction efficiency of the first step is low as there will be a considerable amount of water in the liquid phase. The extraction is continued with a mixture of polar and apolar solvents (acetone plus hexane, or methanol plus dichloromethane). For complete extraction, at least three subsequent extractions are needed and the contact time (24 hours) with the solvent should be sufficient to complete the desorption of the CBs from the sediment.

Wet sediments can also be extracted utilizing a Soxhlet, but this is best done in two steps. First, a polar solvent, such as acetone, is used to extract the water from the sediment. Then the flask is replaced and the extraction is continued with a mixture of, e.g., acetone/hexane.

In both cases, water should be added to the extracts and the CBs should be extracted by an apolar solvent such as hexane.

For dried sediments, Soxhlet extraction is the technique most frequently applied to extract CBs. The use of a mixture of a polar and an apolar solvent (e.g., acetone/hexane) is recommended for sufficient extraction efficiency. A good choice is 25 % acetone in hexane. A higher content of the polar solvent increases the extraction efficiency, but the polar solvent has to be removed prior to gas chromatographic analysis. The extraction can be carried out with a regular Soxhlet or a hot Soxhlet. At least 50 to 60 extraction cycles should be performed (approximately 8 hours for the hot Soxhlet). The extraction efficiency must be checked for different types of sediments by a second extraction step. These extracts should be analysed separately.

Supercritical fluid extraction (SFE) is a relatively new method. The optimal conditions are still under investigation. A new static extraction system applying high temperature and high pressure also seems to be a promising technique.

In principle, all the methods described are suitable for the extraction of CBs from sediments. For dry samples, however, Soxhlet extraction is recommended over mixing methods.

Prior to any concentration step, a keeper (high-boiling alkane) must be added.

5.2. REMOVAL OF SULPHUR AND SULPHUR-CONTAINING COMPOUNDS

The crude extract requires a clean-up as many compounds other than CBs are co-extracted. This extract will be coloured due to chlorophyll-like compounds extracted from the sediment, and it will also contain sulphur and sulphur-containing compounds, oil, PAHs, and many other natural and anthropogenic compounds.

An aqueous saturated Na_2SO_3 solution is added to a hexane extract. In order to allow transfer of the HSO_3-ions to the organic phase, tetrabutylammonium (TBA) salts and isopropanol are added to the mixture. Subsequently, water is added to remove the isopropanol. The aqueous phase is then quantitatively extracted with hexane (Jensen *et al.,* 1977). If the extraction is performed by a polar solvent miscible with water, the Na_2SO_3 solution can be added directly after the extraction. If the extraction mixture also contains an apolar solvent, then, depending on the ratio of the solvents, the addition of TBA and isopropanol may not be necessary. Any excess $Na_2SO_3$and reaction products can be removed by the addition of water and partitioning between the apolar solvent and water.

Japenga *et al.* (1987) developed a column method for the removal of sulphur and sulphur-containing compounds. The column material is made by mixing an aqueous solution of Na2SO3 with Al_2O_3. Some NaOH is also added to improve the reaction with sulphur. Subsequently, the material is dried under nitrogen until a level of deactivation equivalent to 10 % water is reached. Storage must be under nitrogen because sulphite in this form may easily be oxidized to sulphate. Eluting the extract (hexane) through a column filled with this material results in removal of the sulphur in combination with further clean-up of the sediment extract. The sulphur removal properties are somewhat difficult to control.

Mercury, or copper powder, wire, or gauze remove the sulphur directly from an organic solvent. Although mercury is appropriate for removing sulphur, it should be avoided for environmental reasons. Copper can be applied during or after Soxhlet extraction. Ultrasonic treatment might improve the removal of sulphur. If sulphur appears to be present in the final extract, the amount of copper or mercury used was insufficient and the clean-up procedure must be repeated.

Silver ions strongly bind sulphur and sulphur compounds. Loaded on silica, $AgNO_3$ is a very efficient sulphur-removing agent. It can be prepared by mixing dissolved $AgNO_3$ with silica and subsequently drying under nitrogen. Compounds containing aromatic rings are strongly retained, but for CBs this retention is reduced, probably due to shielding of the rings by the chlorine atoms. Retained compounds can easily be eluted by using cyclohexene, or another solvent with double bonds, as a modifier.

Elemental sulphur is strongly retained on a polystyrene divinylbenzene copolymer column as generally applied for gel permeation chromatography (GPC). In addition, this method combines the removal of sulphur with a clean-up.

All of these methods have advantages and disadvantages. Sometimes the use of multiple methods may prove necessary for different samples. Several methods leave some aromatic sulphur compounds in the extract which will elute from the GC column at the same retention time as the lower CBs. The major part of these compounds can be removed by eluting an apolar extract over a column containing silica loaded with concentrated sulphuric acid.

5.3. FURTHER CLEAN-UP

As CBs are apolar, clean-up using normal phase chromatography is the most appropriate technique for their separation from other compounds. Using an apolar solvent, e.g., hexane or iso-octane, as an eluent, CBs normally elute very rapidly. All polar solvents used in the extraction or sulphur-removal step should be removed before further clean-up. The last concentration step is usually performed by evaporation with a gentle stream of nitrogen. Evaporation to dryness should always be avoided.

Deactivated Al_2O_3 (5–10 % water) is often used as a primary clean-up. Al_2O_3 normally gives a sufficiently clean extract for a gas chromatography electron capture detector (GC-ECD) screening of the sample, provided that sulphur has been removed.

Deactivated SiO_2 (1–5 % water) does not retain CBs (including planar CBs) and only slightly retains polycyclic hydrocarbons when eluted with hexane or iso-octane.

For high activity silica (overnight at 180 °C), the retention of CBs is negligible while PAHs are more strongly retained. The CBs and a few organochlorine compounds are eluted with apolar solvents.

When using more polar solvents (e.g., hexane/acetone), some interfering organochlorine pesticides are eluted**.**

When GPC is used for removing the sulphur, the removal of high molecular weight material can also be incorporated into the procedure. GPC does not separate CBs from other compounds in the same molecular range (such as organochlorine pesticides), so additional clean-up is usually required.

For the separation of CBs from lipids or oil components, reversed-phase high-performance liquid chromatography (HPLC) can be used. Due to the use of aqueous solvents in reversed-phase HPLC, the samples have to be transferred several times between polar and apolar solvents.

5.4. CONTROL OF EXTRACTION AND CLEAN-UP

The check of extraction and clean-up can be performed by analysing a reference material. To check the clean-up and concentration steps, it is recommended to pass a standard solution through the entire procedure. This standard solution is used for the determination of the recovery for the sample series. Additionally, an internal recovery standard should be added to each sample before extraction, to check for recovery during the analytical procedures. If major losses have occurred, then the results obtained should not be reported. CB29 is suggested as a recovery standard because, owing to its high volatility, losses due to evaporation are easily detected. CB29 elutes relatively late from alumina and silica columns. Small peaks that may be present in the gas chromatogram at the retention time of CB29 do not hinder the use of this CB because the recovery standard only indicates major errors in extraction or clean-up.

In case GC/MS is applied, labelled CBs can be used as recovery standards. This allows correction for recovery, provided that each chlorination stage is represented.

6. GAS CHROMATOGRAPHY

Because of the large number of CB congeners (a total of 209), high-resolution capillary gas chromatography (GC) is the method of choice for the determination of CBs. The analysis of CBs in sediments should focus on the determination of selected individual congeners. As it is currently impossible to separate all CBs in technical mixtures and to separate them from other ECD-detectable compounds, it is recommended that two columns of different selectivity (polarity) are used for analysis. For more reliable separation of CBs, multidimensional gas chromatography (MDGC) is the preferred method. This technique is especially valuable for specific separations, but still needs basic investigations before routine application is possible.

For all GC methods, parameters have to be optimized.

Column dimensions

Column dimensions for the determination of CBs are:

- length: minimum 50 m, and
- inner diameter: maximum 0.25 mm.

Greater resolution can be obtained by reducing the inner diameter to 0.20 mm or less. Below a diameter of 0.15 mm the carrier gas pressure rises to values greater than 500 kPa, which are not compatible with normal GC equipment. Also, the risk of leakage increases.

The film thickness should be between 0.2 μm and 0.4 μm.

Stationary phases

A wide range of stationary phases can be used for the separation of CBs (e.g., 94 % dimethyl-, 5 % phenyl-, 1 % vinyl polysiloxane, or 7 % phenyl-, 7 % cyanopropyl-, 86 % methyl-siloxane). The use of more polar phases is sometimes limited as their maximum temperatures are not as high as for apolar, chemically bonded phases. Stationary phases that separate CBs on the basis of molecular size, such as the liquid crystal phase, should not be used for monitoring purposes since they do not provide sufficient reproducibility.

Carrier gas

Preferably, hydrogen should be used as the GC carrier gas. When using columns with very small inner diameters, the use of hydrogen is essential. The linear gas velocity should be optimized. Appropriate settings for 0.25 mm i.d. columns range from 20–40 cm s^{-1} and for 0.15 mm i.d. columns from 30–50 cm s^{-1}.

Injection techniques

The two systems commonly used are splitless and on-column injection. Split injection should not be used because strong discrimination effects may occur. Other techniques such as temperature-programmed or pressure-programmed injection may have additional advantages, but should be thoroughly optimized before use. The volume of the liner should be large enough to contain the gas volume of the evaporated injected solvent. When the liner is too small, memory effects can occur due to contamination of the gas tubing attached to the injector. Very large liner volumes can cause a poor transfer of early eluting components, so that peaks due to those analytes will be reduced or even disappear. An auto-sampler should be used. In addition, the use of a light packing of (silylated) glass wool in the liner improves the response and reproducibility of the injection, but some organochlorine pesticides such as DDT may be degraded when this technique is applied.

Temperature programming

The temperature programme must be optimized for a sufficient separation of the CB congeners. An analysis time of 60–120 minutes is inevitable. In addition to a reproducible temperature programme, a fixed equilibration time is important for a correct analysis and constant retention times.

For further details and recommendations, Smedes and de Boer (1998) should be consulted.

Detection

The most frequently used detector for CB analysis is the electron capture detector (ECD). Injection of chlorinated or oxygen-containing solvents should be avoided. The use of a mass selective detector (MSD) or even a mass spectrometer (MS) as a detector for CB analysis is becoming more common and generally applicable. Negative chemical ionization (NCI) is extremely sensitive for penta- to decachlorinated CBs (approximately ten-fold better than ECD). Electron impact ionization (EI) may be used as an alternative ionization method, but for most CBs the sensitivity of this method is ten-fold lower than for ECD.

System performance

The performance of the GC system can be monitored by regularly checking the resolution of two closely eluting CBs. A decrease in resolution indicates deteriorating GC conditions. The signal-to-noise ratio yields information on the condition of the detector. A dirty ECD-detector or MS-source can be recognized by the presence of a higher background signal, together with a reduced signal-to-noise ratio.

Long-term stability

One laboratory reference sample should be included in each series of samples. A quality control chart should be recorded for selected CBs. If the warning limits are exceeded, the method should be checked for possible errors. When alarm limits are exceeded, the results obtained should not be reported.

A certified reference material should be analysed at least twice a year, and each time the procedure is changed. Each laboratory analysing sediments should also participate in interlaboratory studies on the determination of CBs in sediments on a regular basis.

7. IDENTIFICATION

The presence of a single chlorobiphenyl compound is proved if the retention time of the substance corresponds with that of the same compound in the standard solution analysed under the same conditions on both columns. Using a GC/MS system additionally, the molecular mass or characteristic mass fragments (chlorine cluster), is a suitable way to prove the identification of individual CBs.

8. QUANTIFICATION

CB determinations should always be carried out using calibration solutions prepared from crystalline CBs. Preferably, certified CBs should be used. Two independent stock solutions of different concentrations should always be prepared simultaneously to allow a cross-check to be made. Calibration solutions should preferably be stored in ampoules in a cool, dark place. For all containers with standards, the weight loss during storage should be recorded.

After clean-up and before GC analysis, at least one internal standard is added for volume correction.

The ideal internal standard is a CB which is not found in the samples and does not co-elute with other CBs, e.g., CB 29, CB 112, CB 155, CB 198, or all 2,4,6-substituted CB congeners. Alternatively, 1,2,3,4-tetrachloronaphthalene can be used.

Internal standards should be added in a fixed volume or weighed to all standards and samples.

Since the ECD has a non-linear response curve, a multilevel calibration with at least five concentration levels is strongly recommended. A point-to-point calibration is preferred. If that option is not available, a linear working range can be identified, which allows the use of linear regression within this range. Alternatively, a non-linear fit can be used. If regression is applied, the standards should always be recalculated as samples and checked against their nominal values. Deviation from the nominal values should not exceed 5 %.

When the chromatogram is processed by using automated integrators, the baseline is not always set unambiguously, and always needs visual inspection. The use of peak heights is recommended for quantification.

The GC system should be equilibrated by injecting at least one standard or sample omitting any further evaluation prior to a series of samples and standards. In addition, standards used for multilevel calibration should be distributed regularly over the sample series, so that matrix and non-matrix containing injections alternate. A sample series should consist of:

1) a procedural blank;

2) a laboratory reference material;

3) at least five standards;

4) one standard solution that has been treated in the same manner as the samples (recovery determination).

When using a GC/ECD system with two columns of different polarities, the more reliable result should be reported.

The limit of determination should depend on the purpose of the investigation. A limit of 0.1 ng g^{-1} (dry weight, fraction < 2 mm) or better should be reached. The method for calculating the limit of determination should follow the advice in Part B-4.2.3 (COMBINE manual). The limit of determination that can be achieved depends on the blank, the sample matrix, the concentrations of interfering compounds, and the quantity of sediment used for analysis.

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ANNEX B-13 APPENDIX 3. : TECHNICAL NOTE ON THE DETERMINATION OF HEAVY METALS AND PERSISTENT ORGANIC COMPOUNDS IN MARINE SEDIMENT

1. SAMPLING AND SAMPLE HANDLING

The major criterion for successful sediment sampling is to guarantee a fairly undisturbed sample stratification. Of particular interest is the undamaged surface of the sample. Reasonable results are obtained by the application of box corer devices or a multiple corer.

Trend monitoring in sediments requires information about the current trace substance burden in the uppermost sediment layer (e.g., 2 cm). This first centimetres accumulate the deposits of the recent few years and thus are the object of the routine sediment analysis. Only if long-term time series (decades/centuries) of the trace substance burden of the deposit (or background concentration studies) are part of the investigations, is the analysis of deeper sediment layers required.

Immediately after sampling, the first 2 cm of the core is removed and stored. If the entire core is the object of the investigation, it is recommended to dissect the first 10 cm into five 2 cm layers. The deeper part should only be analysed in distinct sections, which cover the ranges: 15–17 cm, 22–24 cm, and 29–31 cm (Perttilä and Brügmann, 1992). Pieces of glass or colourless polyethylene tools are recommended for the sectioning of the core. After each layer has been cut off, the tools should be changed and cleaned. The selected sediment layers (samples) should be placed in separate and clean glass or polyethylene (polypropylene/polystyrene) containers carefully labelled and pre-weighted. The label should contain at least the sample identification number, and the date and location of sampling.

The following procedure is recommended for cleaning the tools and containers for sediment sample handling prior to the sampling campaign. Wash by soaking for 2–3 days in diluted (10%) HNO_3, then rinse with high purity water. During the sampling campaign, the reused tools, the table, and corer components should be carefully cleaned by rinsing with seawater.

The tools and containers must be stored dust-free when not in use. A comprehensive description of cleaning procedures for plastic and glass laboratoryware can be found in Annex B-12, Appendix 1 "Technical notes on the determination of trace metals (Cd, Pb, Cu, Co, Zn, Ni, Fe) including mercury in seawater" of these "Guidelines".

The samples should be deep frozen as soon as possible after packing. Take note that freezing of a large bulk of containers should be avoided; the samples in the centre would take longer to cool and this may result in some loss of mercury. Once frozen, the samples can be stored at temperatures of –20ºC or below.

2. SAMPLE PRETREATMENT; CONTAMINATION CONTROL

Because trace metals are mostly associated with the fine sediment fraction, it is often recommended that a defined grain size fraction of the sediment be considered (<63 µm; <20 µm). Therefore, the sediment samples have pass through a sieving procedure (Smedes et al., 2000; Loring, 1991; Limpenny and Rowlatt, 1994).

Sieving should preferably be carried out on wet sediment using water from the sampling location (Smedes et al., 2000).

Prior to the instrumental detection, sediment samples must be digested. The removal of water from the frozen samples is recommended, preferably by freeze-drying. The freeze-drying can be performed directly on the frozen sediments and without change of the container; the loss of mercury is also thus avoided. The freeze-dried sediments can be then stored almost indefinitely.

During freeze-drying, samples can (and should) be protected from cross-contamination (particles and vapours) by applying a lid with a small hole covered with filter-paper over the sample container.

After drying, the sediments should be carefully homogenized, e.g., using a ball mill.

For the complete digestion of marine sediments, a pressure wet ashing is recommended (Loring and Rantala, 1991; UNICAM, 1991). Since the rate of digestion and efficiency of acid decomposition increase substantially with elevated temperatures and pressure, the closed vessel techniques, using conventional heating or microwave energy, are applied preferably to open systems. The most widely applied technique for sediment mineralization is at present microwave digestion with concentrated acids, mostly nitric and hydrofluoric acids(Loring and Rantala, 1990; McCarthy and Ellis, 1991). Hydrofluoric acid is added to the sediment to remove silica (SiO_2). Al, Li, and Fe are commonly used for normalization of the results of analyses. The normalization procedure gives best results if Al values from partially digested samples are used. More information about the application of normalization procedures can be found in Annex B16 and Smedes et al. (2000).

Further requirements to avoid losses of the determinand or to solve contamination problems are described by, e.g., Boutron (1990) and Schmidt and Gerwinski (1994). The availability of high purity reagents is a prerequisite for reliable determination of heavy metal concentrations. And the first order of priority is a sufficient supply of high purity water. For contamination control, a procedural blank (recommended in triplicate) has to be carried out throughout all the operational steps parallel to the samples.

3. CALIBRATION

For calibration purposes, single standard stock solutions at a concentration of 1000 mg dm^{-3}, purchased from a qualified manufacturer, can be used. Fresh standard stock solutions should be compared with the older standard solutions. Single or mixed working element standard solutions are prepared by dilution of the stock solution using dilute acid, as required, though a mixed standard solution is more convenient in use. The concentrations of particular elements in a mixed standard stock solution can be matched in such a way as to produce a single series of working standard solutions for all elements analysed (with the exception of Al and Fe whose concentrations fall in a different range). All standard solutions have to be stored in polyethylene, borosilicate or quartz volumetric flasks. Standard solutions with lower concentrations, if prepared correctly and controlled in a QA system (checking of old versus new, and checking with standards from a different source), can be kept for a period no longer than one month.

It must be mentioned that plastic materials used for the production of laboratoryware exhibit certain adsorptive or exchange properties. Hence, boundary-surface interactions can be very important when very dilute analytical solutions are handled. It is thus imperative that volumetric flasks, reagent vessels, pipette tips, etc., for handling sample solutions and low level reference or analyte solutions must never be used for transferring or processing stock solutions of analyte or concentrated reagents.

The calibration procedure has to meet some basic criteria in order to give the best estimate of the true element concentration of the sample analysed:

- the concentrations of standards for the preparation of the calibration curve (function) should cover the range of concentrations as related to practical conditions; the mean of the range should be roughly equal to the expected analyte concentration in the sample;
- the required analytical precision should be known and achievable throughout the entire range of concentrations;
- the measured value (instrument signal) at the lower end of the range has to be significantly different from the procedural analytical blank;
- the chemical and physical properties of the calibration standards must closely resemble those of the sample under investigation, i.e., the difference in density between the standard and environmental sample should be minimized (this is of particular importance in flame atomic absorption determinations);
- as a general rule, the analysis of each batch of environmental samples should be accompanied by analysis of a certified reference material (CRM) or at least a laboratory reference material (LRM).

4. INSTRUMENTAL DETERMINATION

Heavy metals appear in marine sediments in low concentrations, ranging from mg kg−1 to µg kg−1 (Szefer, 2002). Stoeppler (1991) provided a comprehensive review of the most frequently used techniques for quantitative analysis of metallic trace elements.

Instrumental determination of heavy metals in the acidic solution obtained is carried out depending on the instrument and manufacturer's specifications. In most cases, i.e., in most marine sediments, Cd and Pb can be determined by GFAAS (Graphite Furnace Atomic Absorption), while Cu, Zn, Cr, Ni, Mn, Al, and Fe can also be determined by the less sensitive flame atomization.

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ANNEX B-14 TECHNICAL NOTE ON THE MEASUREMENT OF PH IN SEAWATER

1. INTRODUCTION

pH is one of the variables that characterize the marine acid-base equilibrium. pH is used as a co-factor in primary production measurements, for the calculation of dissociation constants (of, e.g., trace metals), and for calculations concerning the carbonate system. This technical note describes the main procedures applied when pH is used as a co-factor for biological measurements. More precise measurements may require other routines or equipment.

2. METHODS

Combined electrodes are frequently used to measure pH, and consist of a hydrogen ion selective electrode (normally with an internal Ag/AgCl electrode) and a reference electrode (normally Hg/Hg2Cl2). The electrical contact between the two half-cells is achieved by an internal salt bridge (saturated KCl solution) with the sample as an external liquid junction.

3. SAMPLING

Sampling for pH measurement is done immediately or as soon as possible after samples for oxygen or hydrogen sulphide are taken. For storage and transportation of samples, completely filled and tightly closed polyethylene or glass bottles that have been rinsed with the sample can be used. Samples should be analysed as soon as possible, but can be stored refrigerated in the dark for up to 24 hours.

4. ANALYTICAL PROCEDURE

Equipment maintenance

The measurement of pH depends on the performance of the pH-meter, hence some important aspects of electrode maintenance have to be observed. Frequently, crystallization of KCl causes an increase of electrode resistance or even cuts off the electrical contact. It is therefore recommended before starting up the calibration of the pH-meter to check that the salt bridge is filled with electrode-filling solution (a saturated KCl solution) up to the level designated in the manufacturer's instructions, usually to about 1 cm beneath the inlet. If the filling solution is no longer saturated, i.e., there are no visible crystals of KCl, add the solid salt through the inlet.

It is also recommended to check for the occurrence of any air bubble inside the glass bulb of the electrode. The best procedure for removing air inside the glass bubble is shaking the electrode. If this does not help, warm the electrode cautiously up to about 60 °C in a water bath and repeat the shaking.

Store electrodes in accordance with the manufacturer's instructions. Before use, it is recommended to equilibrate the electrodes by immersing them in a sample for 15 minutes.

Electrodes that have been used during heavy plankton blooms or otherwise need to be cleaned may be submersed in 0.1 M HCl or HNO3 for 30 minutes. Change the inner filling solution and let the electrode condition in storage solution for at least 1 hour before use.

Calibration and measurement

Calibrate the electrodes and pH-meter daily when in use.

Calibrate the pH-meter with two buffer solutions (NBS-scale), according to the manufacturer's instructions. It is important to note that the buffers are not too old (according to the producer statement), and that they are handled properly, i.e., only opened briefly when needed and kept tightly closed. For calibration, commercially available certified buffers of pH 7 (or the electric 0-point of the pH-meter) and pH 9 are recommended, to cover the expected range of the samples to be measured. If the laboratory produces its own buffer solutions, CO2-free reagents and water must be used. CO2 can be removed from the water by bubbling with nitrogen for 10–15 minutes, or by boiling the water for 10–15 minutes and cooling. The calibration and measurement must be performed at 25±2°C, using a thermostatted water bath at 25±1°C. It is important to have a stabilized reading before registering the result. Stirring can be used to speed up the equilibrium. When using an automatic reading, there is a risk of registering results before equilibrium is reached. In this situation, manual reading should be applied. For primary production measurement purposes, the results are recalculated to the *in situ*temperature (Wedborg *et al*., 1999).

During calibration and the measurement of samples, the electrode must be rinsed with distilled water or wiped off before it is inserted into the next solution. It is also important that the electrode membrane is not allowed to come in contact with the sample container walls during measurement.

5. QUALITY ASSURANCE

Check:

- the electrode potential in accordance with the manufacturer's instructions in all analytical series;
- the temperature sensor against a calibrated thermometer twice annually.

During each series of environmental samples, the calibration of the pH-meter should be checked against another certified commercial buffer with a pH similar to that of the samples, e.g., pH 8. It is necessary to check the trueness and stability of a non-certified reference solution, e.g., by checking the solution regularly against a certified reference electrode. Register the control reading of the check sample in an X-chart.

The electrode should be quality checked at regular intervals, using the same buffers as for calibration, according to the following procedure:

1. The EMF value of the electrode, in buffer 7, should be within ±30 mV (for electrodes working according to DIN 19263). Other values may apply, so consult the electrode manual.
2. The difference between the EMF-values in buffer 9 and 7 should be approximately 118 mV (two times the Nernst factor).

3. The calibration slope should be within 0.95–1.05 (this checks the pH meter signal correction).

If the electrode fails to meet these criteria, clean, repair or discard it.

6. REPORTING OF RESULTS

pH values are reported with two decimal digits.

7. PRECISION

For primary production purposes, a total within-laboratory standard deviation of ± 0.1 is usually satisfactory. A precision (total within-laboratory standard deviation) of 0.02 can normally be achieved by this procedure.

8. REFERENCES

ISO. 1994. Water quality—Determination of pH. ISO 10523.

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Wedborg, M., Turner, D.R., Anderson, L.G., and Dyrssen, D. 1999. *In* Methods of seawater analysis, 3rd edition. Ed. by K. Grasshoff*et al*. Wiley-VCH, Germany.

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ANNEX B-15 TECHNICAL NOTE ON THE MEASUREMENT OF TOTAL ALKALINITY IN SEAWATER

1. INTRODUCTION

This technical note is intended to support the analytical quality of marine measurements in the Baltic area. Total alkalinity is a measure of the acid-neutralizing capacity of a sample, and is one of the parameters that characterize the marine acid-base equilibrium. It determines the sum of alkaline components in a sample (hydroxide, carbonate, hydrogen carbonate, and other buffering components). In the COMBINE programme, total alkalinity is used as a co-factor in primary production measurements.

2. METHODS

The method described here is the direct titration method, and it is intended and recommended for seawater analysis. Methods for the determination of the alkalinity of sediments and methods based on back-titration methods are not included in this note. The recommended methods that can be applied are potentiometric or colorimetric (indicator) titration. The result is dependent on the endpoint pH of the titration, where the approximate endpoint is 4.5. The method is described in detail by Anderson *et al.* (1999), APHA (1995), and IS0 9963 (1994).

3. SAMPLING

Samples are collected and stored in gas-tight polyethylene or glass bottles that are completely filled and closed tightly. The sample volume necessary for titration depends on the total alkalinity, and can be up to 200 ml for samples of low alkalinity. Normally, seawater samples are stable for at least two weeks if stored cool and dark. However, samples with significant biological activity cannot be expected to be stable.

4. ANALYTICAL PROCEDURE

The procedure involves titration down to approximately pH 4.5 with hydrochloric acid. The titre of the hydrochloric acid needs to be determined correctly; where possible, the use of commercially available hydrochloric acid with a known concentration is recommended. Reagents shall be prepared using CO2-free water, which can be prepared by boiling purified water for 10–15 minutes followed by cooling, or by bubbling with nitrogen for 10–15 minutes. Alkalinity can be determined by colorimetric titration using an indicator. The exact endpoint depends on the alkalinity of the sample and can be determined on the basis of table values, from Gran titration graphs, or be determined from the inflection point of a titration curve. The most accurate results are normally achieved by the potentiometric titration method with an exact endpoint determination.

Equipment maintenance

For maintenance of the pH-meter, see ‘Technical Notes on pH Measurement in Sea Water’.

Calibration

Regarding pH-meter calibration, see the 'Technical Notes on pH Measurement in Sea Water', but use instead buffer 4 and buffer 10. Regarding the control of the electrode, Hansson buffer can be applied.

Check that the potential measurement of the electrode in Hansson buffer is between 350 mV and 450 mV. If not, repair or discard the electrode. Equilibrate the buffer to room temperature before use. [1]

Preparation of Hansson buffer

	Solution A, 1 litre	Solution B, 1 litre
NaC	l 321.2 mM (18.770 g)	421.2 mM (24.614 g)
KCl	10.5 mM (0.782 g)	10.5 mM (0.782 g)
Na_2SO_4	28.9 mM (4.104 g)	28.9 mM (4.104 g)
$MgCl_2(H2O)_6$	54.4 mM (11.060 g)	54.4 mM (11.060 g)
$CaCl_2(H2O)_2$	10.6 mM (1.558 g)	10.6 mM (1.558 g)
HCl (ampoule)	100.00 mM	

Add HCl as a 9973 Titrisol ampoule (Merck) containing 0.1 mole HCl. Dissolve 0.606 g Tris (2-amino-2-hydroxymethylpropane-1,3-diol) in 25.00 ml solution A. Dilute to 50.00 ml with solution B.

Stability:

- Solution A: 3 months;
- Solution B: 1 month;
- Hansson buffer: 1 week in refrigerator;
- Titrisol ampoule: 1 year.

5. QUALITY ASSURANCE

Check the volumetric equipment for both colorimetric and potentiometric methods. Check the accuracy of titrant addition in automatic titration systems.

Check the pH-meter pH, e.g., with a certified buffer of, e.g., pH 6.

Quality control with the use of X-charts and, if possible, certified reference material, e.g., VKI reference material QC DW[1].

Alternatively, if not used for standardization of the hydrochloric acid concentration, a reference solution of Na_2CO_3 of known concentration, where Na_2CO_3 has been dried to constant weight at 270–300 ^oC for at least 2 hours, can be used in quality control. Check the accuracy of this sample by comparing with measurements of a sample with a known and documented alkalinity, e.g., a certified reference material. A stock Na_2CO_3 solution of 40 mM is stable for 3 weeks, whereas the working solution (e.g., 2000 µM) should be prepared freshly every day.

6. Reporting

Results are normally reported in mmol [HCO3 2–]/dm3 (equals meq/dm3) or in mg $CaCO_3/dm_3$. Formulas for calculation are (APHA, 1995):

Total Alkalinity (meq/dm_3) = 1000 ´ v_{HCl} ´ t_{HCl}/v_b

Total Alkalinity (mg $CaCO_3/dm^3$) = 50 000 ´

vHCl ´ tHCl/vb, vHCl is the volume of HCl in ml, tHCl is the concentration of HCl in mol/dm^3 , and v_b is the volume of the sample in ml.

7. Precision

The precision obtained in a proficiency test (APHA, 1995) was 5 mg l^{-1} between laboratories in samples with a total alkalinity of 120 mg l^{-1}.

8. References

Anderson, L.G. *et al*. 1999. *In* Methods of seawater analysis. Ed. by K. Grasshoff *et al*. Wiley-VCH, Germany.

ISO. 1994. Water Quality—Determination of alkalinity, Part 1: Determination of total and composite alkalinity. ISO 9963-1.

APHA (American Public Health Association) *et al*. 1995. Standard methods for the examination of water and wastewater.

[1] 1 Information regarding the nearest dealer of VKI Reference Materials can be obtained from ProLab, DK (tel no.: (45) 45 76 79 76, fax no.: (45) 45 76 26 02)

Last updated: 29.10.2012 (Annex number changed from Annex B 16 to Annex B 15)

ANNEX B-16 TECHNICAL NOTE ON CO-FACTORS ANALYSIS

(after revision by the MCWG 2000 meeting)

1. CO-FACTORS, DEFINITION AND USE

A co-factor is a property in an investigated sample, which may vary between different samples of the same kind, and by varying may affect the reported concentration of the determinand. Thus the concentration of the co-factor has to be established in order to compare the determinand concentrations between the different samples (e.g. for the purpose of establishing trends in time or spatial distribution) by normalisation to the co-factor.

By the definition given above it is understood that the correct establishment of the co-factor concentration is just as vital to the final result and the conclusions as is the correct establishment of the determinand concentration. Thus the co-factor determination has to work under the same QA system, with the same QA requirements and the same QC procedures, as any other parts of the analytical chain. It is also vital that QA information supporting the data contains information on the establishment and use of any co-factors.

2. CO-FACTORS IN BIOTA ANALYSIS

Dry weight

Freeze-drying or heat drying at 105 C can be used. Dry to constant weight in both cases. By constant weight is meant a difference small enough not to significantly add to the measurement uncertainty.

Lipid content

The method by Smedes (1999), which uses non-chlorinated solvents and has been demonstrated to have high performance, is recommended. This method is a modification of the Bligh and Dyer (1959) method, and can be performed using the same equipment. The two methods have been shown to give comparable results.

Physiological factors

Age, sex, gonad maturity, length, weight, liver weight etc. are important co-factors for species of, for example, fish. For more information see Section D5 of the COMBINE manual.

3. CO-FACTORS IN WATER ANALYSIS

Particulate material

Determined by filtration through filter according to the ISO 11923:1997 standard.

Organic carbon

The method recommended is described in Annex C-2 of the COMBINE manual.

Salinity

Salinity (and temperature) may be defined as a co-factor in investigations where mixing of different water masses is studied or takes place. The same standard oceanographic equipment as described in the technical note on salinity is used, and the performance requirements will also be the same.

4. QA INFORMATION TO SUPPORT DATA

When reporting data that has been normalised to a co-factor, or where the co-factor data is reported along with the results, always supply the following information:

- Type of co-factor (parameter)
- Analytical method for the co-factor
- Uncertainty in the co-factor determination
- How the co-factor has been used (if it has)
- Results from CRMs and intercomparison exercises (of the co-factor)

REFERENCES

F. Smedes, Determination of total lipid using non-chlorinated solvents, The Analyst, 1999, 124, 1711

E.G. Bligh and W.J. Dyer, Can. J. Biochemical Physiol., 1959, 37, 911

ISO 11923:1997 Water quality -- Determination of suspended solids by filtration through glass-fibre filters

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ANNEX B-17 TECHNICAL NOTE ON THE DETERMINATION OF ORGANIC CARBON IN SEAWATER

SGQAC 2001/WP 5.3

Particulate matter

The particle size of the organically bound carbon of particles (POC) generally ranges between 0.45 µm and 300 µm. This includes both living organisms, such as phytoplankton, yeasts, bacteria, and microzooplankton, and detrital particles and aggregates. The production and decomposition of biogenic particles as well as their fractional removal to the deep sea control the distribution of most trace elements in the oceans. Microbial decomposition, desorption, and dissolution of suspended or sinking marine particles can release elements associated with labile (e.g., organic) fractions back to the sea water. On the other hand, particles can scavenge trace elements from the dissolved phase and thereby transport them to sediments. Analysis of the composition and distribution of the particulate fractions in the oceans is therefore required to understand the behaviour and geochemical cycling of, e.g., trace elements.

Dissolved matter

Among the different carbon reservoirs, dissolved organic matter (DOM) has the greatest mass, representing about 1000 ´ 10^{15} g of carbon, and not least because of its importance for the global climate there is a need to obtain accurate and comparable data on dissolved organic carbon (DOC) concentrations. Methods for the determination of DOC developed at a rather slow pace due to difficulties related to the composition of sea water. While DOC concentrations are around 1 mg dm^{-3}, sea water usually contains more than 35 g dm^{-3} of salts and more than 25 mg dm^{-3} of inorganic carbon as CO_2, HCO_3^-, and CO_3^{2-}.

1. SAMPLE HANDLING

The sample should be handled and transferred between containers as little as possible to avoid contamination during the steps between sampling and analysis.

It is important to obtain a representative sample, which during certain circumstances, e.g., during heavy algal blooms, can be achieved by shaking the water sampler immediately before taking the sub-sample. The homogeneity of the sample may be verified, for example, by separately analysing sub-samples from the upper and lower layers of the bottle.

For POC determinations, suspended particles are collected on filters. Since organic carbon is to be measured, filters must be made of inorganic material, e.g., glass fibre or metal foil (precombusted for 4 hours at 450 °C). Whatman GF/F glass fibre filters are recommended.

The determination of DOC implies that the samples are filtered. The limit between dissolved and particulate organic carbon is determined by the filter porosity (generally 0.45 µm).

If the water samples are not filtered, the organic carbon content analysed would represent TOC, i.e., the sum of organically bound carbon present in water, bonded to dissolved or suspended matter.

2. STORAGE OF SAMPLES

Filters containing particulate matter collected for POC analysis should be dried under vacuum for at least one day and stored dry in a desiccator with silica gel or, alternatively, temporarily stored in a freezer and later dried in a drying oven at 60 °C for 30 min.

A major potential problem for DOC analysis of samples of sea water is contamination. A particular problem for DOC samples is contamination by volatile water-soluble compounds such as ketones and alcohols. Exposure of the sample to the laboratory atmosphere should be limited and this type of work should have dedicated areas away from potential contamination sources.

The water sample should be stored in a refrigerator (2–5 °C), and analysed within one week. If a longer storage time is needed, the water sample could be stored frozen (–15 °C to –20 °C) for several weeks. One way to prevent contamination during storage is to store the water samples in sealed glass ampoules.

3. SAMPLE PRETREATMENT

If only DOC is to be determined, the sample should be filtered through a suitable filter, with a nominal pore size of 0.45 µm.

4. APPROPRIATE CHEMICAL ANALYTICAL METHODS

For POC analysis, a variety of similar instruments currently appear on the market. In particular, Carlo Erba and Hewlett-Packard CHN analysers have been frequently used. The main components of the analysers are basically the same, with an autosampler, a combustion column reactor, a reduction column, a gas chromatographic separation system, the detector unit, and an output device for the analytical results. Helium is used as the carrier gas. In the combustion reactor, oxygen gas and other oxidizing and catalysing reagents support the completeness of high- temperature combustion of organic carbon and nitrogen compounds to carbon dioxide, elemental nitrogen, and N-oxides. Elemental copper in the reduction column reduces nitrogen oxides to N_2 and binds excess oxygen. Water and the combustion products CO_2 and N_2 are separated by gas chromatography, and N_2 and CO_2 are detected and quantified by thermal conductivity detectors (TCD).

The analytical strategy for determinations of DOC in sea water typically comprises three stages: (1) initial removal of inorganic carbon species, (2) oxidation of the organic material into carbon dioxide, and (3) quantification of the carbon dioxide produced. The most difficult and controversial step in DOC determinations has been the oxidation. The oxidation method has to quantitatively transform the carbon bound in very complex mixtures of organic molecules into carbon dioxide, without formation of artefacts. Organic carbon is oxidized to carbon dioxide by combustion, by the addition of an appropriate oxidant, by UV radiation or any other high-energy radiation.

5. CALIBRATION AND THE BLANK

Analysis of POC is most often carried out together with analysis of PON (particulate organic nitrogen). For POC and PON determinations, the instrument is calibrated with high purity acetanilide (analytical-reagent grade). Acetanilide is used because its elemental composition matches the elemental composition of particulate material obtained from sea water, i.e., C:N = 8. At least ten filters should be analysed to determine the procedural blanks and the standard deviations from the mean values. These filters are treated in the same way as the sample filters, but the same water which is used for rinsing the sample filters (filtered sea water or artificial sea water) is filtered through the blank filters.

The DOC and TOC determinations are calibrated by analysing potassium hydrogen phthalate standard solutions of adequate concentrations. As a control of the DOC filtration, the carbon content of the filtrate after washing blank filters with dilution water should be determined and taken into account. The TOC of the water used for dilution and for preparation of the calibration standards should be sufficiently low to be negligible in comparison with the lowest TOC concentration to be determined.

6. INTERNAL QUALITY ASSURANCE AND CONTROL

The internal quality control should be carried out to check the operational performance of the system, by regularly analysing control samples and duplicate samples. If acetanilide is used as a control sample for POC and PON, it should be from another batch and preferably bought from another company than the calibration standard. For DOC and TOC analysis, copper phthalocyanine is suitable as a control sample solution. The control samples should be analysed with each series of samples and duplicate samples should be analysed regularly. These results should be plotted on control charts in order to verify the accuracy of the results, and estimate the measurement uncertainty.

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Grasshoff, K., Kremling, K., and Ehrhardt, M. (eds.) 1999. Methods of seawater analysis. Weinheim, New York.

ISO. 1999. Water quality – Guidelines for the determination of total organic carbon (TOC) and dissolved organic carbon (DOC). ISO 8245.

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Last updated: 29.10.2012 (Old Annex B 4 on Measurement uncertainty has been deleted and is now included as chapter B.4.2.6. Subsequently the Annexes in this section have been renumbered)

PART C PROGRAMME FOR MONITORING OF EUTROPHICATION AND ITS EFFECTS

C.1. Introduction
C.2. Sampling stations
C.3. Sampling programme as committed by the Contracting Parties
C.4. Directives for sampling and analysis of hydrographic, chemical and biological variables

Annexes

C.1. INTRODUCTION

The programme on eutrophication and its effects considers short and long term variations in hydrographic conditions and in chemical and biological variable.

More specifically the aims of COMBINE mean for:

Hydrographic variations:

aim: to set the background for all other measurements related to the identification and quantification of the effects of anthropogenic discharges/activities, the variables providing an indication of natural fluctuations in the hydrographic regime of the Baltic Sea must be monitored on a continuous basis

Core variables:

- temperature, salinity, oxygen and hydrogen sulphide
- light attenuation

Main variables:

- current speed and direction

Problems related to eutrophication (chemical and biological variables):

aim: to determine the extent and the effects of anthropogenic inputs of nutrients and organic matter on marine biota, the following variables must be measured:

a) Concentrations of nutrients

Core variables:

phosphate, total phosphorus, ammonia, nitrite, nitrate, total nitrogen and silicate, to quantify the changes in the nutrient pool. In CMP nitrate and nitrite may be measured together.

Main variables (In CMP supporting studies):

- Particulate and dissolved matter (carbon, nitrogen and phosphorus). These parameters are all essential for budget calculations and the contracting parties are recommended to include these in their programmes in all areas.

- Humic matter is an important source of nutrients in the Baltic Sea, especially in the Gulf of Bothnia and in its estuaries and should be incorporated into the programme there.

b) The response of the different biological compartments:

Core variables:

- chlorophyll-*a*, as an equivalent of the standing stock of phytoplankton;
- phytoplankton species composition abundance and biomass, to indicate a response in the biodiversity and a possible change in the food chain composition (e.g., introduction of alien species or increase in toxic species that are harmful to other organisms, and to indicate changes in the stock of primary producers;
- zoobenthos species composition, abundance and biomass (increase in biomass indicates eutrophication) and species composition (reduced species diversity). Excessive levels of eutrophication can result in low concentrations of oxygen in the bottom waters, resulting in damage to or death of zoobenthos.

Main variables (In CMP supporting studies, except zooplankton and phytobenthos):

- to measure the change in the rate of production, i.e. the first response of phytoplankton to the nutrient loading;
- zooplankton species composition, abundance and biomass, as changes can result, e.g. from changes in phytoplankton biomass and species composition. Especially in coastal waters zooplankton indicates different water masses, salinity fronts and other hydrological events.
- sinking rate of particulate matter;
- vertical profiles of chlorophyll a fluorescence, to give detailed information on vertical distribution of phytoplankton;
- phytobenthos, response to light climate and nutrient concentration results in depth distribution and species composition.

Supporting studies:

- Bacterial numbers and production are important in the cycling of nutrients in the Baltic Sea ecosystem. Especially in the Gulf of Bothnia, the role of bacteria is of major importance in the energy cycle, since the ratio of pelagic primary production to inputs of allochtonic organic matter is high. At least these bacteria should be a part of the high frequency sampling programme. However, bacteria are also of major importance in other areas of the Baltic Sea.
- Semi-quantitative analysis of phytoplankton can be used in addition to quantitative analysis to reveal temporal and spatial changes in phytoplankton communities.
- Microzooplankton plays a dominant role in certain shallow regions, and gives additional information on the functioning of the ecosystem.
- satellite imagery, as a tool for monitoring the spatial distribution of phytoplankton biomass in the surface layer, especially the accumulations of blue-green algae;

- annual primary production studies: important in assessing the changes in cycling of organic matter;
- fast repetition fluorometry, to record primary productivity with high resolution;
- flow cytometry, to describe the plankton community with an automatic method;
- HPLC pigment analysis, to get fast information of the phytoplankton pigment composition as indicator of the taxonomical composition;
- grain size distribution of sediment in relation to studies of macrozoobenthos;
- denitrification and nitrogen fixation, to describe the processes in the biological nitrogen cycle.

c) Integration and evaluation of results:

* Numerical and statistical models: It is essential that different kinds of models become part of the monitoring system, on equal terms with actual field measurements. The use of models also provides an opportunity to test the reliability of data. There are several uses of models;

- Real-time evaluations: if the monitoring should function as some kind of early-warning-system it is only with models in connection with measurements that we can assess the real time conditions.
- Budget calculations: models are necessary when interpolating/extrapolating measured data and are thus indispensable when making budget calculations.

An assessment of the results from the programme should be able to detect regional trends in hydrographical parameters, in nutrient concentrations, in phyto-, mesozooplankton, phytobenthos and macrozoobenthos abundance and species composition (where potentially toxic and/or alien species should be of particular concern) and in oxygen/hydrogen sulphide concentrations. For the assessment of the eutrophication status it is also important that the programme can resolve anthropogenic and climatological effects.

In order to meet the requirements of the strategy identified, the programme for the open sea, within each separate sub-basin, must be able to account for:

1. the winter pool of nutrients,
2. annual cycles of hydrographical parameters,
3. regional distribution and long-term changes in phyto- and zooplankton populations,
4. the spatial distribution of oxygen/hydrogen sulphide concentrations in the bottom water (in critical areas, especially during late summer/autumn),
5. spatial and long-term variability of macrozoobenthos,
6. occurrence of alien species which might have marked effects on the ecosystem,
7. events (e.g. toxic algal blooms) of importance for human health, recreational values or other economically important sectors, and

8. water exchange and nutrient fluxes between the Baltic Sea basins and between the Baltic Sea and the North Sea

C.2. SAMPLING STATIONS

To be able to fulfil these requirements, the programme should at least consist of:

- mapping of the winter pool of nutrients at least once per year before the onset of the phytoplankton growth period;
- mapping of oxygen/hydrogen sulphide and nutrient conditions in the near bottom waters a few times per year. It is important that this is carried out in late summer or autumn in certain critical areas.
- mapping of zoobenthos at least once a year;
- high frequency sampling which is needed especially for the pelagic variables and for monitoring water exchange between the various basins and between the Baltic Sea and the North Sea. This is obtained by visiting selected open sea or coastal stations frequently (preferably weekly measurements during the vegetative period), by using ships-of-opportunity sampling and automatic fixed stations. Automatic fixed stations are also needed for measurement of sinking rate of particulate matter.

Thus the COMBINE programmes comprises mapping stations and high-frequency stations. The BMP sampling stations presented by the Contracting Parties are shown in Figs. A.1-A.10 and Annex C-1.

*MAPPING STATIONS

1. Hydrography and nutrients:

The choice of stations during mapping surveys should be governed by the objectives of the survey, except that the frequent stations in each region always should be included in a mapping. Consequently, a fixed network of mapping stations is not considered since the need will vary due to varying physical/biological/chemical conditions. However, the objectives with the different mapping surveys should be identified and clearly stated.

Sampling frequency:

A few times per year:

- mapping the winter pool of nutrients
- mapping the oxygen/H_2S conditions, particularly in critical areas and season (e.g. the late summer/autumn).

Core variables:

- temperature and salinity

- O_2 and H_2S
- PO_4 and Tot-P
- NO_2, NO_3, NH_4 and Tot-N
- SiO_2

2. Macrozoobenthos

For studies of spatial and long term variations in macrozoobenthos, abundance biomass and species composition.

Sampling frequency:

Once or few times per year;

Core variables:

macrozoobenthos

Main variables:

- temperature and salinity,
- O_2 and H_2S in the near-bottom water
- weight-loss of ignition,
- smell (H_2S),
- depth of oxygenated layer in the sediment

Note: grain-size is listed on p. 2 of Part C

*HIGH FREQUENCY SAMPLING

1. Cruise stations

Sampling frequency on sample stations should be >12 times per year (basically monthly sampling but weekly in the vegetative period)

Core variables:

- temperature and salinity
- O_2 and H_2S
- PO_4 and Tot-P
- NO_2, NO_3, NH_4 and Tot-N
- SiO_2
- Chlorophyll-*a*

- Phytoplankton

Main variables:

- Primary production
- pH and alkalinity
- Zooplankton

2. Ship-of-opportunity sampling

Unattended recording and sampling on ferries and other commercial ships with regular schedules gives a possibility to collect data with high temporal and spatial resolution in the surface layer of the sea with large spatial extent. These kinds of measurements supply information important especially for the real time monitoring, and early warning system of, e.g. toxic algal blooms, and can also serve as reference and calibration for satellite images.

Sampling frequency:

The sampling frequency should be about every 200 m and every 1-3 days for temperature, salinity and chlorophyll a fluorescence. For phytoplankton and nutrients about every 10 km and every 1 - 3 weeks.

Core variables:

- Temperature and salinity
- chlorophyll *a*
- PO_4 and Tot-P
- NO_2, NO_3, NH_4 and Tot-N
- SiO_2
- phytoplankton

3. Automatic fixed stations:

These stations make it possible to collect high frequency data on temperature, salinity, oxygen, light attenuation and current speed/direction. Data from such stations are essential in frontal areas as e.g. the Belt Sea for evaluation of the water exchange. These stations also give access to real time data as input to numerical models (dispersion models) and are thus an important part of a system giving on-line information on certain events (e.g. inflows of North Sea water, potentially toxic algal blooms, oil spill accidents). Automatic stations with high sampling frequency will also improve our understanding of the dynamics of the marine system. High-frequency sample stations should be located close to the fixed stations.

Sampling frequency:

Temporal sampling frequency range between minutes and hours (days and weeks for the sinking rate of particles)

Core variables:

Temperature and salinity

Main variables:

- current velocity and direction
- sinking rate of particles

C.3. SAMPLING PROGRAMME AS COMMITTED BY THE CONTRACTING PARTIES

DENMARK

The preliminary Danish marine monitoring programme consists of:

- 2 automatic stations to record current speed and direction as well as temperature and salinity;
- 7 high-frequency hydrography/hydrochemistry stations where measurements are made annually 30-47 times. Additionally 4 high frequent stations are temporarily established in the Sound area as part of the control monitoring programme for the construction of the link across the Sound;
- 3 high frequency pelagic biology stations (annual sampling 26 times). Plus 1 frequent station (BMP-K2) in the Bornholm Basin;
- 27 mapping stations: the existing BMP hyrdography/hydrochemistry stations in the Kattegat, Sound and the Belt Sea already included in the Danish monitoring programme (15 st.), 2 BMP-stations in the Kiel and Mecklenburg bights, respectively. Plus some national stations (10 st.). Mapping of winter nutrients in February (1 cruise). Mapping of oxygen each month August-November (4 cruises). The cruises will be coordinated with Sweden and Germany. At all cruises and stations hydrography, hydrochemistry, oxygen and chlorophyll-*a* will be measured.

ESTONIA

- January (or February) - 30 stations covering whole area; measured variables: nutrient concentrations (PO_4-P, tot-P, NO_2-N, NO_3-N, NH_4-N, tot-N, SiO_2-Si), temperature, salinity, Secchi depth, O_2 (or H_2S), chlorophyll-*a*;
- June - 20 stations, measured variables: macro-zoobenthos, temperature, salinity, Secchi depth, O_2, nutrient concentrations (PO_4-P, tot-P, NO_2-N, NO_3-N, NH_4-N, tot-N, SiO_2-Si) and chlorophyll-*a*;
- October-April once a month, May-September every second week - 7 stations covering 2 high-frequent areas, measured variables: temperature, salinity, Secchi depth, O_2, nutrient concentrations (PO_4-P, tot-P, NO_2-N, NO_3-N, NH_4-N, tot-N, SiO_2-Si), chlorophyll-*a*, primary

production, phytoplankton (species composition and semi-quantitative abundance), zooplankton (biomass and species composition) and colony-forming bacterioplankton.

- August - phytobenthos observations at chosen transects in each high-frequent area and at additional reference areas.

FINLAND

The Finnish BMP programme includes:

- large number of stations with low sampling frequency (normally once a year) to map the winter pool of nutrients and the oxygen conditions in the near bottom water. The number and the positions of the mapping stations may vary slightly from year to year. The variables are temperature, salinity, Secchi depth, O_2, H_2S, PO_4-P, tot-P, NO_2-N, NO_3-N, NH_4-N, tot-N and SiO_2-S;

- large number of fixed stations for one annual (May-June) macrozoobenthos sampling including basic hydrography. The number of stations may vary slightly from year to year;

- high frequency sampling using ship-of-opportunity technique for temperature, salinity, chlorophyll-*a*, phytoplankton species composition and their semi-quantitative abundance as well as for PO_4-P, tot-P, NO_2-N, NO_3-N, NH_4-N, tot-N, and SiO_2-Si. Additionally, phytoplankton is determined quantitatively with lower frequency.

- satellite imagery to monitor the extent of the blue green algal blooms;

- several fixed near coastal stations in each sub-basin with a sampling frequency of ca 20 times per year. The variables are temperature, salinity, turbidity, colour, pH, O_2, PO_4-P, tot-P, NO_2-N, NO_3-N, NH_4-N, tot-N, SiO_2-Si, alkalinity and chlorophyll-*a*;

- about 100 near coastal or coastal mapping stations where temperature, salinity, turbidity, colour, pH, O_2, PO_4-P, tot-P, NO_2-N, NO_3-N, NH_4-N, tot-N, SiO_2-Si, alkalinity are measured in March and July-August and chlorophyll-*a* in July-August.

GERMANY

The German programme for monitoring eutrophication and its effects includes:

A. fixed sampling stations in the open sea for measuring:

- nutrients and oxygen conditions. The variables are temperature, salinity, Secchi depth or light attenuation, O_2, H_2S, PO_4-P, tot-P, NO_2-N, NO_3-N, NH_4-N, tot-N and SiO_2-Si. PH and dissolved as well as particulate carbon and nitrogen are supplementary variables.

- the pelagic biology variables chorophyll-*a*, phytoplankton species composition, abundance and biomass as well as mesozooplankton species composition and abundance.

- the macrozoobenthos variables species composition, abundance and biomass.

- the sinking rate of particulate matter with atomated sediment traps.

- hydrographic variables temperature, salinity, O_2 and current speed and direction at autonomous mooring stations.

B. a larger number of fixed near coastal sampling stations for measuring:

- nutrients and oxygen. The variables are temperature, salinity, O_2, H_2S, PO_4-P, tot-P, NO_2-N, NO_3-N, NH_4-N, tot-N and SiO_2-Si.
- the pelagic biology variables chlorophyll-*a*, phytoplankton species composition, abundance and biomass.
- the macrozoobenthos variables species composition, abundance and biomass.

C. supporting studies to develop novel, efficient monitoring techniques at selected stations for:

- HPLC determination of pigments, particle counting by flow cytometry and shipborne bio-optical and video techniques for use in phytoplankton and benthos analyses in the open sea.
- autonomous nutrient measurements at different depths at one of the mooring stations (FB).
- phytobenthos investigations along the coastline on selected transects.

Detailed information about locations and sampling frequencies of investigations under A and B are given in Annex C-1.

LATVIA

The Latvian marine monitoring programme for monitoring the eutrophication and its effects includes:

The Gulf of Riga

- *mapping stations*

1. winter pool of nutrients - 7 stations once in February.
2. oxygen/hydrogen sulphide and nutrient conditions - 12 stations once in August. Measured variables are: temperature, salinity, Secchi depth, O_2, H_2S, PO_4-P, tot-P, NO_2-N, NO_3-N, NH_4-N, tot-N, SiO_2-Si.
3. pelagic biology - 4 stations once in August. Variables are: chlorophyll-a, phytoplankton (species composition, abundance, biomass), mesozooplankton (species composition, abundance, biomass).
4. macrozoobenthos species composition, abundance and biomass - 19 stations once in August.

- *frequent stations*

1. hydrography and nutrients - 9 stations sampled 6-9 times per year (February - November). Measured variables are: temperature, salinity, Secchi depth, O_2, H_2S, PO_4-P, tot-P, NO_2-N, NO_3-N, NH_4-N, tot-N, SiO_2-Si.

2. pelagic biology - 7 stations sampled 7-8 times per year (February - November). Variables are: chlorophyll-a, phytoplankton (species composition, abundance, biomass), mesozooplankton (species composition, abundance, biomass), bacterioplankton.

- *high-frequency stations*

1. 2 stations sampled 20-21 times per year (February - December). Variables measured are: temperature, salinity, Secchi depth, O_2, H_2S, PO_4-P, tot-P, NO_2-N, NO_3-N, NH_4-N, tot-N, SiO_2-Si; chlorophyll-a, phytoplankton (species composition, abundance, biomass), mesozooplankton (species composition, abundance, biomass), bacterioplankton (1 station).

Eastern Gotland Basin

- *mapping stations*

1. hydrography, nutrients, oxygen/hydrogen sulphide - 7 stations sampled 3 times per year (February, May, August).). Measured variables are: temperature, salinity, Secchi depth, O_2, H_2S, PO_4-P, tot-P, NO_2-N, NO_3-N, NH_4-N, tot-N, SiO_2-Si.

2. pelagic biology - 4 stations sampled 3 times per year. Variables are: chlorophyll-a, phytoplankton (species composition, abundance, biomass), mesozooplankton (species composition, abundance, biomass).

3. macrozoobenthos species composition, abundance and biomass - 13 stations once in August.

- *frequent stations*

1. hydrography, nutrients, pelagic biology - 6 stations sampled 5 times per year (May - September). Measured variables are: temperature, salinity, Secchi depth, O_2, H_2S, PO_4-P, tot-P, NO_2-N, NO_3-N, NH_4-N, tot-N, SiO_2-Si; chlorophyll-a, bacterioplankton.

LITHUANIA

In the BMP Lithuania will investigate hydrography, hydrochemistry and hydrobiology as follows:

- 4 BMP stations (J1, J2, K1, L1) and 10 open sea (deep water) stations (46, 46a, 2c, 64a, 5b, 5c, 6b, 6c, D6, 43); sampling 4 times per year,
- 15 coastal zone stations (1, 1b, 2, 2b, 3, 4, 4c, 16, 64, 5, 6, 7, 20, 20a, 20b); sampling frequency 6 times per year,
- 3 "hot spot" stations (1K, 4K, 7K); sampling frequency 16 times per year

POLAND

The Polish monitoring programme comprises of the following measurements:

In the hydrological programme the variables are:

- water temperature and salinity, Secchi depth, O_2, H_2S, and sea currents

In the hydrochemical programme the variables are:

- PO_4-P, tot-P, NO_2-N, NO_3-N, NH_4-N, tot-N, SiO_2-Si

The biological programme comprises microbiology (in the coastal zone), chlorophyll-*a*, primary production, phyto- and zooplankton species composition, abundance and biomass, zoobenthos species composition, abundance and biomass and fish species composition, size distribution and diseases in selected area of the coastal zone.

The Polish monitoring programme is to be carried out on the basis of the following number of the stations:

Open sea stations (sampling at least 6 times per year, except macrozoobenthos - once a year), including:

- - hydrology, hydrochemistry - 4 stations
- - biology (pelagic and benthic) - 3 stations

22 coastal stations including:

- hydrology and hydrochemistry - 21 stations
- microbiology - 10 stations, 2 times a year
- pelagic biology - 12 stations, 4 times per year
- macrozoobenthos - 5 stations, once a year
- macrophytobenthos - 4 stations, 2 times per year
- 4 high frequency stations including hydrology, hydrochemistry and pelagic biology - 12 times a year

In the Polish programme reference points for each sampling area have been defined. Three stations (SK, L7, R4) are located within the identified BSPA areas while two other (ZP 6, P 102) lay close to the BSPA.

RUSSIA

[Information is missing.]

SWEDEN

The Swedish proposal for the BMP comprises

- 52 stations with low sampling frequency (1-2 times per year) to map the winter pool of nutrients and the oxygen conditions in the near bottom water, especially in late summer or autumn in the Kattegat, the Arkona, Bornholm and Gotland basins. The variables are temperature, salinity, Secchi depth, O_2, H_2S, PO_4-P, tot-P, NO_2-N, NO_3-N, NH_4-N, tot-N and SiO_2-Si;
- 19 stations with a sampling frequency of at least 12 times per year. The variables are temperature, salinity, Secchi depth, O_2, H_2S, PO_4-P, tot-P, NO_2-N, NO_3-N, NH_4-N, tot-N and SiO_2-

Si. At a subset of stations alkalinity, pH, chlorophyll, humic matter and phytoplankton abundance and biomass will be measured;

- 5 (2 coastal and 3 open sea) stations with high sampling frequency (20-30 times per year) with weekly sampling during the vegetative period. The variables are temperature, salinity, Secchi depth, O_2, H_2S, PO_4-P, tot-P, NO_2-N, NO_3-N, NH_4-N, tot-N, SiO_2-Si, alkalinity, pH, chlorophyll a, phytoplankton abundance and biomass, primary production. Zooplankton should be a supplementary variable at the high sampling frequency stations. In addition, supplementary variables are included depending of the local needs. These include particulate and dissolved carbon and nitrogen, bacteria, sedimentation and humic substances;
- one automated buoy station to monitor fluxes of water, salt, and nutrients between the Baltic Sea and the Skagerrak (North Sea);
- 139 soft bottom macrofauna stations are collected annually (May-June) from off-shore areas and in the coastal zone including basic hydrochemistry and sediment description;
- phytobenthos samples are collected annually once a year (August) from Baltic Proper (one area from coastal zone and one area from open sea which is further divided into 4 subareas). Totally 26 stations are visited. Variables to be measured are abiotic, plants and animals.

C.4. DIRECTIVES FOR SAMPLING AND ANALYSIS OF HYDROGRAPHIC, CHEMICAL AND BIOLOGICAL VARIABLES

Directive on the sampling methods and the procedure for analysis of eutrophication variables are given in Annexes C-1 to C-12:

Annex C-1 Tables listing sample stations
Annex C-2 Hydrographic and hydrochemical variables
Annex C-3 Sediment traps
Annex C-4 Phytoplankton chlorophyll-*a*
Annex C-5 Phytoplankton primary production
Annex C-6 Phytoplankton species composition, abundance and biomass
Annex C-7 Mesozooplankton
Annex C-8 Soft bottom macrozoobenthos
Annex C-9 Guidelines for monitoring of phytobenthic plant and animal communities in the Baltic Sea
Annex C-10 Guidelines for coastal fish monitoring
Annex C-11 Guidelines concerning bacterioplankton growth determination
Annex C-12 Guidelines concerning bacterioplankton abundance determination

In order to increase the number of plankton samples from open Baltic Sea areas and to improve the quality of plankton data from such pelagic biological station, the Contracting Parties have split the responsibility for working up plankton samples from different sub-areas among them. Whenever, a Contracting Party passes a pelagic biological station which is intended to be a high-frequent open sea station (see Annex C-1) quantitative phyto- and zooplankton samples should be collected and sent to the Contracting Party responsible for working up plankton samples from the specific sub-area as listed below. Each of the laboratories will have to decide the amount of resources it can allocate to the analysis. The sampling and working up of samples shall strictly follow the present manual concerning plankton samples.

TABLE C.1. Responsibilities of the Contracting Parties for working up plankton samples

Area	**BMP area**	**Responsible Contracting Party**	
		Phytoplankton	**Zooplankton**
Bothnian Bay	A	Sweden 10)	Finland 3)
The Quark	B	Sweden 10)	Finland 3)
Bothnian Sea	C	Sweden 10)	Finland 3)
Åland Sea	D	Finland 3)	Finland 3)
Archipelago Sea			
Gulf of Finland; eastern part	F	Russia 8)	Russia 8)
- central part		Estonia 2)	Estonia 2)
- western part		Finland 3)	Finland 3)
Gulf of Riga	G	Latvia 5)	Latvia 5)
Northern Baltic Proper	H	Sweden 11)	Finland 3)
Western Gotland Basin	I	Sweden 11)	

Eastern Gotland Basin	J	Sweden 11)	Germany 4)
		Lithuania 6)	Lithuania 6)
Southern Baltic Proper	K	Poland 7)	Poland 7)
		Sweden 11)	Germany 4)
Arkona Basin		Germany 4)	Germany 4)
Bay of Mecklenburg	M	Germany 4)	Germany 4)
Kiel Bight	N		
Little and Great Belt	P	Denmark 1)	Denmark 1)
Kattegat East	R	Sweden 9)	Denmark 1)
Kattegat West		Denmark 1)	Denmark 1)

Responsible laboratories:

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3) Finnish Institute of Marine Research
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Last updated: 31.03.2006

ANNEX C-1 TABLES LISTING SAMPLE STATIONS

The following table is an MS Excel (5.0) file. It conatins two worksheets:

1. Combine Stations 2003 contains all the sampling stations sorted by country. The data can be filtered by country.

2. MAP shows the All/Filtered stations.

COMBINE Stations

CMP stations are partly missing or are not confirmed by all Contracting Parties.

The latitude and longitude values are expressed as DD MM dd

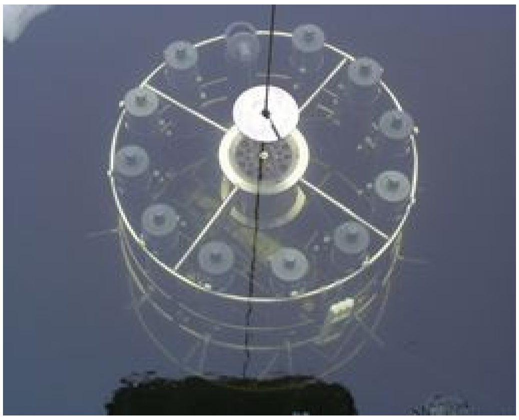
D=Degree

M=Minute

d=desimal of minute

(e.g. 50 25 67 = 50°25,67')

ANNEX C-2 HYDROGRAPHIC AND HYDROCHEMICAL VARIABLES



1. Introduction
2. Purpose
3. Sampling strategy, equipment and techniques
4. Procedures for observation or analysis
    4.1 Temperature
    4.2 Salinity
    4.3 Oxygen
    4.4 Hydrogen sulphide
    4.5 Nutrients
    4.6 Particulate and dissolved matter
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    4.8 Light attenuation
    4.9 pH
    4.10 Alkalinity
    4.11 Current speed and direction
5. Quality assurance
6. Reporting requirements
References
Technical Annex I

1. INTRODUCTION

This guideline is to fulfil the aims of the HELCOM monitoring programme. Following variables are measured:

Core variables:

- temperature
- salinity
- oxygen

- hydrogen sulphide
- nutrients: phosphate, total phosphorus, ammonia, nitrate, nitrite, (In the CMP nitrate and nitrite may be measured together), total nitrogen, silicate
- light attenuation

Main variables (In the CMP supporting studies):

- particulate and dissolved matter: POC, PON, POP, DOC, DON, DOP
- humic matter
- pH and alkalinity (only in combination with primary production measurements)
- current speed and direction

2. PURPOSE

As to set the background for all other measurements and to quantify the effects of anthropogenic activities the hydrographic/hydrochemical measurements shall be able to account for

- the winter pool of nutrients,
- annual cycles of hydrographic and hydrochemical parameters,
- space-temporal variations in the distribution of hydrographic and hydrochemical variables below the halocline,
- spatial distribution of salinity, oxygen/hydrogen sulphide and nutrient concentrations in the bottom water,
- water transport between the Baltic and the North Sea as well as between Baltic sub-basins and its effects on hydrographic and hydrochemical parameters,
- the supply of nutrients and nutrient limitation in coastal waters.

3. SAMPLING STRATEGY, EQUIPMENT AND TECHNIQUES

Different sampling techniques are necessary to resolve temporal and spatial variations of different variables. Master information such as position, date, time, weather conditions shall be recorded according to the reporting requirements (see below).

The sampling techniques can be grouped as follows:

Attended measurements: Sampling carried out at the stations with fixed position.

Data on the variables listed above may be collected using a CTD system which is attached to a rosette sampler or a cast of reversing water samplers (e.g., Niskin or Nansen bottles) equipped with reversing thermometers. It is strongly recommended that CTD system is equipped with the fluorometer for

recording of chlorophyll-a measurements. The depths at which sampling should take place are as follows (in metres): 1, 5, 10, 15, 20, (25)*, 30, 40**,** 50, 60, 70, 80, 100, 125, 150, 175, 200, 225, 250, 300, and 400 metres, and as close to the bottom as possible (preferably less than 1 metre from the sediment surface to get near bottom oxygen concentration.) In the CMP, samples should be taken from 1 m below the surface or an intergrated sample from 1m to the pycnocline (spring layer) and as close to the bottom as possible (less than 1 m). At least two samples should be collected. For unstratified water less than 10m depth, samples will be taken from 1m or an integrated sample is taken. The regional conditions and circumstances have to be considered, when choosing the sampling depth. National programmes should be coherent.The general description how to take water samples is given in**Technical Annex I**.
[*) 25 m obligatory in the Kattegat and the Belt Sea]

The sampling depths for pH and alkalinity are those selected for primary production sampling.

Unattended measurements: Sampling carried out at automatic stations or onboard of ships-of-opportunity.

Basically these measurements shall comply with the same standards as the attended measurements. Preferably one or more of those sampling depths mentioned above should be used. However, it is recognised that technical or other reasons, e.g. dynamic conditions, also influence where the samples are taken.

4. PROCEDURES FOR OBSERVATION OR ANALYSIS

4.1 Temperature

Temperature is to be determined by temperature sensors as on a CTD (as well as in a flow-through system onboard ships-of-opportunity or on an automatic station) or by reversing thermometer. The measurements can be done from different platforms, i.e. research vessels, ships-of-opportunity and fixed platforms.

If CTD system is used, both temperature profile and temperature values at the moments when water samplers are tripped should be stored. Handling and calibration of CTD is described in Technical Annex I. Reversing thermometers should be calibrated using standard facilities at least once every two years. Mercury thermometer readings should be corrected using the Sverdrup formulae, as given by La Fond (1951). If digital reversing thermometers are used, one should follow the manufacturer's recommendations.

Unattended measurements require special consideration according to (UNESCO, 1993; Rantajärvi and Leppänen, 1994).

The required accuracy for thermometers and sensors used is ± 0.05°C, in CMP 0.1°C.

4.2 Salinity

Salinity is to be determined by conductivity and temperature sensors as on a CTD (as well as in a flow-through system onboard ships-of-opportunity or on an automatic station) or from water samples by a laboratory salinometer. The measurements can be done from different platforms, i.e. research vessels, ships-of-opportunity and fixed platforms.

Handling and calibration of CTD is described in Technical Annex 1 to this document and in Annex B-9 Appendix 1. Samples for the determination of salinity must be collected with care, in order to ensure that no salt crystals are trapped in the cap. Sample bottles which ensure negligible evaporation must only be used, this has to be validated by each laboratory. Samples should be analysed using a salinometer that has been carefully standardised using IAPSO Standard Seawater. It is important that other kinds of standard seawater should be calibrated against the IAPSO Standard Seawater and not against a KCI standard on which IAPSO Standard Seawater is calibrated. It is also important to follow closely the manufacturer's recommendations on salinometer use.

In order to compute salinity values from salinometer conductivity ratio values, only the International Oceanographic Tables Vol. 3 (UNESCO, 1981) or related algorithms (UNESCO, 1988) should be used. The values produced by using either of these publications are derived from the Practical Salinity Scale 1978, and are, therefore, in practical salinity units. The algorithms should also be used to compute density values from the Equation of State for Seawater 1980. Density and other derived physical quantities may also be obtained using the Tables published in (UNESCO, 1987).

Salinity determinations from water samples can be carried out on shore, provided that the analyses are carried out within a few weeks after sampling.

The required accuracy for salinity is 0.05 psu, in CMP 0.1 psu.

4.3 Oxygen

The basic method for the determination of oxygen concentration is the Winkler method (Grasshoff et al., 1983). The oxygen sensors may be used, however it is highly recommended to take water samples in areas with low oxygen concentration (below 2 cm3/dm3).

Samples for the determination of oxygen concentration should be collected using appropriate water samplers, especially when collecting near bottom samples to ensure that sample is taken within one metre from the sediment surface. Oxygen sample must be the first one taken from the water sampler and the reagents should be added immediately.

Oxygen samples may be stored for up to 24 hours after adding the reagents and after complete fixation. The bottles should be kept in the dark and any change in temperature should be avoided. The bottles can be stored under a waterlock for up to one month.

If oxygen sensors are used (e.g., attached to the CTD), regular checks of the reproducibility of the sensor have to be carried out by titration of water samples by the Winkler method. At least the surface reading has to be calibrated. Oxygen sensors cannot be properly calibrated against moist air (100% saturation). Oxygen sensors may be poisoned by hydrogen sulphide. If a sensor has been lowered into hydrogen sulphide containing water, it must be checked immediately.

Minimum analytical requirements are as follows; detection limit 0.02 cm3/dm3, accuracy 0.03 cm3/dm3, for CMP 0.1 cm3/dm3.

4.4 Hydrogen sulphide

For small amounts, use the colorimetric method (Fonselius, 1983) and for large amounts, use dilution or volumetric titration according to Fonselius, 1983.

Hydrogen sulphide samples of moderate concentrations (<300 µM) may be stored for several days after adding the reagents. The bottles should be kept in dark and any change in temperature should be avoided.

Minimum analytical requirements are: simply note, by smelling at the water sample, if hydrogen sulphide is present or not.

The concept "Negative Oxygen" is a convenient way to express the amount of oxygen utilized for producing hydrogen sulphide by reduction of sulphate ions $2\ (CH_2O) + SO_4^{2-}\ 2CO_2 + 2H_2O + S^{2-}$. Assuming simple stochiometry ($S^{--} + 2O_2\ SO_4^{--}$) sulphide may be converted into "negative oxygen" in order to get comparable equivalents by multiplying the hydrogen sulphide result by two (one molecule of H_2S is equivalent with two molecules of oxygen). To convert from µmol/l of sulphide to cm^3/dm^3 of oxygen, multiply the µmol -result by the factor -0.044. This simplification, however, may not completely reflect natural conditions, because other oxidizable sulphur compounds may possibly be produced in significant amounts by microbial sulphate reduction under anoxic conditions.

4.5 Nutrients

The determination of nutrients is based on colorimetric methods (c.f. Grasshoff *et al.*, 1983, Kirkwood, 1996).

Minimum analytical requirements are as follows:

- Detection limit umol/l Accuracy requirements (IZI score <2)
- medium-high-low concentration
- phosphate 0.02 12% 25%
- nitrite+nitrate 0.1 12% 25%
- nitrite 0.02 12% 25%
- ammonia 0.1 12% 25%
- silicate 0.1 12% 25%

Low concentration can be defined as being within a factor of 20 of the respective detection limits.

The use of IZI score was established during QUASIMEME LPS (Laboratory performance studies). A IZI=2 was considered a minimum requirement for a satisfactory analysis. A IZI score 1 should be attainable.

It is recommended that interlaboratory intercomparison tests for nutrients sshould be carried out on a regular basis. Standard stock solutions containing concentrations > 1 mmol/dm^3 may be stored for about an year if kept in DURAN glass ampoules, except for nitrite and silicate. Stock solution for nitrite may be stored for maximum half a year and glass ampoules should not be used for silicate.

It is strongly recommended that all laboratories participate in QUASIMEME II Proficiency Testing Programme.

Nutrient standard solutions are also recommended to be compared on a regular basis to either "Sagami Industrial Standards" or to primary standards which have been confirmed using Sagami standards or by intercalibration.]

Total nitrogen [Total nitrogen means nitrogen compounds measured after peroxo-disulphate oxidation.]

The peroxo-disulphate oxidation (Koroleff, 1983) with consecutive determination of the nitrate based on colometric methods (cf. Grasshoff *et al*., 1983, Kirkwood, 1996) should be used.

Total phosphorus and total nitrogen

As an alternative for the methods listed above for total nitrogen, use digestion according to Valderrama (1981) and Koroleff (1983), for both total nitrogen and total phosphorus.

Silicate

Preference should be given to the ascorbic acid method by Koroleff (1983). Other methods intercalibrated within QUASIMEME can also be used.

Corrections for turbidity, salinity or hydrogen sulphide

Details are given in Part B, Annex B-9 and Annex B-10.

When nutrients are analysed using various kinds of autoanalyzers, the effect of sample turbidity is often omitted. The cuvettes of autoanalyzers are often too short to register the small error caused by turbidity. In precise work with cuvettes longer than 2-3 cm, the turbidity of the sample should be measured when analysing phosphate and nitrite and also nitrate if a cadmium coil is used as reductor instead of cadmium grains.

4.6 Particulate and dissolved matter

Particulate organic carbon and nitrogen (POC and PON), concentrated on filters, is measured using the high-temperature combustion technique after filtration on a 0.7 µm filter. Recommended filtered amount is 500 cm^3 for open sea samples. Filters have to be burnt off at 450C for 4 hours prior to use. In order to avoid sedimentation and adsorption of particles onto the wallsof the sample bottle, filtration within 2 hours is recommended. Blank filters are prepared by filtering water of the highest available purity under the same conditions as the samples. For calibration purposes dry acetoanilide standards, containing a well defined amount of carbon and nitrogen, are prepared.

The dissolved fraction (DOC/DON) is measured using a TOC analyzer (HTCO method according to Sugimura and Suzuki (1988) either directly on a filtrate (preferable technique) or on the untreated sample and calculated as the difference between the total and particulate fractions. In both cases inorganic carbon has to be removed by acidification to pH 4 and purging with an inert gas for 10 minutes prior to analysis.

4.7 Humic matter

Humic matter is measured on ordinary water samples using fluorescence spectroscopy at 350/450 nm excitation/emission wavelength (Coble et al, 1990; Wedborg et al, 1994). Samples can be stored in the dark for at least 2 weeks. It is strongly recommended that calibration is always carried out with a quinine sulphate solution in 0.5 M H_2SO_4 in order to ensure comparability between different projects. If true humic substance units are sought, a humic substance standard has to be prepared for every sea area.

4.8 Light attenuation

Vertical light attenuation is measured using a Secchi disc or an irradiance metre (400-700 nm).

Secchi depth should be measured at all stations whenever possible, i.e. in day light and when the sea is relatively calm. Light attenuation shall always be measured if primary production measurements are performed. The methods for measuring light attenuation are described in Annex C-5, Phytoplankton Primary Production, Chapter 7.

4.9 pH

pH should be measured, by the electrometric method with glass electrodes. Measurement should be made according to Grasshoff et al. (1983), Poisson et al. (1990) or UNESCO (1987) using thermostated samples. The temperature correction should be made using the temperature coefficient by Gieskes (1969). pH sensors (e.g., attached to a CTD) are also allowed.

Remarks: No depth correction should be applied because the pressure coefficient is not precisely known.

4.10 Alkalinity

Alkalinity should be determined by potentiometric titration. Today most methods are according to Bradshaw et al. (1981) or a similar method, where the evaluation is done by curve fitting either before or after the equivalent point. The definition of the alkalinity is shown in Dickson (1981).

4.11 Current speed and direction

The information on water transport between the Baltic and the North Sea as well as between Baltic sub-basins is of great importance for the budget calculations. Therefore it is recommended to perform current measurements in the transition areas. Preferably measurements from fixed platforms or at buoy stations should be carried out and appropriate current meters should be used. Manufacturer recommendations must be followed. Data treatment according to UNESCO (1991; 1993).

5. QUALITY ASSURANCE

The QA programme should ensure that the data are fit for the purpose for which they have been collected, i.e. that they satisfy detection limits and levels of accuracy compatible with the objectives of the monitoring programme (c.f. text above).

Concerning QA and sampling c.f. Technical Annex I.

Concerning QA and chemical variables c.f. Part B.

6. REPORTING REQUIREMENTS

To be provided at a later stage.

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TECHNICAL ANNEX I TEMPERATURE AND SALINITY MEASUREMENTS OF SEAWATER

ACQUISITION OF CTD DATA AND SAMPLING WITH ROSETTE CTD SAMPLER

Introduction

Temperature and salinity can be considered the most important parameters in Physical Oceanography because of their conservative characteristics. By means of these two parameters one can identify the water masses, their evolution and propagation, even though other parameters such as chemical, biological or sedimentological, are extremely useful.

Ocean circulation is still, to a large extent, deduced from the density field which is defined by the temperature and salinity fields. Particular care must be taken in measuring these two parameters and especially the salinity whose variations, at low temperatures, have a greater influence on the density determination.

Previous method for measuring salinity was the Knudsen titration method, based on the assumption of the almost constant composition of sea water. The chlorinity of the sample was determined by titration with silver nitrate, and then the relationship $S = 0.030 + 1.8050\ Cl$ gave the salinity expressed in grams per kg seawater. This method was used until the 1950's and after that was gradually substituted with the laboratory salinometer. With this instrument the salinity is determined by measuring the conductivity of the sea water sample and comparing it, directly or indirectly, to the conductivity of the "standard seawater".

In the 1960's and 1970's the era of "in situ" measurements started by means of profilers, called CTDs because the standard configuration is formed by Conductivity, Temperature and Depth(pressure) sensors. The introduction of CTD systems introduced new problems in computing salinity from the observed variables, conductivity, temperature and pressure. This led to the development of a new algorithm for salinity, "The practical salinity scale 1978"(PSS 78) and further "The International Equation of State of Seawater 1980" (UNESCO, 1981).

Salinity determination by titration is no longer scientifically acceptable. The bench salinometers are still used, and moreover they are recommended to be used for the control and calibration of the CTD conductivity sensor during cruises.

Until a few years ago, temperature measurements were done with reversing mercury thermometers. Now, generally, CTD profilers are used together with reversing digital thermometers with platinum thermoresisters. In some CTD systems, a combination of a thermistor, which has a rapid response time, with a platinum thermometer, which has a slower but more stable response and is inherently more accurate, is employed.

Methods have been developed to compensate for different time constants of the sensors of the CTD system, mainly by low pass filtering. Guidelines on how to handle CTD data have been introduced (UNESCO, 1988, UNESCO, 1991).

In conclusion, the T and S measurements changed from a discrete to an almost continuous type. Generally the data acquisition speed is 24 Hz, which means 24 samples of each parameter every second. In this way, high resolution in the description of the thermohaline structure of water masses is obtained.

The gain in the accuracy of the measurements is from +/-0.02 PSU for titrations to +/- 0.005 PSU for CTDs and from +/-0.02 ° C for mercury reversing thermometers to +/- 0.004 ° C for CTDs.

Particular mention must be made of the automatic temperature and salinity acquisition systems, for the great quantity of information (time series) that they give, although there is a certain loss in precision. These systems are normally used in moorings together with current meters, sediment traps, etc. Particular instruments in this category are the thermo-salinometers. These instruments can achieve good quality measurements, and since data acquisition is autonomous, they can be used much more widely, for example, on board passenger and commercial ships but for surface samples only.

Materials required

Equipment

A CTD probe preferably equipped with double sensors of temperature and conductivity since this configuration will be a guarantee of no loss of data and a quality check of the data. A broad- beamed altimeter or a bottom switch is recommended in order to be able to go very close to the bottom but still ensure security of the instrumentation.

Other sensors recommended are the oxygen sensor, fluorometer, transmissometer, scatterometer and PAR (Photosynthetically Available Radiation).

A Rosette multisampler with capacity of 12 or, better, 24 bottles of at least 1.7 l volume.

Water sampling bottles. The water samplers shall be clearly marked with individual numbers. This will facilitate logging of any malfunctions, in most cases leakages.

A laboratory salinometer with thermostatic bath and an accuracy better than ± 0.002 PSU.

At least two reversing digital platinum thermometers with traceable calibration.

Personal Computers for the data acquisition and for the data processing.

An analogical tape recorder for a playback of the cast (optional).

Supplies

Bottles for salinity samples. Glass bottles with stoppers with plastic under-stoppers are recommended. Particular care must be taken in closing the salinity bottle samples. Do not completely fill the bottles since thermal expansion can cause damage to the stopper or bottle. Before storage wash the bottles with fresh water.

Vials of standard seawater for the salinometer calibration (35 PSU, P series) and for the in house quality control (10 PSU, H series).

Procedures

Data acquisition

There are many protocols for CTD measurements (WOCE, 1991, UNESCO, 1994, UNESCO, 1988). Starting from what is suggested by the previous protocols and taking into account the field experience from the BMP, the following protocol is proposed.

When the CTD is on deck turn on and note the CTD pressure and temperature on the log book (see the CTD station log sheet given in**Attachment 1**).

The CTD and Rosette package must be lowered a few meters below the sea surface for at least two minutes before starting the measurements. This is of special importance if using a oxygen sensor. After a few minutes the CTD is brought back to near the surface and the measurement starts. If the sea state is rough it is recommended to start the downcast from a few meters below the sea surface to prevent the bubbles of the breaking waves entering the conductivity cell.

It is recommended to keep the lowering speed as constant as possible and between 40 and 120 cm/s.

If the CTD is equipped with an Altimeter or a bottom switch , lower the CTD and Rosette package as close to the bottom as possible. Enter in the log book the distance to bottom, CTD depth and sonic depth and all the other information required by the CTD log.

Preferably the rosette bottles should be fired at the selected depths during the up-cast as to be able to obtain an undisturbed ctd profile during the down-cast.

The first bottle, to be closed near bottom, must be equipped with two reversing thermometers. It is recommended to use the new reversing digital platinum thermometers, and in this case, waiting for at least 15 seconds before starting the recovery.

Almost all the data acquisition software creates a Bottle file (a file with pressure, temperature and conductivity values measured at the moment of firing the bottle, or an average around that moment), nevertheless it is recommended to write on the CTD log the bottle number and the pressure, temperature and salinity displayed by the unit, at the moment of firing the bottles as to ensure a correct bottle firing sequence.

When the CTD is back on the deck, note on the CTD log the pressure and temperature. The pressure value must be approximately the same as that read before the cast, differences are due to thermal and mechanical hysteresis of the pressure sensor. Do not use deck pressure as offsets to correct pressure.

Deck pressure should only be used as consistency check against laboratory-measured historical drift. Flush the sensors with freshwater and deionized water to prevent formation of salt crystals. The conductivity cell must be kept as clean as possible. Do not touch the inside walls of the conductivity cells or the electrode surface with hands or any other physical means. Protect the CTD from the direct sun radiation and each sensor according to the specific manufacturer's instructions.

Before samples are drawn from a water sampling bottle, one must make sure that it is not leaking. This is done by opening the tap without opening the air went on the top of the bottle. The bottle is tight if no water comes out of the tap. Leakages should be logged.

When all samples have been taken the water sampling bottle must be emptied for remaining seawater and the CTD be flushed again with fresh water.

Note on the CTD log all particular events that happened during cast.

Make a backup of the data immediately at the end of the cast, before carrying out another operation.

Maintenance

At the termination of a cruise the CTD underwater unit must be thoroughly washed with freshwater. The water sampling bottles must be filled with (or if possible dipped in) freshwater as to prevent any formation of saltcrystals inside the bottles.

Between cruises (and whenever environmental conditions require it) it is important that the CTD underwater unit and rosettsampler are stored in a way that prevents contamination.

Calibration

Temperature, conductivity and pressure sensors must be calibrated before and after a long cruise, or at least once a year by sending them to the manufacturer or to a calibration centre. It is very important to keep the calibration series of each conductivity and temperature sensor to reconstruct the drift history of each sensor.

During the cruise a check of the temperature sensor must be made at every cast by means of a pair of reversing thermometers mounted on a bottle fired in a well mixed layer. A number of salinity samples (preferably from all water bottles or at least from well mixed layers) must be taken to cover the range of temperatures, salinities and pressures encountered on each cast in order to check the conductivity sensor which drifts sufficiently to require field calibration.

The salinity values obtained by the laboratory salinometer must be inverted to an *in situ* conductivity using the CTD temperature and pressure. The conductivity values so obtained will be compared to the CTD conductivity values (for more details see the UNESCO, 1988).

Data Processing

Attention must be paid to data processing in order to consider the change of temperature standard from T68 to T90 (T68 = 1.00024 T90). Software, for the CTD data acquisition and processing, that manufacturers give to the customer together with the instruments, generally follow the UNESCO recommendations (UNESCO, 1988).

They can be summarised in the following steps.

1. Convert the digitised voltages or frequencies measured by the sensors to physical units.

2. Correct the data by applying the calibration coefficients, the pressure offset and the "slop" and "offset" correction coefficients deduced from laboratory calibrations and salinity bottles samples.

3. Time lag correction. The sensors have very different time responses, the pressure and conductivity are fastest, followed by the temperature, while the oxygen is slowest. Oxygen must be advanced for a few seconds relative to pressure. Similarly, the temperature must be advanced relative to pressure in order to reduce salinity spikes. Particular care must be taken in this operation.

4. Mark "bad" the scans with values which deviate by more than a given number of standard deviations. Also the scans reversing values of pressure or with a CTD velocity less than a given limit must be marked "bad."

5. Apply different kinds of filters to smooth spikes in the data. Particular care must be taken to avoid displacement in the data.

6. If in the CTD configuration there is the oxygen sensor, compute the oxygen.

7. Average the data every 0.1 decibar.

8. Compute salinity, density and other oceanographic parameters.

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Attachment 1: CTD station log

Country Ship Station name Station number
File name
Cruise Date Operator CTD nr

CTD offset control	On deck press	On deck temp
Before lower		
After lower		

Position	Latitude	Longitude	Time	Sonic depth
Start				
Bottom				
End				

Water samples CTD raw data from deck unit	

Btl depth	Btl nr	press	temp	salinity	salinity samples nr	Thermom. nr	Temperature

Comments : ________________________________

ANNEX C-3 SEDIMENT TRAPS

1. INTRODUCTION

Due to increased new production during the process of eutrophication export of organic matter from the euphotic zone to the sediment surface is enhanced. Concomitantly species composition in the pelagic community may undergo changes, which will be reflected in the composition of vertical flux.

2. PURPOSE

The purposes for recording the time varying vertical particle flux with sediment traps at some selected stations in the Baltic Sea are:

- to measure the seasonal patterns in the flux and its interannual variability
- to record trends in quantity and composition of the flux
- to reconstruct pelagic events such as blooms and occurrence of species from year round fluxes.

3. SAMPLING STRATEGY OF VERTICAL PARTICLE FLUX

A clear pattern of seasonal flux events can be expected related to events of primarily new production with little pelagic consumption such as after spring and fall blooms as well as sporadic blooms. Year round sampling of vertical particle export will allow to identify time and duration of bloom events as well as phytoplankton species composition from frustules and/or biochemical markers such as alkenons or pigment products. Moreover, given a trap deployment in anoxic waters, to where no living zooplankton penetrates, trap collection will allow an estimation of zooplankton mortality. The fate of cyanobacterial blooms, whether they are consumed in the pelagic foodweb or are largely exported to the seafloor, will be followed. Enumeration and possible identification of resting stages will supplement species analysis from water column samples.

The main strategy of sediment trap deployment is to obtain a year round record on proxies for pelagic events which normally escape detection by frequent ship sampling alone. Emphasis should be given to qualitative aspects, as in the narrow Baltic Sea basins resuspension and lateral transport may falsify quantitative estimates during some periods. The Bornholm and Gotland basins are chosen for sampling sites.

An ideal sediment trapping protocol would define rigorous guidelines on trap design, deployment, sample collection methods, sample processing, analytical methods and calculations. However, there is a general consensus that due to the paucity of data which enable one to quantitatively compare the relative merits of different designs and techniques the present state-of-the-art does not yet justify strict protocols. Further, it is hardly to envisage a rigorous protocol for the different environments of deployment and complex variability of sample types.

4. SAMPLING

Cylindrical or funnel-shaped traps should be used, which fulfil the basic hydrodynamic requirements that particles which have entered the trap will not be washed out of the trap. Cylinders with a minimum aspect ratio of 5:1 and funnel-shaped traps equipped with a baffle of the same aspect ratio will be suitable. Multi-traps with a turn-table for several sample containers for which individual sampling time can be preset for any time interval from days to months are preferable.

The traps should be deployed below the halocline and sufficient distance to the sea-bottom to minimise the effects of resuspension.

The minimum temporal resolution should allow a continuous recording during the deployment period and provide sufficient material for the respective analysis of the samples. A monthly resolution for winter months and a bi-weekly resolution for the pelagic growth period is recommended as a minimum. Trap design allowing a weekly resolution is preferable at the beginning and towards the end of the growth period.

Sampling containers of the trap should be filled with filtered water from the deployment depth.

During long deployment times a preservative is essential to avoid microbial degradations of the samples and to prevent activities of swimmers. Formaldehyde appears to be the most suitable compromise in terms of effectiveness and prevention of swimmer fragmentation.

5. POST-DEPLOYMENT PROCEDURES

5.1 Storage

Immediately after trap recovery sampling containers are stoppered tightly and are kept under refrigeration in the dark until separation of particles from the supernatant solutions.

5.2 Swimmer-handling

By definition swimmers are organisms, i.e. zooplankton, that have entered the trap actively rather than by passive sinking. In trap collections from oxic waters swimmers cannot be differentiated from passive animal fluxes. These organisms must be picked from the samples before further handling of trap material for flux estimates.

Zooplankton picked from trap samples from deployment in anoxic waters can be expressed as zooplankton flux provided that the existence of living zooplankton in the anoxic layer above the trap can be excluded by regular water column sampling with nets.

5.3 Optical inspection of trap collection

Before subdivision for various analyses the wet sample is inspected and qualitatively described using a dissecting microscope.

5.4 Subdivision of samples

No rigorous protocol can be followed for the splitting procedure. This will largely depend on the results of the optical inspection. Sieving of samples for size-fractionating should be avoided as fragile particles are destroyed by these procedures and thus fractionation is of no use.

Any subsamples should be a discrete aliquot of the entire sample, the amount will be dependent on the minimum quantity required for the respective analysis.

For microscopical analysis wet splitting is to be conducted. For bio-chemical particle characterisation, such as dry mass, carbon, nitrogen and phosphorus species, biogenic silicate, a large wet subsample should be desalinated and freeze dried with subsequent subsampling by weight.

6. ANALYSIS

Analyses from trap samples should include at least the determination of

- dry weight
- total particulate C, N and P
- microscopic investigation of particle composition including phyto- and zooplankton

(UNESCO, 1994) and is to be carried out according to the BMP Manual unless composition of trap material dictates otherwise

7. CALCULATIONS

The downward vertical flux of any particle and particulate component is defined as the quantity of that particle or that component through a horizon in a given time. Flux is calculated from the sample diameter of a given trap and exposure time of the respective sampling container and expressed in units of quantity per horizontal area per time.

REFERENCE

UNESCO, 1994. Protocols for the Joint Global Ocean Flux Study (JGOFS). Core Measurements. IOC Manuals and Guides 29: 170 pp.

ANNEX C-4. PHYTOPLANKTON CHLOROPHYLL *A*

1. INTRODUCTION

Nutrient enrichment/eutrophication may give rise to increased phytoplankton biomass, increased frequency and duration of phytoplankton blooms and increased primary production. Chlorophyll-*a* is used as an estimate of phytoplankton biomass.

2. PURPOSE

The purposes for measuring chlorophyll-*a* concentration in the Baltic Sea Area are:

- to describe the spatial distribution and frequency of phytoplankton blooms;
- to describe temporal trends in phytoplankton biomass and in the frequency and duration of phytoplankton blooms.

3. SAMPLING STRATEGY

Increased chlorophyll-*a* concentrations mainly occur in nutrient-enriched waters and in the deeper layers of stratified water. To describe the spatial distribution of chlorophyll-*a* concentrations, horizontal and vertical sampling at an appropriate number of stations is required. In order to describe the spatial distribution and surface coverage of phytoplankton blooms, regular spatial surveys could be carried out using ships of opportunity or aerial visual and remote sensing devices, accompanied by regular water sampling. In order to give exact information on the vertical distribution it is recommended to make profiles with a fluorometer.

Chlorophyll-*a* concentrations vary substantially during the growth season and may vary considerably from year to year as a consequence of many factors (e.g. meteorological/hydro-graphic conditions). Thus, it may be difficult to describe temporal trends. For this reason, sampling needs to cover the entire growth season. This leads to the possibility of assessing mean values for the spring season and for the entire growth season.

The sampling strategy for sea-truth measurements follows the general HELCOM strategy of shared ship cruise measurements on agreed stations in the Baltic Sea. The following text describes only ship cruise measurements of chlorophyll-*a* and phaeopigments.

4. SAMPLING

For the open sea, the standard sampling depths for chlorophyll-*a* are in the upper water column the same as for nutrients: 1 m, 5 m, 10 m, 15 m and 20 m. In CMP, the sample from 1 m or an integrated sample (1-10 m) could be analysed. Additional sample(s) should be obtained from chlorophyll maxima present at other depths. Such maxima are found using a profiling fluorometer/ CTD. Chlorophyll should also be analysed from the same sample used for phytoplankton and primary production analyses.

For ships-of-opportunity and helicopter sampling a single sample from the mixed surface layer can be taken.

5. STORAGE OF WATER SAMPLES

It is important that the water is filtered immediately after sampling.

6. VOLUME DETERMINATION

6.1 Spectrophotometric determination

Due to the seasonal variations of chlorophyll-*a* concentrations in the Baltic Sea area the sample volumes have to be optimized to fit the volume of the extraction solvent and the cuvette length:
- at a concentration of 0.1 mg x m^{-3}:

10 cm^3 extraction solvent and 5 cm cuvette length needs 1200 cm^3 of water to be filtered to get a spectrophotometric absorbency of about 0.05.
- at a concentration of 1 mg x m^{-3}:

10 cm^3 extraction solvent and 1 cm cuvette length needs 600 cm^3 of water to be filtered to get a spectrophotometric absorbency of about 0.05.

6.2 Fluorometric determination

The sensitivity of the fluorometer is about ten times higher compared to the spectrophotometer. Thus, the required volume could be reduced ten times.

7. FILTRATION

- The samples shall be filtered in subdued light.
- The filtration should be carried out immediately after sampling.
- Filter to be used: GF/F
- Suction pressure must not exceed 3 x 10^4 Pa.
- Filtration time must not exceed 30 min for the volumes needed for the spectrophotometric method and 3 min for the fluorometric method.

8. DRYING OF FILTERS

The filter should be drained under suction before removal from the filtration equipment. The filter should be dried in darkness at room temperature before extraction.

9. STORAGE OF FILTERS

Extraction and analysis should be done without delay. If necessary, filters may be stored deep-frozen up to 1 month before extraction and analysis (Jeffrey et al., 1997).

10. EXTRACTION

All work with the chlorophyll extract shall be carried out in subdued or green light.

After the filtration and drying, the folded filter is placed in a graduated centrifuge tube with proper stopper, to be able to adjust the volume and to avoid evaporation.

- 96% ethanol should be used as solvent.
- Extraction volume: 10 cm^3 added with a calibrated dispenser/pipette, smaller amount could be used to get the optimal concentration (see Chapter 6.1).
- Extraction time: 6-24 hours at room temperature.
- Mixing: Constant or by shaking the tubes a few times during the extraction time.
- The extraction tube should be closed immediately after adding ethanol and should be kept tightly closed during extraction, storage and centrifugation.

11. CENTRIFUGATION

Using spectrophotometric measurements centrifugation is necessary.

If necessary, the extract volume is adjusted to 10 cm^3 before centrifugation. The stoppered centrifuge tube is shaken vigorously to get a homogeneous distribution of the chlorophyll in the extraction solvent.

The sample is centrifuged for 10-20 minutes at about 10,000 $m\ s^{-2}$, in order to reduce the spectrophotometric blank reading (750 nm) which should not exceed 0.005 for a 1 cm cuvette.

12. STORAGE OF EXTRACT

The measurements shall be made immediately after centrifugation. If this is not possible the extract may be stored in a deep freezer ($-20^{o}C$) for no more than 24 hours. The volume must be adjusted before reading.

13. CHLOROPHYLL-A MEASUREMENT PROCEDURE

The measurements of chlorophyll-*a* may either be made by a spectrophotometer or a fluorometer. The measurements of phaeopigments are tentative and shall only be made using a fluorometer.

13.1 Spectrophotometric readings

The spectrophotometer should be calibrated in terms of absorbency and wave-length at least once a year.

- Bandwidth: 2 nm
- Wavelength: 750 and 663-665 nm (at the peak)
- Reference: 96% ethanol
- Cuvette: flexible, due to concentration (note the length)

The zero correction of the baseline and a cell to cell blank must be done in order to define the zeropoint of the absorbency in the extraction medium.

Calculations

To calculate the chlorophyll-*a* content using the spectrophotometric technique, the following equation should be used:

$$\text{Chl.a (mgm}^{-3}) = \frac{10^3 \, e \, A\,(665\ k)}{83\, V\, l}$$

e = volume of ethanol, cm^3

A(665 k) = absorbency at 665 nm (the peak) minus the absorbency at 750 nm after correction by the cell-to-cell blank
l = length of cuvette, cm
V = water volume filtered, dm^3
83 = absorption coefficient in 96% ethanol

The volume of chlorophyll sample, volume of ethanol and the length of cell (cuvette) must be chosen to give absorbency at 663-665 nm of 0.05-0.8, i.e. the optimum range of the spectrophotometer.

13.2 Fluorometric reading

It is necessary to make sure that the concentration of the extraction is at the optimum range of the instrument used. Synthetic detergents should be avoided when cleaning the cuvettes, as they may interfere with the fluorescence.

- Excitation setting: 425-430 nm
- Emission setting: 663-665 nm

Filter fluorometers must be equipped with a blue lamp corresponding to GE F4T5B, red-sensitive photomultiplier and primary filter corresponding to Corning 5-60 and secondary filter corresponding to Corning 2-64.

The fluorometer gives relative values which have to be converted to chlorophyll-*a* concentration using a calibration factor. The calibration factor is determined from the spectrophotometric reading or directly from fluorometric reading, in both cases using certified reference material.

For the measurement of phaeopigment the extract shall be acidified with 1 M HCl (0.06 cm^3 to 5 cm^3 of extract) after the first reading. 0.5-3 minute after acidification, a new reading should be made.

Calculations

Use the equation:

$\text{Chl.-}a\ (\text{mgm}^{-3}) = RfseV^{-1}$

R = fluorescence reading
f = calibration factor
s = slit correction

e = volume of ethanol (cm^3)
V = volume of filtered water (dm^3)

The calibration factor is determined as follows:

$f = KR^{-1} Ve^{-1}$

K = concentration of chlorophyll-*a* (mgm^{-3}) determined spectrophotometrically as described by Arvola, 1981.

When phaeopigment is to be calculated use the equation:

Phaeopigment ($mg.m^{-3}$) = $f_a ((r R_a) - R) s e V^{-1}$

R_a = fluorescence reading after acidification
r = ration R/R_a obtained from an extract free from phaeopigment
$f_a = f r (r^{-1})$
f, R, s, e, V = see above

14. ANALYTICAL QUALITY ASSURANCE

The general aspects of quality assurance for chlorophyll *a* are covered under Part B, B.5.3.1.

15. DATA REPORTING

For data reporting ICES environmental data reporting format should be used.

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ANNEX C-5 PHYTOPLANKTON PRIMARY PRODUCTION

Introduction

Primary production is the only regular rate measurement in the Baltic Monitoring Programme. From these measurements it is possible to calculate the amount of organic material formed from light, carbon-dioxide and nutrients. Primary production has important links to eutrophication and sedimentation and, consequently, to deep water oxygen concentrations.

Purpose

The measurement of primary production in water is carried out for, inter alia, the following purposes:

- to measure the ecophysiological response on different nutrient availability;
- to describe temporal trends in primary production.

Method

Primary production should be measured with the "P/E - method", in an incubator. With this method the uptake rate of carbon is measured at a range of irradiance levels in order to get a relationship between photosynthesis and light. Pmax (maximum photosynthetic rate) and (initial slope of the P-E relationship) and E_k (the light saturation irradiance) can be calculated using this method.

The advantage of the method is that ecophysiological information of the phytoplankton assemblage can be derived from the P/E - relationship. It is also possible to calculate the daily production per m^3 from these measurements after calculation and incorporation of the vertical attenuation coefficient and solar irradiance, and with some assumptions the annual primary production.

Sampling and analytical procedure

WORKING MANUAL AND SUPPORTING PAPERS ON THE USE OF A STANDARDIZED INCUBATOR-TECHNIQUE IN PRIMARY PRODUCTION MEASUREMENTS

(Version 22nd, February 1999, filename MANUAL4.doc)

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PREFACE

The ^{14}C method for the measurement of primary production in the sea has been used for more than 45 years. The database is considerable and it seems, as the method will continue to be an important tool in the monitoring of the status of the marine pelagic ecosystem. A major problem in the comparison of productivity data is, however, the use of different measuring methods. The differences stem from both conceptual and practical reasons. Within ICES long discussions have been held to create a database on primary production. However, the fear that the data were not comparable resulted in a workshop, where the methods applied by different laboratories were intercompared. Very significant differences in results were found between laboratories (Richardson, 1991). During its meeting in 1988 the ICES Working Group on Primary Production found that there was a need for a standardized method for primary production measurements to be used in monitoring studies of which the data were to be stored in the ICES data bank. It was decided to make a strict protocol for primary production measurements performed in an incubator. The intention was to make the incubator inexpensive and the method with as few steps as possible. Over the years that have passed since this decision, there have been long detailed discussions, but also fruitful tests of the incubator developed by Colijn et al (Annex 1, Annex 2). This manual with supporting papers is meant to serve as the protocol for future monitoring of primary production in the ICES area, and hopefully far beyond.

Although the initially intended simplicity has been left due to the wish to be able to measure full P-E relations, we still have given emphasis to obtain a concise and strict protocol, which does not leave much room for alternatives. Sometimes we have given alternatives where these do not affect standardization. However, in order to produce comparable data for a data bank we were obliged to keep the alternatives to a minimum and enable a rigorous quality assurance.

In summary, the purpose of this manual is to provide a strict protocol of the monitoring of Primary Production. Following this manual will ensure comparable data in the ICES database.

INTRODUCTION

The P-E curve method should be used (for terminology we refer to Sakshaug et al., 1997). With this method the ^{14}C uptake is measured at a range of irradiance levels in the incubator, in order to get an estimate of the photosynthesis rate versus irradiance. This can then be parameterized and give values of P_{max} (maximum photosynthesis), a (maximum light utilization coefficient measured as the slope of the linear increase of photosynthesis against irradiance), E_k (the saturating irradiance) and, after calculation and incorporation of vertical attenuation and solar irradiance, the daily primary production per m^2. Together with data on chlorophyll-a, P_{max} can be normalized to obtain assimilation numbers.

The method for estimating primary production by the "ICES Incubator" given in this manual (cf. Annex 1) is intended for monitoring purposes. Measurements should be possible from small, as well as from large vessels. Because of this, simplifications from what could be considered to be the ideal method have been introduced. It should be pointed out that the "ICES Incubator" method is not meant as a replacement of other "P-E techniques". It has been designed to provide a reliable measurement of primary production, using a simple incubator and a standard protocol.

The incubator is a rectangular perspex tank (33 x 33 x 9 cm) with a turning wheel on which a maximum of 12 experimental bottles can be clamped. 10 fluorescent tubes (TLD 8W J8, no 33) illuminate it. The full description of the incubator is given in Annex 1. The standard protocol is presented here. The incubator is manufactured by HYDROBIOS, Kiel, Germany (see List of Manufacturers).

Standardization of the method involves strong reduction of the number of alternatives. However, a few are indicated (see text in*italics*) but the standard method is to be used to obtain quality assured data for the ICES data bank.

SAMPLING STRATEGY

Mixed water columns

In areas where the euphotic zone is mixed and the phytoplankton community is uniformly distributed, **one** representative sample, obtained at 5 m depth is sufficient. It is important, however, to make sure that the water layer is mixed. This is easiest done with a CTD and fluorescens profile.

As an alternative an integrated sample can be taken with a hose (0-10 m) (Lindahl, 1986 and Annex 3). Mixed discrete samples from 0 to 10 m depth can also be used.

Stratified water columns

In stratified waters, where the phytoplankton community is not homogeneously distributed, a water sample should be obtained with a hose (see Appendix), covering the water column of interest. This single sample is treated as a mixed water sample.

If preferred, samples from different depths can be taken and incubated separately at temperatures similar to temperatures from the sampling depths. In that case more incubators may be needed, or subsequent incubations are to be made.

The hose sampling method can also be used as an alternative to sampling with water bottles, as the complete sample can easily be divided by depth for individual incubations by using clamps (Lindahl, 1986).

In conclusion, measurements of primary production in stratified water bodies are more complicated and will normally fall beyond "simple" monitoring strategies.

MEASURING PROTOCOL (SEE FIG. 1)

⚠ **Experts to add figures**

General Preparation

1. PLACEMENT OF THE INCUBATOR.

The incubator must be placed so that light conditions outside the incubator do not disturb the light climate inside the incubator. The incubator needs to be thermostatically controlled, to give the same temperature as the water sample. For samples from stratified waters differing in temperature two separate incubators should be used, or two consecutive incubations should be performed. The second water sample(s) should be kept dark and at the original temperature during the first incubation.

2. INCUBATION FLASKS.

Tissue culture flasks (see also 3.) of about 50 ml should be used. These flasks also work as paddles for the water-jet driven rotation of the flask-wheel. After each incubation, the flasks and the caps should be rinsed with diluted HCl (10%) and then several times with distilled water to avoid contamination. The flasks should be dried at 70 °C.

3. IRRADIANCE LEVELS IN THE INCUBATOR (FOR DETAILS SEE ANNEX 3)

A set of incubation flasks with different transmission levels, from 0 to 100% should be used (for manufacturer of special prepared bottles see Appendix). It is important that there should be enough measuring points to obtain a good measurement of P_{max} and a. With the special prepared 12 bottles this is not a problem and after some experimentation with the incubator normally a series of 6 bottles will suffice to measure a reliable P-E relationship. The exact irradiance in each bottle should be measured, despite the transmission the manufacturer gives percentages. This can be done with a small sensor, which can be introduced into the bottles (a manufacturer of this calibrated sensor can be found in the Appendix). To obtain irradiance saturated photosynthetic rates (P_{max}) a minimal irradiance of $500\ \mu E \cdot m^{-2} \cdot s^{-1}$ should be available. This is achieved by using 10 fluorescent lamps (TLD 8W J8, no 33). In case $500\ \mu E \cdot m^{-2} \cdot s^{-1}$ is not reached, a mirror behind the lamps and possibly on the other side of the tank will increase the irradiance flux.

4. ^{14}C SOLUTION.

Dilution of commercially available ^{14}C solution should be avoided due to the risk of contamination. The standard activity of every batch of ^{14}C solution should be controlled by the liquid scintillation technique (see point 11). It is recommended to use ampoules which contain the whole amount of ^{14}C needed for one incubation series. This reduces the number of measurements of the added ^{14}C activity.

In case ^{14}C solutions are prepared 'home-made' high grade chemicals and UHQ water must be used.

The final carbonate concentration of the solution should agree with the average carbonate concentration of the sea area being studied and the pH of the solution should be in the range of 9.5 - 10.0.

5. ACCOMPANYING FIELD MEASUREMENTS.

In order to obtain a representative sample of phytoplankton it is important to have knowledge of the vertical distribution of the algae. This is easiest accomplished by a CTD-cast combined with an *in situ* chlorophyll-fluorescence cast. Measurements of the under-water irradiance in at least 5 different depths, in order to calculate the vertical irradiance attenuation coefficient are also necessary. If the daily production is going to be calculated, the daily surface irradiance must also be measured in hourly intervals.

6. SAMPLING.

Non-transparent and non-toxic sampling devices must be used. Sampling should take place in daylight, to avoid strong interference of inequality due to diel rhythms of the phytoplankton (Annex 1, Gargas et al., 1979).

After sampling but before incubation subsamples are taken for chlorophyll (Fig.1, Step 1) and TCO_2 analysis (Fig. 1, Step 2).

The incubation should start as soon as possible, preferably within half an hour after sampling. All transfers of water samples should take place in subdued light, in order to avoid light-shock of the phytoplankton.

7. TOTAL CO_2 CONCENTRATION. (FIG. 1, STEP 2)

Total CO_2 concentration should be calculated according to other standard methods, using titration of carbonate (Strickland and Parsons, 1972). In brackish waters, such as the Baltic Sea, the CO_2 concentration can be calculated by the formulas of Buch (1945). In both cases temperature, salinity and pH must be measured.

8. ADDITION OF ^{14}C (FIG. 1, STEP 3).

The ^{14}C solution is added to the whole volume of sample needed to fill all the flasks. After thorough mixing, the flasks are filled. This procedure minimizes errors compared to pipetting the radioactive tracer to every individual incubation bottle.

Incubation bottles are filled with a measuring cylinder. The flasks should be filled up to the neck, leaving an air bubble in the flask. One dark flask from each original sample should be incubated.

The ^{14}C solution should be added to the sample in such concentrations that statistically sufficient counts of the radioactivity in the phytoplankton can be obtained. A triplicate measurement of the added activity is needed (Fig.1, Step 4). These samples should be counted immediately to avoid loss of activity. Therefore in case direct counting is impossible the inorganic ^{14}C should be mixed with ethanol-amine by pipetting 0.25 ml of sample with added activity together with 0.25 ml of ethanolamine. Scintillation cocktail can be added later and radioactivity determined.

As an alternative incubation flasks are first filled and then the ^{14}C solution is added to every flask. It is important that the added volume is small and that a precise, calibrated micropipette is used.

9. INCUBATION. (FIG.1, STEP 5)

The incubation time should be about 2 hours and the rotation speed should be approximately 10 rpm. Start and end of the incubation should be given in the protocol so that the precise incubation period (in minutes) can be used for the calculation. To achieve an unhampered rotation of the samples all positions on the wheel need to be filled (e.g. by using flasks filled with water).

10. END OF INCUBATION. (FIG. 1, STEP 6)

After incubation the flask contents are filtered immediately. In case of high algal biomass or high sedimentation load it might be needed to filter a subsample. A defined portion should be taken and filtered.

Glass-fibre filters (GF/F, Ø 25 mm) should be used, since these filters are cheap, become opaque and are known not to disturb the counting procedure of the radiotracer. To avoid any contamination of the filter edges, prewetted filters should be used. The suction pressure should not exceed 30 kPa during filtration. The filters should be rinsed once with a small volume (5 ml) of filtered seawater from the original sample (use filtrate of the chlorophyll-a measurement!).

After filtration the filters should be placed in scintillation vials and dried at room temperature for 24 hours. Following addition of scintillation liquid, the samples should be kept in dark for at least 3 hours to reduce chemiluminescence.

As an alternative to filtration many scientists use the bubbling method to obtain the total (dissolved and particulate) primary production.

From each incubated sample a sub-sample of 10 ml (exactly) is pipetted into a scintillation vial and 0.2 ml of 80 % HCl is immediately added. In a ventilated cupboard, the vials are then bubbled with a fine jet of air bubbles for 20 minutes, or are left open for 24 hours. 10 ml of scintillation cocktail is added and the vials are shaken by hand for some seconds before scintillation counting.

11. COUNTING OF THE RADIOACTIVITY. (FIG.1, STEP 8)

The liquid scintillation technique should be used when counting the uptake of ^{14}C. In order to get a statistically accurate measurement, 40 000 DPM, or counting for 10 minutes is needed to get a result with 1-% accuracy. Quench curves for different amounts of chlorophyll should be established and adding an internal standard, e.g. ^{14}C -hexadecane or toluol, should check the measuring efficiency of the liquid scintillation counter. Normal counting efficiency calculation is done by using the channel ratio method. Modern scintillation counters are equipped with programs to facilitate efficiency calculations. The user is referred to the instructions of the manufacturer.

12. CALCULATION OF CARBON UPTAKE (FIG. 1, STEP 9).

The total carbon uptake is calculated from the equation:

$$dP/dt\ (\mu g C \cdot L^{-1} \cdot hr^{-1}) = \frac{dpm\ (a) \cdot total^{12}CO_2\ (c) \cdot 12\ (d) \cdot 1.05\ (e) \cdot k1 \cdot k2}{dpm\ (b)}$$

Where

(a) = Sample activity (minus back-ground), dpm
(b) = Total activity added to the sample (minus back-ground), dpm
(c) = Total concentration of $^{12}CO_2$ in the sample water, µmol/L (or µM)
(d) = The atomic weight of carbon
(e) = A correction for the effect of ^{14}C discrimination
k1 = subsampling factor (e.g. sample 50 ml, subsample 10 ml: k1=subsample factor 50/10= 5)
k2 = time factor (e.g. incubation time 125 minutes: k2= 60/125= 0.48)

The results will be given as $\mu g\ C \cdot L^{-1} \cdot h^{-1}$ per irradiance level and as well as the photosynthesis at light saturation (P_{max}), the maximum light utilization coefficient (a), and light saturation parameter E_k , from the P-E curve (see below).

13. CALCULATION OF DAILY PRIMARY PRODUCTION

In order to calculate the daily primary production a number of parameters are needed. These include:

1. Vertical attenuation (extinction) coefficient, in m^{-1}, measured with a calibrated irradiance meter.

In case no attenuation can be measured, Secchi disc values can be used by conversion. The attenuation coefficient is calculated as

Att. Coef. = x / Secchi depth (m)

where x is 1.7 - 2.3 (1.7 (Raymont, 1967), 2.3 (Aertebjerg and Bresta, 1984), 1.84 (Edler, 1997)). This factor changes with sea area. In principle, it increases with decreasing salinity.

2. Insolation (Hourly measurements of incoming radiation between 400 and 700 nm (PAR), in $E \cdot m^{-2} \cdot s^{-1}$

3. P_{max}, E_k, and a.

The transformation of the hourly production corrected for dark uptake into daily production, which is the ultimate ecological goal, should follow the protocol outlined in Appendix 2.

A simple computer program for the calculation of the daily production is available (see list of manufacturer). After giving the raw data to the protocol, the software will calculate the daily production and combine the data in a database for the ICES data bank.

14. QUALITY ASSURANCE

General

In order to produce specified and confident data on primary production the performance of the measurement and the analytical procedures must follow a high quality system and operate in a state of statistical control. The method used shall be validated to meet the required specifications related to the use of the results. The validation includes selectivity, sensitivity, range, limit of detection and accuracy.

A high quality is maintained by using experienced and well trained personnel. Ring tests and intercomparisons ought to be conducted regularly.

Selectivity

The ^{14}C tracer method is used to measure the incorporation of the added isotope in the form of $NaH^{14}CO_3$ as an estimate of the photoautotrophic growth, measured as photosynthesis of phytoplankton. The method is highly selective but nonphotosynthetic incorporation of ^{14}C and non-biological fixation takes place simultaneously. This is measured as the dark uptake. It can not, however be used as respiration value as in the oxygen method. In the scintillation counting procedure interference may occur from the background values. They are, however, always subtracted from the uptake values.

Sensitivity

Apart from the high selectivity the method is also very sensitive. Uptake rates of 0.05 µg $C \cdot L^{-1} \cdot hr^{-1}$ can easily be measured. There is no actual upper and lower limit of the method. The sensitivity can be improved by adding more ^{14}C to the samples and/or by counting the incorporated radioactivity of the phytoplankton over a longer time which will improve the counting statistics.

Detection limit

The detection limit is set by the background radiation, the use of a zero time blank and dark incubations. The lower limit of detection is a sample should be defined as having activities at least three times the background values.

Range

As mentioned above a range virtually does not exist. Uptake rates between 0.05 and 250 µg $C \cdot L^{-1} \cdot hr^{-1}$ can easily be measured. At very high uptake rates an increase in pH may occur. This would affect the distribution and availability of the bicarbonate ion. Under such conditions the incubation period should be reduced. Under natural marine and brackish water conditions this does not happen.

Accuracy

Random as well as systematic errors occur in this method. Random errors should be kept to a minimum by adopting the standard procedure with only few experimental steps in the whole process from sampling to scintillation analysis. Systematic errors may occur with the light source, irradiance levels, filtration technique and the scintillation counting. These errors should be kept to a minimum. Regular control of handling and function of the instruments used, as well as calibration of the instruments are necessary tools to control the errors. The use of an independent analytical method to measure the systematic error is not possible, since there is no better independent analytical method available as an alternative for the 14C tracer method. Certified reference material (CRM) exists for the calibration of the scintillation counter, or for the calibration of the counting procedure with the original samples by using the internal standard method (adding standard to a sample). Participation in intercomparison exercises is one of the possibilities to test the comparability and therefore the precision and error propagation in this method. In general, however, most variability of this method will be caused by the biological nature of the material. Therefore strict procedures for the measuring protocol are needed to obtain the best possible results.

The quality assurance should ensure that the data are fit for the purpose for which they have been collected, i.e. that they satisfy the detection limits and levels of accuracy compatible with the objectives of the monitoring program.

15. DATA DELIVERY:

In order to have the possibility to check and recalculate daily productivity data it is important that all laboratories deliver their data in the same format and that this includes the fixation rates for all irradiances. Data should be delivered in the ICES, Biological Reporting Format.

ACKNOWLEDGEMENT

To be included

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List of Manufacturers

Incubator:

HYDROBIOS, c/o H. Fischer, Am Jägersberg 5-7, 24161 KIEL, Germany, Tel. +49-431-3696011, Fax: +49-431-3696021, E-mail: hydrobios@t-online.de

Incubation flasks and light sensor:

ZEMOKO, c/o, ing. Jan de Keyzer, Dorpsplein 40, 4371 AC Koudekerke, the Netherlands, Tel/Fax: +31-118-551182
Working Manual ICES Incubator
Irradiance Differentiation and Control in the ICES incubator.

Calculation program:

SMHI, Oceanographic Services, Nya Varvet 31, SE-426 71 V. Frölunda.
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APPENDIX: DESCRIPTION OF HOSE SAMPLING METHOD FOR PHYTOPLANKTON MEASUREMENTS

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In order to get a true integrated sample of phytoplankton the mixing of discrete water bottle samples is not adequate. To overcome this the hose method should be used (Lindahl, 1986).

An armoured PVC hose is suitable. The inner diameter of the hose should be c. 20 mm, giving a sampled volume of c. 3 L with a 10 meter hose. The length of the hose should cover the 10 upper meters of the sea to be sampled, as well as the distance from the sea surface to the boat deck, from where it is operated.

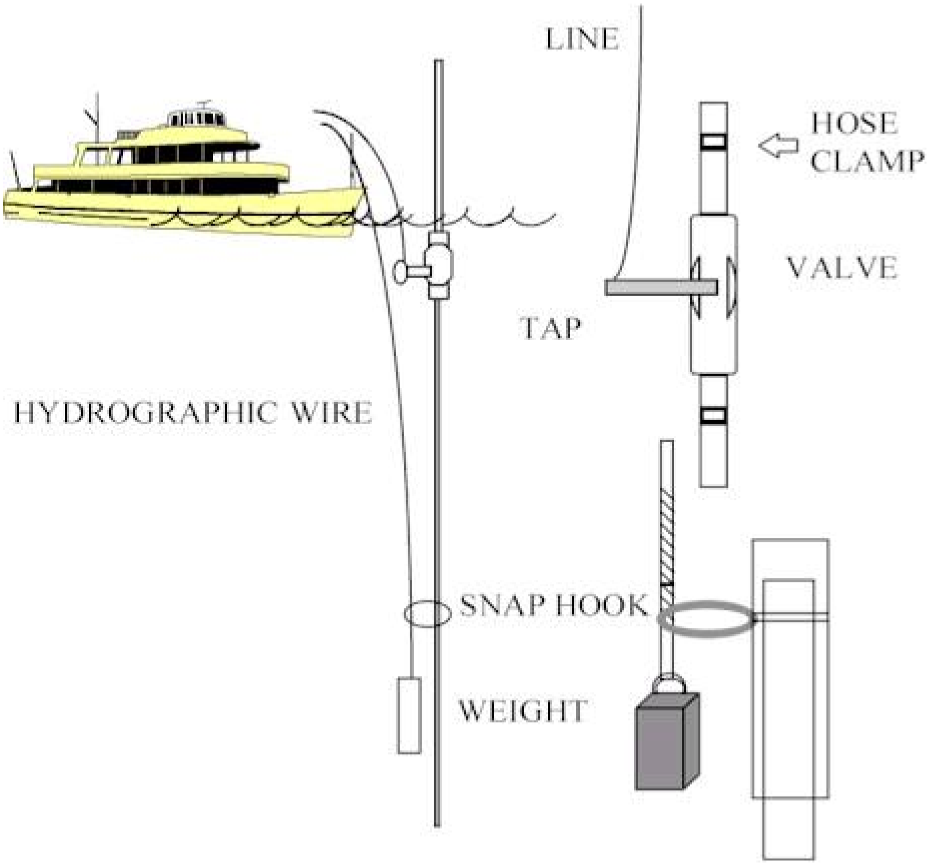
In the end of the hose a PVC tube should be placed and secured with a hose clamp. A snap hook should be fastened at the hose clamp. Ten meters up, corresponding to the surface of the sea, a valve should be placed and secured with hose clamps. At the end of the tap a line should be connected. It must be possible to operate the line from the boat deck.

When sampling, the lower end of the hose is connected to the hydrographic wire with the snap hook. The end of the hose should be below the wire weight in order to avoid contamination. The valve must be open when lowering the hose. The hose is lowered slowly until the valve has reached the sea surface. The valve is closed by pulling the string connected to the tap. The hydrographic wire is then elevated. The valve is then opened and the content of the hose is filled into a bucket and mixed. Subsampling bottles are filled from the bucket.

After sampling the hose is rinsed carefully with fresh water and stoppers are put in both ends in order not to pollute the inside of the hose. A thorough cleaning with diluted HCl or other detergents should be made at the end of a cruise and the hose should be dried.

Reference

Lindahl, O., 1986. A dividable hose for phytoplankton sampling. ICES; C.M. 1986/L:26, Annex 3.



Primary production protocol

Annex 1 Colijn, F., Kraay, G.W., Duin, R.N.M., Tillmann, U. and Veldhuis, M.J.W., 1996. Design and test of a novel Pmax incubator to be used for measuring the primary production in ICES monitoring studies. ICES CM 1996/L3. (Annex 1 is a modified version of the original paper)

Annex 2 L.P.M.J. Wetsteyn[1], L. Edler[2], M.M. Steendijk[1], G.W. Kraay[3], F. Colijn[4] & R.N.M. Duin[5]. Light measurements and intercalibration of standard ICES incubators
(second draft).

Annex 3 de Keyzer, J.. 1994. Irradiance Differentiation and Control in the ICES incubator. Zemoko, Maritiem (unpubl. report)

(revised version 4.December 1997)

ANNEX 1 DESIGN AND TESTS OF A NOVEL P-I INCUBATOR TO BE USED FOR MEASURING THE PHYTOPLANKTON PRIMARY PRODUCTION IN ICES MONITORING STUDIES

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Acknowledgements:

This paper has been developed during a period of several annual meetings of the ICES Working Group on Phytoplankton Ecology and its predecessors. Field and laboratory studies were made in successive years. We would like to acknowledge many colleagues who gave the possibility to test the method on board their ships or in their labs, like the meeting in Helsinki, Finland in 1989; the additional measurements made in the Indian Ocean in 1992-1993; and in the German Wadden Sea in 1995.

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2. Description of the incubator
3. Results of test runs on five locations
4. Discussion, and recommendations
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Abstract

An inexpensive and simple incubator for primary production measurements is presented along with a protocol (see Working Manual) to achieve strictly comparable and reliable ^{14}C-fixation rates of phytoplankton. The incubator, based on Steemann-Nielsen and Aabye Jensen (1957), is comprised of incubation bottles revolving in a temperature controlled water bath at a fixed irradiance. The recommended protocol and incubator have been tested in different water types, such as Dutch , German and Finnish coastal waters, in the North Sea and in the Indian Ocean, and give reliable estimates of the photosynthetic rate at the fixed irradiance used. Coefficients of variation were between 0.6 and 7.6 in incubation experiments with three and five samples. No difference between P_{max} measured in the Baltic incubator and the ICES incubator was observed.

The incubator has been used as a P-I incubator during cruises in the Indian Ocean by providing a series of bottles with different transmittance levels. These experiments show that actual P-I relations can be measured with a good fit of the P-I curve parameters, like initial slope ±, I_k, I_{opt} and P_{max} values.

A series of measurements were performed for a period of one year at a monitoring station in the German Wadden Sea. These measurements showed the typical characteristics of P-I incubations with almost stable alpha values and temperature controlled P_{max}levels. Correlations between chlorophyll and primary production were high.

Daily primary production values have been calculated based on the P-I relations after integration over time and depth on selected series of data and compared with a simple empirical equation based on P_{max}, attenuation coefficient, daylength and daily insolation. The agreement between both methods was rather poor, and variable. Dependent on the calculation mode all values were roughly 1.5 to 2 times too high as compared to the integrated values based on one of the fitted P-I curve parameters. Further work needs to be done to improve this empirical formulation. The three equations used to calculate the daily primary production were comparable. Calculations not based on a sinoidal light function but on a rectangular mean irradiance level were 5-20 % higher.

Based on the measurements in the German Wadden Sea daily primary production has been calculated according to a strict format. This format will be proposed to standardize calculations.

Application of a known irradiance in the incubation bottles is still one of the most difficult parameters in this method, whereas the application of the tracer method itself is easy and straightforward.

1. INTRODUCTION

Results of the Hirtshals intercalibration (c.f. Richardson, 1991) were discussed during the workshop of the ICES Working Group on Primary Production in Copenhagen (June 1988). The meeting adopted the following recommendation: "... that there is a need for a standardized primary production method to be used in monitoring studies with special coded data in the ICES data bank". The authors have accepted to comply with the request by building a simple and inexpensive incubator and proposing an appropriate protocol.

At present several procedures are available to measure daily depth-integrated primary production ($mgC.m^{-2}.d^{-1}$). Most of these methods are based on measurement of P (photosynthesis) vs. I (irradiance) relationships, of vertical attenuation coefficients, and solar irradiance (Aertebjerg Nielsen & Bresta, 1984; Gargas & Hare, 1976; Richardson, 1987).

The results of the Hirtshals intercalibration workshop (Anonymous, 1989; Richardson, 1991, 1993) have shown that calculation of integral daily primary production may contain a whole series of errors or assumptions which cause large differences in the final result. Substantial errors arise from handling of samples, incubation time, incubation handling, liquid scintillation counting, and calculation methods, but the main difference was due to the different types of incubators used (measurement of irradiance, differences in light quality etc.). Therefore, data offered to the ICES data bank are not comparable and therefore were never stored. This paper describes the use of an incubator and develops a strict protocol with as few steps as possible, and contains recommendations about the use of materials, to reach highest comparability of results.

Originally, our task, however, has been limited to this specific point and therefore no attempt has been made to propose a method to calculate integral daily production from single P_{max} ($mgC.m^{-3}.h^{-1}$) measurements, assuming that the incubator has the possibility to measure P_{max} at light saturation within a large range of irradiances. Several other assumptions have to be made to calculate daily primary production, including a vertically homogeneous distribution of algal biomass, similar photosynthetic characteristics of the phytoplankton and the same species composition throughout the water column. Also data on vertical attenuation and daily irradiance should be available. As shown by Riegman and Colijn (1991) calculations based on surface samples alone can underestimate areal primary production by 17%. As pointed out by Platt and Sathyendranath (1988) oceanic primary production might be well estimated from an irradiance model based on measurements of P_{max} and ±, and a remotely sensed biomass field. Such estimates might be possible for the North Sea within the near future if both P_{max} and ± are known.

Stimulated by the discussions and comments in the ICES WG we finally have attempted to use the ICES-incubator as a P-I incubator and to compare daily primary production values measured in the ICES-incubator with fully integrated values over time and depth, using P-I relations.

2. DESCRIPTION OF THE INCUBATOR.

The incubator strongly resembles the one originally used by Steemann Nielsen & Aabye Jensen (1957), (cf. Postma & Rommets, 1970; Cadée & Hegeman, 1974). It is constructed of a rectangular perspex tank (h x b x w= 33 x 33 x 9 cm) with a turning wheel (max. 12 rpm, 18 cm in diameter) on which experimental bottles (max. 12) are clamped. Illumination is provided by 10 Philips 8 W fluorescent tubes (TLD 8W J8, no.33) which can be switched off/on separately (Figure 1). Irradiance should in all cases be measured with an appropriate light sensor (e.g. LICOR, $\mu E. m^{-2}.s^{-1}$ or $W.m^{-2}$) or the special sensor developed by de Keijzer (1994) (Annex 3). Our experimental set up gave a mean irradiance of 360 $\mu E.m^{-2}.s^{-1}$, providing a saturating ^{14}C fixation rate (see results section). However, the light field is not homogeneous but ranged from 140 to 530 $\mu E.m^{-2}.s^{-1}$ depending on position of the flasks during revolution. The homogeneity of the light field can be easily improved by using a backscattering white polystyrene foam layer opposite to the fluorescent tubes. These irradiance measurements were done with a 2À-sensor and therefore are substantially lower than the earlier measurements in the incubator during the Indian Ocean cruise with a spherical sensor: with 10, 8, 6, 4 and 2 tubes and this polysterene layer we measured 1100, 850, 650, 300 and 250 $\mu E.m^{-2}.s^{-1}$, respectively as maximum irradiances. A full description of the irradiance distribution is given in (Annex 2) where several options for illumination were tested.

During one of the meetings it was discussed whether this incubator could be used to measure P-I relations. Indeed, this can be done by covering the incubation bottles with neutral density filters (e.g. Flash Light Lee), available in several transmission classes. An alternative is painting the bottles in different black intensities. Such tests have been performed during cruises in the Indian Ocean in 1993. However, this procedure did not fall into our primary goal as stated above in the recommendations of the 1988 meeting. Thus the incubator now no longer acts as a simple incubator again introducing several of the "old" uncertainties and errors, especially as far as irradiance levels in the bottles is concerned. During a later stage the problem how to obtain different irradiance levels in the incubation bottles has been solved by using a epoxy-resin layer of different attenuation (Annex 2).

Incubations are carried out in disposable tissue (ultraclean) culture flasks (e.g. Greiner, tissue culture flasks, 690160) containing 50 ml of sample. These flasks can be used several times without deterioriation of the vessel walls and are suited to adhere the epoxy-resin layers.

Temperature is controlled to within ± 0.1 °C by a suitable thermostat with enough capacity (Lauda, Colora). Water is recycled within the bath by an extra pump which also causes the revolution of the wheel, with the flasks acting as paddles. If only a few samples are incubated the open positions should be filled with flasks containing water to attain a constant turning of the wheel. A running seawater system on board the ship could be used instead of the thermostated water bath. The complete system is built by Hydrobios (Kiel, Germany), whereas the calibrated incubation bottles are sold by ZEMOKO (the Netherlands) (full adresses of both companies are given at the end). The cost per unit can be reduced if several incubators are built/ordered simultaneously.

3. RESULTS OF TEST RUNS ON FIVE LOCATIONS.

Several tests by independent workers have been conducted with the apparatus in its former and improved form.

3.1. Test at the Netherlands Institute of Sea Research (NIOZ).

During the typical spring bloom of phytoplankton in Dutch coastal waters (plankton dominated by the diatoms Biddulphiaaurita, B. sinensis, Coscinodiscus concinnus, Skeletonemacostatum and colonies of Phaeocystis sp.), an incubation experiment was performed, according to the protocol (see Appendix). Incubation periods of 1 and 2 hours were tested, along with two filter types: Whatman GF/F (approximate pore size 0.7 µm, 47 mm) and Sartorius cellulose acetate 11106 (pore size 0.45 µm, 47 mm).

After filling the experimental bottles, 0.1 mL $NaH^{14}CO_3$ (Amersham) from a stock solution prepared with superclean distilled water containing one pellet of Ultrapure NaOH (pH =9), was added. Ampoules have been cleaned with 6N HCl. Total activity added, to be determined for each experiment, was $11.46 .10^6$ dpm/ 0.1 ml. Precautions should be taken to use a pure ^{14}C-bicarbonate solution, especially when release of extracellular dissolved organic carbon has to be measured (Bresta et al., 1987).

After incubation, samples were filtered within a few minutes through the two filter types. After fuming over concentrated HCl for 5 min in a desiccator, samples were counted in 10 ml Instagel in 20 ml glass scintillation vials. Cells on the filters were disrupted in a Bransom Ultrasonic device during 15 min. Without this disruption, counts can be up to 50% lower. Cpm's were converted into dpm's with a quench curve and the external standard channels ratio method. Results of the first experiment are compiled in Table I.

The results show a good reproducibility of the ^{14}C fixation rates, an almost linear uptake over the 2h period, and a lower recovery and a higher variability of ^{14}C on Sartorius cellulose acetate filters compared with GF/F filters (cf. Hilner & Bate, 1989). Dark values were about 2% of the light values.

3.2. Test at the Finnish Institute of Marine Research (Helsinki)

During an ICES workshop, the new incubator was tested on board the research vessel Aranda by making a direct comparison between the ICES incubator and the Baltic Sea incubator on July 6, 1989. A surface water sample containing cyanobacteria and several other species without dominance of a particular one was taken from the Baltic and divided into 14 bottles. To each bottle 0.1 ml of 2 mCi

$NaH^{14}CO_3$ was added. Samples were incubated 2 h 25 min and filtered onto GF/F filters, and fumed over concentrated HCl for 10 min. Filters were disrupted by sonification and counted as above. Five samples were incubated in the ICES incubator, 5 in the Baltic Sea incubator at full light (400 $\mu E.m^{-2}.s^{-1}$), and another four samples were incubated at 50%, 25%, 10% and 5% of full light, respectively. Reduction of irradiance was obtained with neutral density filters.

Results are given in Table II. The full light samples in both incubators showed the highest fixation rates. The reproducibility was very high in both incubators. The single point measurements at the attenuated irradiances showed a good linearity, indicating that in this case four measurements suffice to estimate the photosynthetic efficiency a. Despite the difference in maximum irradiance in the two incubators, the same maximum fixation rate was measured, suggesting that photosynthesis was saturated at an irradiance of about 300 $\mu E.m^{-2}.s^{-1}$.

3.3. Tests in the North Sea by the National Institute of Coastal and Marine Management (RIKZ), formerly Tidal Waters Division at Middelburg (NL)

A similar but completely independent set of experiments was conducted during one of our regular sampling surveys of the North Sea within the EUZOUT (Eutrophication of the North Sea) project. Samples were taken at different stations in the North Sea (Fig. 2), covering both coastal and offshore waters, up to 370 km from the Dutch coast during a cruise from 25 to 27 July, 1989. Surface, thermocline and subthermocline samples were also incubated at the stratified stations. To 50 ml samples 10 µCi in 0.1 ml was added. In this case the results are also compared with P_{max} values calculated from P-I measurements on the same samples incubated simultaneously but in another incubator (Peeters et al., 1991; Klein & van Buuren, 1992). Two comparisons of short (2 h) versus long (6 h) incubation times were made. All samples were filtered onto Whatman GF/F filters; after addition of 10 ml HCl, samples were bubbled with air for 20 min and counted as described in Peeters et al. (1991).

The results are given in Table III. Depending on the station a wide range of photosynthetic activities was observed. Coastal eutrophied stations showed rates up to 40 times higher than in the oligotrophic central part of the North Sea. Vertical profiles showed high rates in the thermocline or subthermocline layers. The long-term incubations showed an almost linear uptake over the 6 h period. Duplicate incubations generally showed a maximum difference of 10%.

Comparison of the P_{max} in the ICES incubator with the P_{max} in the P-I incubator shows that the ICES P_{max} is somewhat higher than the latter P_{max}. This confirms our findings in Helsinki which also showed that the ICES incubator measures a value close to P_{max}. However, samples in the P-I incubator were run for about 6 h instead of 2 h in the ICES incubator.

3.4. Tests during Indian Ocean cruises (JGOFS) in 1992-1993 by NIOZ (Texel) east of African coast off Somalia and Kenya

During these cruises of which the results will be presented elsewhere a series of experiments were performed with bottles painted black with different degrees of transmittance resulting in a range of c. 4% to 100%. Irradiance in all individual bottles however had to be measured. Thus the incubator has now been used as a real P-I incubator. To increase the irradiance levels the backside of the incubator was covered with white polystyrene foam which gave a range of 40 to 1100 $\mu E.m^{-2}.s^{-1}$ in the bottles. A large sample of about 10 l has been taken from the surface during an evening cast at 18 h. LT. From this sample a P-I relation has been measured for 2h, including chlorophyll-a concentrations. Part (about 7 l) of the sample has been stored overnight in a dark cool box and incubated in a similar way the next morning at 6.00 h LT. The results of three of such series are given in Fig. 3 and Table IV. The P-I curves were analysed according to equations given by Jassby & Platt (1976), Platt et al. (1980) and Eilers & Peeters (1988). The first two equations showed comparable results whereas the third one showed higher P_{max} values for both incubations. The former P_{max} values were within 5% difference. Calculation of daily production also showed good agreement for the former two equations. However the P_{max} and daily production values showed large differences between the two incubations (evening vs. morning) , mainly due to the higher P_{max} values of the morning incubation due to a circadian rhythm (chlorophyll had slightly increased during the storage period) whereas also the initial slope a increased by 25%. More data of the Indian Ocean cruises are available but will be published elsewhere (Veldhuis and Kraay, in prep.).

3.5. Tests at the Station Büsum, along the German Wadden Sea in 1995

Within the framework of our monitoring studies in the German Wadden Sea, weekly incubations were made using the standard incubators, kindly provided by Mr. Bert Wetsteijn of the RIKZ in Middelburg. Contrary to the standard procedure, we used a direct cooling of the incubator in the lab by a Lauda cooler instead of the closed circuit with the copper tubing. This was done to be able to obtain very low incubation temperatures during winter time and does not have any further consequences for the measurements. The samples were illuminated from both sides to obtain sufficiently high irradiances up to 800 $\mu E.m^{-2}.s^{-1}$ for proper P_{max} determination. Throughout these measurements we used the new incubation bottles and the improved irradiance setup as described in Wetsteijn et al. (1996). As a standard incubation time 2 hours were used, but in winter during low activities up to 4 hours were used. TL tubes were arranged to perform a homogeneous light field. Irradiance was measured inside the incubation bottles with the same equipment as developed by Wetsteijn et al.(1996). Mean irradiance values were based on twelve measuring points during one revolution of the wheel. The special incubation bottles prepared by ZEMOKO (see Wetsteijn et al., 1996) were used throughout the measurements. For one P-I measurement 8 bottles including one dark were used. Dark values were low but always subtracted from the light values. Added activity ranged from 0.5 to 3 µC in winter (volume 50 to 300 µl). Samples were filtered over 0.45 µm membrane filters (not GF/F) under reduced suction pressure (200 mm Hg), washed with 10 ml 'cold' filtered seawater and dried. Counting took place in Filter-count (Packard). Added activity was counted after dilution in 55 ml of sample and pipetting 50 µl of the mixture in counting vials. Calibration occurred according to the external standard ratio procedure of the liquid scintillation counter.

Primary production values were normalised to chlorophyll-a measured spectrophotometrically according to Lorenzen (1967).

The results are presented in figures 4 to 6. In Fig. 4 four representative examples of P/I curves are shown from different seasons. Curve fitting and calculation of P/I parameters was made according to the equation of Platt and Gallegos (198). The seasonal variations in P-I parameters is shown in Fig. 5. Chlorophyll specific maximum photosynthetic rates (P^{b}_{max}) ranged from 2.0 to 9.9 µG C./ µg Chlor/ h^{-1} and showed a large variation over the year and was highly significant correlated with water temperature (Fig. 6). In contrast, the slope of the P/I curves ranged from 0.0150 to 0.0375 µg C/ µg Chlor. h^{-1}/ $\mu E.\ m^{-2}.s^{-1}$ (Fig. 5) and proved to be less variable and irrespective of water temperature. During the whole year no strong light inhibition at high irradiances could be observed. I_k values, used as a parameter of light adaptation, were relatively high throughout the year varying between 81 and 453 $\mu E.\ m^{-2}.s^{-1}$(Fig. 5). Thus in spite of the low light conditions in the Wadden Sea due to high turbidity, no signs of low light adaptation of the phytoplankton could be detected. Further we conclude that based on the measured high P^b_{max} values and the natural mean low light levels in the Wadden Sea , the phytoplankton of the turbid inner parts is light limited and not nutrient limited throughout the year.

The results of these P-I measurements will be used, in combination with irradiance and attenuation measurements to calculate the daily and annual primary production at station Büsum (Tillmann et al., in prep.).

4. DISCUSSION, RECOMMENDATIONS AND PROBLEMS.

To a great extent the task accepted during the 1988 ICES meeting in Copenhagen has been fulfilled: a simple and inexpensive incubator has been built and tested. The tests so far show that the incubator works well, that it is simple to use, and that it also has the potential to measure P-I curves. However, it is not recommended as a P-I incubator, due to the fact that these already exist in a wide variety with more sophisticated irradiance regulation. Reproducibility and linearity of uptake rates are within the expected limits. Problems arising from different photosynthetic characteristics like a daily disparity or circadian rhythm in the same sample can not be solved. Because such differences can be quite large there is no simple solution except to incubate samples several times during the day. To reduce this kind of variability a practical and pragmatic solution could be to incubate all samples around noon.

The series measured at Station Büsum during 1995 show the consistent results which can be obtained with the incubator. Apart from minor changes such as the cooling device at low temperatures, we followed the protocol as described for the continuously mixed water mass. The series will be used to calculate the annual primary production, whereas we intend to continue the measurements to get a series for several years to see whether nutrient reductions influence the primary production in this part of the Wadden Sea. At the moment light limitation is the most important regulating factor.

Apart from the results obtained so far, there is a need for concurrent work with two types of incubators: an ICES type of incubator for monitoring studies and a more sophisticated type where P-I relations can be measured for physiological studies. Comparisons between this simple and maybe more complex types of incubator should be made by the individual scientists as part of an intercalibration study. Nevertheless, the limited amount of methodological steps is of great advantage and reduces several of the common errors. If the Working Manual is followed, data obtained in this way are directly comparable.

Discussions both in the ICES working group and with several colleagues have shown that there is a need for a further standardization step leading to the calculation of values per m^2 from these P_{max} measurements. As a first approach, empirical formulations like the one used by Cadée & Hegeman (1974) and DiToro et al.(1971) are useful. In Helsinki we decided that such a formulation should be derived, which then could be used to calculate a value per m^2. A first attempt has been made to use such an empirical equation by comparing daily primary production calculated by integration and based on P-I parameters with this empirical estimate of daily primary production. The results (not given) showed that daily primary production calculated according to the equations given by Eilers and Peeters, Jassby and Platt, and Platt and Gallegos and in all cases with a sinusoidal irradiance give almost equal results. If daily primary production is calculated with a rectangular light distribution using one mean irradiance level the daily values are about 5 to 20% higher. If we use the empirical equation of Ditoro et al. (1971) we obtain values up to 1.5 to 2 times as high. Probably the calculation is not yet very realistic and we have further evaluated this procedure and finally present a calculation mode with the Working Manual. The calculation is also available on disk through SMHI in Sweden.

One should, however, realize that in all cases this value is only an estimate, due to physiological characteristics of phytoplankton (Neale & Marra, 1985; Savage, 1988; Vandevelde et al., 1989), and to an uneven vertical distribution of phytoplankton in the sea (Riegman & Colijn, 1991). Calculation of primary production under such circumstances can only be achieved if samples from different depths are incubated and their light-, temperature- and time-dependant fixation rates are known.

Based on a larger data set comprising P_{max} data and simultaneous P-I measurements, we have calculated the daily primary production in Büsum as an example. The same calculation has been suggested to ICES for the calculation of primary production per m^2 in different areas. A further step in modelling primary production could be the incorporation of time-dependent adaptation responses as described by Neale and Marra (1985). However, this was not the primary goal of the working group and therefore falls beyond the scope of this paper.

A recent paper of McBride (1992) also compiles several equations to calculate daily photosynthesis, one of which may be adopted by ICES as an alternative to the standard. The present method to calculate daily primary production is based on an numerical integration over time and depth which is very rapid and simple with modern PC's.

A problem which is not solved sofar is the irradiance needed to measure P_{max}. In our opinion a procedure should be developed to relate the saturating irradiance for P_{max} to the geographical latitude and the time of the year. Then a standardized incubation irradiance could be prescribed. A moment there is uncertainty because we have not tested it.

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Table I. Fixation rates of sample from the Marsdiep tidal inlet (Wadden Sea, cf. Cadée & Hegeman, 1974).

Filter type	Incubation time (h)	DPM	x ± sd cv
GF/F	1	45163	43724 ± 1757 4.0
GF/F	1	44243	
GF/F	1	41765	
GF/F	2	78384	79183 ± 2469 3.1
GF/F	2	81953	
GF/F	2	77212	
Sartorius	2	71228	67348 ± 5154 7.6
Sartorius	2	69316	
Sartorius	2	61500	

GF/F	2 (in dark)	1142	1424
Sartorius	2 (in dark)	1706	1424

Table II. Samples from the inlet to the Helsinki harbour.

ICES Incubator	Baltic Incubator
CPM/h x ± sd cv	**CPM/h x ± sd cv**
2486	2565
2530	2515
2518 2514 ± 17 0.6	2602 2553 ± 78 3.1
2523	2441
2514	2541
	CPM/h irradiance
	257 5%
	425 10%
	991 25%

	1967 50%

Mean irradiance in ICES incubator: 297 mE.m^{-2}.s^{-1};
Full irradiance in Baltic incubator: 400 mE.m^{-2}.s^{-1}.

Table III. Results from the North Sea cruise (25-27 July 1989); for location of stations see Fig.2. s=short term(c. 2 h), l=long term(c. 6 h) incubation; sur=surface, ther=thermocline, subther=subthermocline sample; P_{max} derived from P-I measurements based on 6 h incubations

DPM/2 h				DPM/2 h			
Station	**ICES**		**P_{max}**	**Station**	**ICES**		**P_{max}**
NW100 sur	3385	4656	2242	TS100 sur	4590	5282	3982
NW70 sur	6951	6777	5532	TS100 ther	7541	7293	6213
TS370 sur/s	3293	2970	2699	TS100 subther	1478	1415	1085
TS370 sur/l	2755	---	---	TS10 sur	6583	6646	3454
TS275 sur/s	1741	1624	---	TS4 sur	59062	56027	53403
TS275 sur/l	1897	1870	1503	NW20 sur	17984	20844	15411
TS175 sur	2336	1906	1328				

TS175 ther	2565	2740	1452*				
TS175 subther	7712	8141	3527*				

* samples showed strong photoinhibition

Table IV. Example of results of experiments conducted in the Indian Ocean, location off Kenya and Somalia (Veldhuis & Kraay, in prep.) to show daily inequality.

Same sample was used for both incubations; parameters estimated by the equation of Platt et al. (1980). Calculation of daily primary production is based on ke = 0.1, daylength = 12 hrs., and mean surface irradiance = 1000 mE.m-2.s-1. SSE is the error sum of squares of the fitted model.

	Evening Incubation	Morning Incubation	Unit
P_{max}	3.55	5.93	mgC.m^{-3}.hr^{-1}
I_{opt}	802	1319	mE.m^{-2}.s^{-1}
I_k	294	290	mE.m^{-2}.s^{-1}
a	0.012	0.021	mgC.mgChl-a^{-1}.hr^{-1}
SSE	1.055	1.633	
Daily Production	260	465	mgC.m^{-2}

Legends to figures

Figure 1. Photograph of ICES incubator (see text).

Figure 2. Map showing location of sampling stations during the July cruise in the North Sea (Peeters et al., 1991).

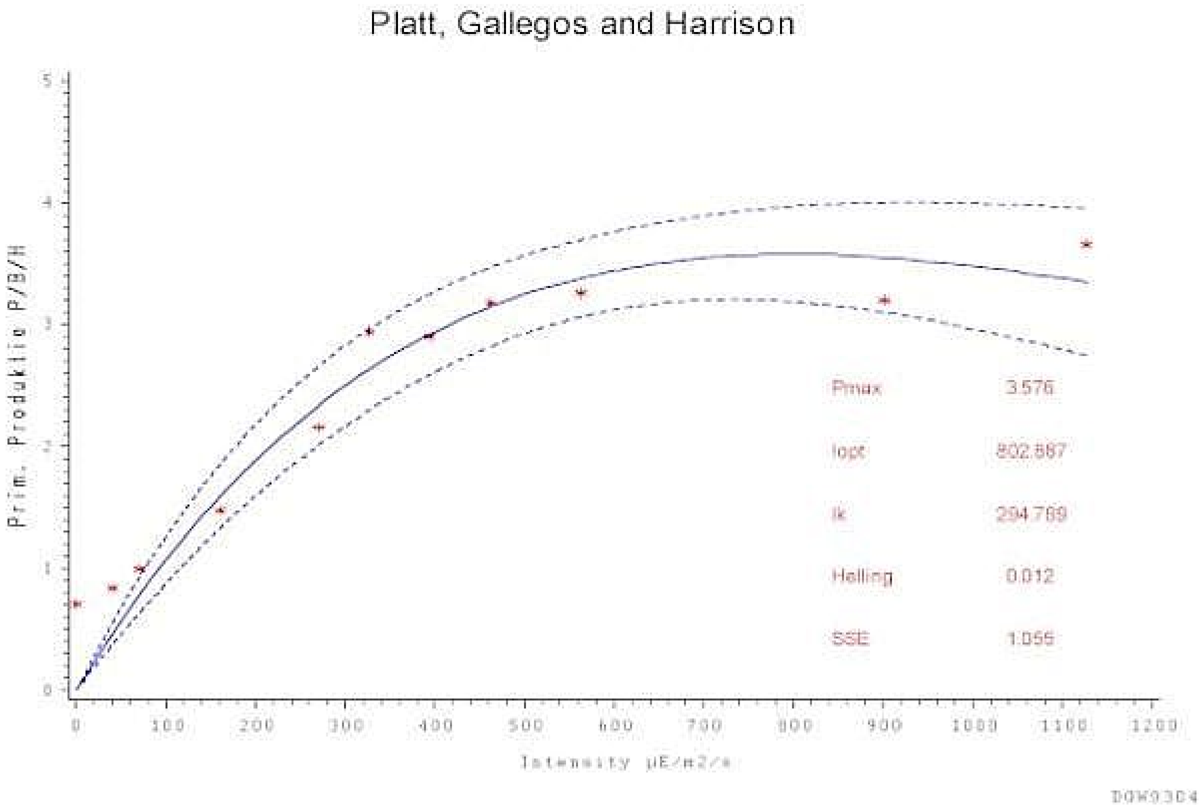
Figure 3. P-I curves for two incubations on the same sample; a) in the evening, b) in the morning. Fitted curve is equation of Platt et al. (1980).

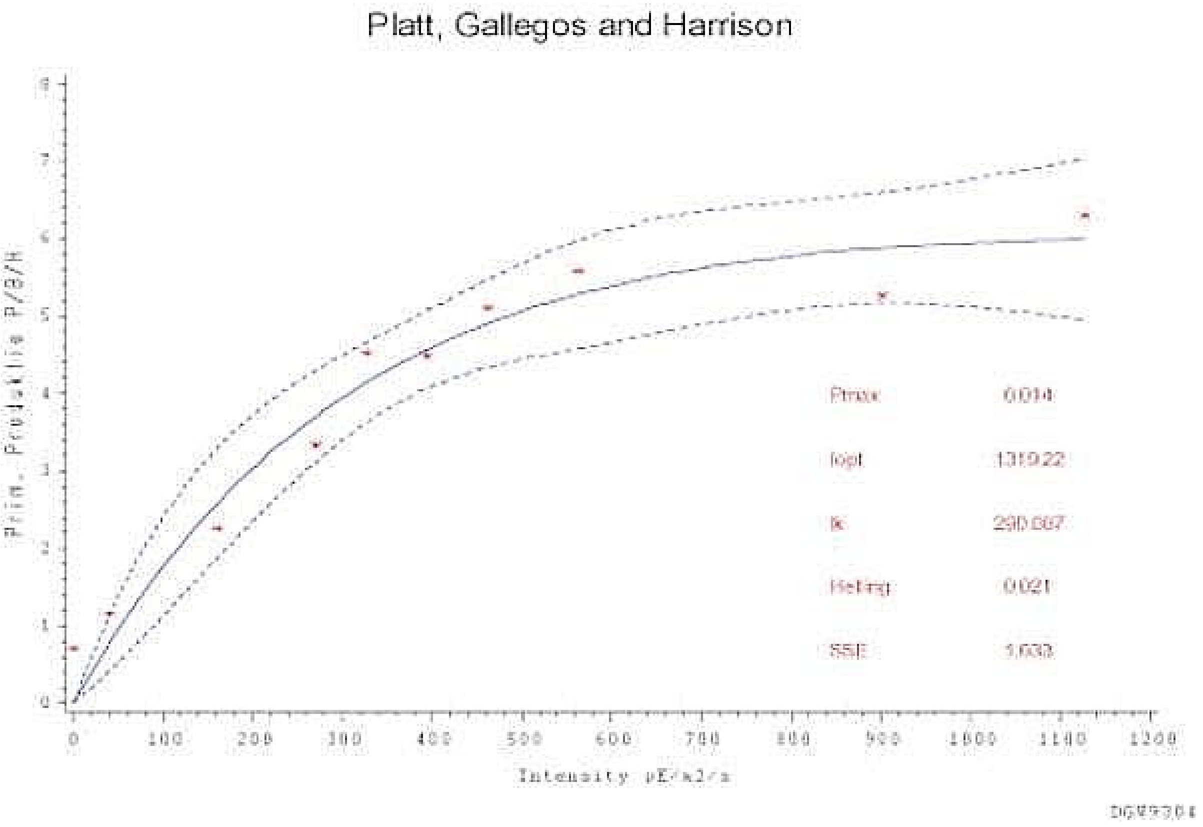
Figure 4. Examples of P-I curves measured at Station büsum; all curves were normalised to chlorophyll-a; fits were made with the equation of Platt (1980)

Figure 5. Seasonal course of P-I parameters at Station Büsum in 1995; all parameter calculations based on Platt et al. (1980)

Figure 6. Relation between assimilation number (P^b_{max}) with temperature for the measurements conducted in Büsum in 1995

Figure 7. Calculation of daily and annual primary production based on daily insolation , vertical attenuation and P-I curves from the ICES incubator





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ANNEX 2 LIGHT MEASUREMENTS AND INTERCALIBRATION OF STANDARD ICES INCUBATORS (SECOND DRAFT)

Contents

Introduction
Material and methods
Results and discussion
References
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(Results from earlier performed light measurements in standard ICES incubators and from a workshop held on 9-11 March 1994 in Middelburg, presented at the meeting of the ICES WG on Phytoplankton Ecology in Copenhagen, 23-26 March 1994; additional revisions made after the meetings in Copenhagen, 23-26 March 1994 and in The Hague, 29-31 March 1995)

INTRODUCTION

Since 1987 some of us have worked in a changing configuration on the construction and experimental performance including a standard protocol of a newly designed 'simple' and inexpensive incubator for primary production measurements. The original term of reference was to develop a simple and inexpensive incubator for use in monitoring studies.

During one of the meetings of the former ICES WG on Phytoplankton and the Management of their Effects, the original set-up was criticized because no P-I relations were measured. Therefore the design was adapted enabling the measurement of P-I relations at a range of 12 (including dark) irradiance levels. The incubator has been used as a P-I incubator during Indian Ocean cruises in 1992-1993 by NIOZ-workers (some results were presented in Colijn et al., 1993).

In the last report of the WG on Phytoplankton and the Management of their Effects (C.M.1993/ENV:7 Ref.:L) it was stated that the Dutch workers would be asked to explore the possibility of convening an evaluation workshop in The Netherlands. One of the objectives of this workshop would be to evaluate the reproducibility of measurements using the standard incubator and protocol in the hands of different users. At the end of 1993 funding for the manufacturing of four incubators, four filter/flask series (each with an irradiance gradient), some irradiance sensors and the execution of light measurements by an optical expert became possible, giving the opportunity to perform a reproducibility experiment before the next meeting.

In this report we will present 1) information on the used epoxy resin coating, 2) information on the used irradiance sensor, 3) some results from earlier performed extensive light measurements in the standard incubators and 4) the results from an intercalibration experiment with four incubators to check the comparability of identical incubators and the variability due to manipulation of the samples by different users. Information with respect to 1), 2) and 3) was taken from ZEMOKO (1994).

MATERIALS AND METHODS

Incubators and incubation bottles

A short description of the incubator has been taken from Colijn et al. (1993). The incubator is constructed as a rectangular perspex tank (h*b*w=33*33*9 cm) with a turning wheel (max. 10 rpm, 18 cm in diameter) on which 12 experimental bottles (Greiner, tissue culture flasks, ca. 55 ml, 690160) are clamped. Water is recycled within the incubator by an aquarium pump causing the revolution of the turning wheel, with the bottles acting as paddles. On board ship the incubator should be closed accurately with a perspex cover to avoid overflowing and short-circuiting.
Illumination is provided by 10 Philips 8 W fluorescent tubes (TLD 8W J8, no. 33) which can be

switched off/on separately.
Water temperature can be controlled using an external cooling device or with a running seawater system. Because we wanted to cool 4 incubators simultaneously a copper tube outside the light field along the narrow vertical walls and the bottom of each incubator was used; the copper tubes were parallel connected to the thermostat (Colora). In this way we reached similar levels of water temperature in the 4 incubators (see Table 1) without the risk of contaminating the cooling device or the 4 incubators at the same time.

Sensor construction and calibration

Knowledge on irradiance measurements is of great importance for P-I measurements. Therefore, a new small spherical irradiance sensor was constructed, consisting of a Si photodetector in front of which a green filter is mounted and surrounded by a spherical collecting element made of diffuse epoxy-resin. With a stopper, through which the wire passed, it can be fixed in the centre of an incubation bottle.
Detailed information of the measured typical spectral and spatial sensitivity of this type of sensor is given in ZEMOKO (1994).
For the absolute calibration of the sensor in W.m^{-2} or mmol.photons.m^{-2}.s^{-1} a spectroradiometersystem was used, consisting of a spherical collecting element, an optical fiber, a Jarrell Ash gratingmonochromator and a Si photodetector. Furthermore a standard tungsten striplamp as a wellknown radiance source was used.

The obtained calibration factors (multipliers to get W.m^{-2} or mmol.photons-m^{-2}.s^{-1}) hold only for the combination of this sensor and TLD33.

With the sensor clamped to the turning wheel it was easy to make a complete rotation-angle of 360° and to calculate the average irradiance and standard deviation. The 4p sensor was calibrated using a tungsten strip lamp and a LICOR-1000 lightmeter. The obtained calibration factors (multipliers to get W.m^{-2} or mmol.photons.m^{-2}.s^{-1}) hold only for the combination of this sensor and TLD33.

Neutral density filtercoating

Different levels of irradiance were created by applying different layers of epoxy-resin (in which dark pigments are mixed in different ratios) as neutral density filters on the surfaces of the incubation bottles. The side walls and the necks of the bottles were covered with black epoxy-resin. The reason that we chose this material is our experience that nettings, grids, and even some neutral density filters seriously influence the relative transmission between 400-700 nm. Determination of transmission values in the 400-700 nm range was performed by means of a halogen lamp with daylight-filter and a monochromator. The tubes have the lowest absolute irradiance in the blue and green parts and the highest absolute irradiance in the yellow and orange parts of the 400-700 nm range (data not presented here).
Four series of bottles were available with the following transmission values (in %):

0 1.0 2.5 9.4 18.0 22.9 28.5 31.5 42.5 51.0 70.6 100
0 1.1 2.6 9.8 18.9 23.5 28.7 31.6 42.8 51.5 71.0 100
0 1.5 2.9 9.9 19.1 23.6 30.5 32.9 43.2 53.1 72.1 100
0 1.5 2.9 9.9 19.3 24.3 31.4 35.7 43.3 54.1 72.9 100

Figure 1 shows the relative transmission of 3 and 1.5 % filters of the used epoxy-resin. This material is most suitable in the very low transmission range (thick epoxy-resin layer). In the high transmission range (thin epoxy-resin layer) it must be even better.

The procedure to make the desired epoxy-resin/dark pigment composition and to fix the layers on the incubation bottles is not given here. The reason is that this work was done by a consulting firm that spended some research on this subject. On request the firm is willing to construct on a commercial basis (a restricted number of) series of incubation bottles with known irradiance levels (ZEMOKO, Maritiem technisch bureau, Dorpsplein 40, 4371 AC Koudekerke, The Netherlands, Tel/Fax 0031-0118-551182).

Irradiance measurements

Figures 2-5 give examples of light measurements performed with the 4p sensor. In these figures rotation-angle 0 corresponds with the highest position on the turning wheel. The small and negligible nipple-shaped structures at the tops in Figures 2-5 are measured when the 4p sensor approaches the vertical parts of the copper tubing. Figure 2 illustrates the insignificant difference between the four TL-sets (with coated bottles and white polystyrene foam against one of the outer walls). Figure 3 gives the absolute irradiance distribution with clear bottles and with and without polystyrene foam. It can be seen that using the polystyrene foam substantially increases the amount of available irradiance in the incubator. Surprisingly, however, the difference between minimum and maximum values increased. Figure 4 illustrates the light-absorbing effect of all coated bottles in position on the turning wheel with 2, 4, 6, 8 and 10 TL tubes used. The most flat irradiance distribution was obtained using 6 TL tubes. Finally, Figure 5 gives the results with coated bottles and two sets of 10 TL tubes in parallel and crossed position. In parallel position the mean irradiance during one rotation is ca. 940 mmol.photons.m^{-2}.s^{-1} and in crossed position ca. 960 mmol.photons.m^{-2}.s^{-1}, see Table 3 in ZEMOKO (1994). It should be preferable to have also one or two higher irradiance values in the more inhibiting part of the P-I curve. Higher (and more uniform distributed) irradiance values might be obtained by using circular fluorescent tubes at both sides of the incubator. Using a white epoxy-resin instead of black epoxy-resin to reach higher irradiance values might be possible. In that case attenuation is achieved by diffuse scattering/reflection instead of absorption. However, the spectral properties (relative transmission in the 400-700 nm range, see also Figure 1) of black epoxy-resin seem to be better than those of white epoxy-resin.

Incubations

A series of 3 consecutive incubations were performed in all 4 incubators with changing users per incubator. A culture of *Phaeodactylum tricornutum*, grown in a 2000 l indoor pond with enriched seawater under continuous light (6 * Philips 60 W) at Chl-a concentrations of ca. 150 mg/l, was used. It was diluted tenfold with 0.2 mm filtered Oosterschelde water 24 hours before the experiment. Water temperature in the indoor pond was ca. 11°C, but is known to fluctuate during day and night. At the experimental day nutrient concentrations were P-o-PO$_4$: < 0.03 mM; Si-SiO$_2$: 18 mM; N-NH$_4$: 1.5 mM and N-NO$_3$+NO$_2$: 48 mM. The low phosphate concentration and very high N/P and Si/P ratio's suggest phosphate-limited conditions.

Protocol

For the experimental procedure we followed the standard protocol with a few modifications due to the lab facilities. Thus the incubation bottles were filled with 55 ml of the sample and to each 20 ml with 2 mCi was added. The bottles were always incubated for two hours. After incubation the samples were filtered over 47 mm GF/F at a reduced suction pressure of < 15 kPa. The filters then were put in scintillation vials. Up till here all manipulations were done by the different users; the rest (preparing the scintillation vials) by one user. To each scintillation vial 10 ml demineralized water was added. After addition of 0.5 ml 2 N HCl they were bubbled with air for 20 minutes. Previous experiments had shown that this period is long enough to remove all the inorganic ^{14}C. After addition of 10 ml Instagel^R the samples were counted for 10 minutes or to 1 % accuracy. Added activity was counted in the same mixture without addition of HCl.

ADDITIONAL METHODS

In all samples a Chl-a value was determined using the HPLC method of the laboratory in Middelburg. Filtration was done over 47 mm GF/F at a suction pressure of < 12.5 kPa. SCO_2 was measured by titration according to standard procedures; the measured SAlkalinity in some of the samples was 2.263. From each sample 20 ml was taken for cell counts (if needed) and preserved with 50 ml acid Lugol's solution.

Experimental set-up

The objective was 1) to examine the error in measured primary production parameters if a certain protocol was used by different users working in identical incubators and 2) to check the reproducibility of a measurement.

When determining the error one should take account of different sources of variability:

- variability as a consequence of subsampling,
- variability by the use of different, but in principle identical incubators,
- variability introduced by the inevitable differences in times of starting the incubations (Exp1-3, see below),
- variability by different users.

To attain the first objective a standard Latin Square Design as experimental set-up was chosen. This set-up can be illustrated with the following scheme:

	Inc1	Inc2	Inc3	Inc4
Exp1	A	B	C	D
Exp2	B	C	A	D

Exp3	C	A	B	D

A, B, C and D are the different users. Inc1, Inc2, Inc3 and Inc4 the different incubators and Exp1, Exp2 and Exp3 the 3 successive experiments. Allocation of the incubators (except Inc4) was ad random as was also the case with the distribution of the samples between the users. With this set-up it is possible to take full account of possible error effects within incubators and within experiments, in such a way that a possible user effect can be distinguished.

The first series of measurements (Exp1) started between 9 and 10 a.m., the second (Exp2) between 12 and 13 p.m. and the third (Exp3) between 15 and 16 p.m. In between samples were kept in the dark in cool boxes.

The photosynthetic parameters P_{max}, I_{opt}, I_k and a were derived after fitting the data to the equations of Eilers & Peeters (1988), Jassby & Platt (1976) and Platt et al. (1980). Dark values were not subtracted in the productivity calculations; all dark values except one were ca. 1 % of the maximal photosynthetic rate.

To attain the second objective, reproducibility of a measurement, one user (D) always used the same incubator during Exp1-3 (see scheme above). Unfortunately these results deviated so much from the results of the other three users that a separate consideration was necessary.

RESULTS AND DISCUSSION

Some general information on water temperatures and speed of the turning wheels during the experimental day is given in Table 1. It follows that these characteristics hardly changed during the experimental day.

The mean chlorophyll-a concentration of the nine used samples was 25.6 mg/l and the coefficient of variation 6 %. We thus can conclude that subsampling did not contributed much to variability.

From the analysis of the Latin Square Design it appeared that (except for the slope a determined with the Platt-Gallegos-Harrison model) the incubator (INC) effect was not significant ($p>0.05$) as was also the case for the time (EXP) effect. After correction of the 'disturbing' factors incubator and time there was no user effect ($p>0.05$). This means that for determination of the magnitude of the different parameters from the different P-I models the general mean can be used and that the magnitude of the error can be calculated from all measurements. The results (averaged values for all users) are depicted in Table 2.

Furthermore it appeared that differences could be found in a derived from the three P-I models both according to the number of the experiment and the number of the incubator; see Table 3. This table presents the averaged values for all users. The differences are small, but can be demonstrated with a design like this. For the other parameters the variation after correction for the 'disturbing' factors is to such an extent that differentiation is not possible.

From Table 2 it appears that Pmax has the smallest coefficient of variation and thus can be determined most accurately. Iopt is most variable, while Ik seems to be much more stable; especially for the Platt-Gallegos-Harrison model. The values for Pmax, Ik and a are reasonably comparable for the different P-I models.

Table 4 gives the results of the fourth user. Comparison with Table 2 shows clearly that this user's measurements differed from those of the other three. Only during the third measurement results were similar.

Table 5 gives the mean values with the standard errors and coefficients of variation for all P-I models used. These results were obtained from Table 2.

The general conclusion is: by handling of a fixed protocol a very precise production measurement can be performed.

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Table 1. General information on water temperatures and speed of the turning wheels during the experimental day.

Water temperature (°C)				Speed (rpm)		
	Mean	**SD**	**n**	**Mean**	**SD**	**n**

Inc1	11.48	0.04	12	8.6	0.6	3
Inc2	11.54	0.08	12	7.8	0.3	3
Inc3	11.72	0.07	12	7.5	0.5	3
Inc4	11.78	0.11	12	8.9	0.9	

Table 2. Mean values, standard errors and coefficients of variation (defined as mean/standard deviation) of several measured parameters. pe=Ei- lers-Peeters model; jp=Jassby-Platt model; pgh=Platt-Gallegos-Harri- son model. Pobs is measured maximal production. Pmax and Pobs in mgC.mg^{-1}Chla.h^{-1}; Iopt and Ik in W.m^{-2}; a in mgC.mg^{-1}Chla.h^{-1}.W^{-1}.m^{2}.

	Mean	Standard error	CV (%)
Pmaxpe	1.7	0.045	8
Pmaxjp	1.67	0.052	9.4
Pmaxpgh	1.69	0.047	8.3
Pobs	1.75	0.045	7.7
Ioptpe	102.3	12.2	35.8
Ioptpgh	179.9	92.9	154.9
Ikpe	21.1	2.79	39.5

Ikjp	27.6	1.65	17.9
Ikpgh	22.2	1.27	17.2
ape	0.089	0.0089	29.9
ajp	0.061	0.0027	13.4
apgh	0.076	0.0041	16

Table 3. The slopes of the P-I curves calculated for the different experiments and incubators. EXP stands for the number of the experiment and INC for the used incubator. The measurements are arranged in order of magnitude (except for the incubators under ape, these gave a different result when compared with the two other models). All values are mean values for the three users. Legend: see Table 2.

	ape	ajp	apgh
EXP2	0.109	0.068	0.0873
EXP1	0.094	0.062	0.0777
EXP3	0.064	0.055	0.0677
INC1	0.087	0.066	0.0827
INC3	0.104	0.064	0.082
INC2	0.076	0.054	0.068

Table 4. The results of the fourth user. * points to a very high value resul- ting from not-saturated P-I curves. The figures are based on three measurements performed simultaneously with the three other users. Legend: see Table 2.

	Mean	Standard error	CV (%)
Pmaxpe	2.163	0.221	17.7
Pmaxjp	2.027	0.27	23.1
Pmaxpgh	2.142	0.357	28.9
Pobs	1.86	0.069	6.5
Ioptpe	*	*	*
Ioptpgh	180	67.9	65.4
Ikpe	54.6	21.5	68.2
Ikjp	63	22.6	62.2
Ikpgh	58.7	24.5	72.3
ape	0.051	0.0141	48.2
ajp	0.038	0.0094	42.4

apgh	0.046	0.0012	45.4

Table 5. The mean values for the three different users and the different P-I models used. Legend: see Table 2.

	Mean	Standard error	CV (%)
Pmax	1.68	0.048	8.6
Iopt	141.1	66.25	140.9
Ik	23.6	2.01	25.6
a	0.075	0.0059	23.6

Figure 1. Relative transmission of 3 and 1.5 % epoxy-resin filters in the 400- 700 nm range.
Figure 2. Absolute irradiance distribution of four different TL-sets, 10 TL tubes, with polystyrene (PS) foam layer and with coated bottles.
Figure 3. Absolute irradiance distribution with and without polystyrene (PS) foam layer, clear bottles and 10 TL tubes.
Figure 4. Absolute irradiance distribution with polystyrene foam layer, with coated bottles and 2 (xxoxxxxoxx), 4 (xoxoxxoxox), 6 (xoxooooxox), 8 (xooooooox) or 10 TL tubes.
Figure 5. Absolute irradiance distribution with coated bottles and two 10 TL- sets parallel (P) and crossed (C).

Experts to add figures

ANNEX 3 IRRADIANCE DIFFERENTIATION AND CONTROL IN THE ICES INCUBATOR

Needed in order to be able to accomplish reliable P-I measurements with respect to phytoplankton primary production studies.

Performed by ZEMOKO, specialized in Radiometry and Marine Optics. By order of RIKZ, March 1994.

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Introduction
Neutral density filtercoating
Sensor construction and calibration
Tolerances and imperfections
Irradiance measurements in four incubators
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INTRODUCTION

A full discription of this incubator is given by Colijn et al. (1993). In essence the incubator consists of a TL-illumination set with ten TL33 fluorescent tubes inside, in front of which twelve rectangular shaped incubation flasks, with the flaskneck fixed on a turning wheel, are rotated. The whole flask assembly (see photo 1) is immersed in a ectangular clear perspex water tank. In principle a single-side illumination is foreseen, using a TL-illumination set at one side of the tank (see photo 2) and for higher efficiency a diffuse reflecting polystyrene foamlayer at the other side. [A higher irradiance can be achieved by a dual-side illumination, using one TL-illumination set at each side of the tank]. In both cases, even with all fluorescent tubes switched on, the irradiance distribution in the plane of the flasks is not uniform. That means that during revolution a time\position depending irradiance exists in the flasks and a time\position averaged value should be determined.

Photo 1. Flask assembly. Twelve rectangular shaped incubation flasks with the flaskneck fixed on a turning wheel to be immersed in the rectangular clear perspex water tank of the incubator. In the basic concept of the ICES incubator the possibility of irradiance differentiation is not provided. Only a maximum level can be chosen by the number of fluorescent tubes switched on. For measuring P-I relations an irradiance gradient or a proper sequence of different irradiance values is needed. Changing the basic concept of the incubator as less as possible, the easiest way to achieve this irradiance differentiation is making a sequence by coating the incubation flasks, each in a different transmission density (see section 2). The light attenuation using such a density filtercoating should be preferably neutral, otherwise one needs a proper quantum irradiance measurement including a photosynthetic action response.
When neutral attenuation is achieved, the irradiance measurements can be done with an irradiance sensor having in fact any spectral response within (or partly in) the TL33-spectrum. For measuring total (scalar) irra- diance, a sensor with spatially uniform sensitivity which is small enough to pass

through the neck of an incubation flask, is needed (see photo 3). Absolute irradiance measurement is attained by a proper calibration of the immersed irradiance sensor (see section 3). Photo 2. The complete incubator. In principle a single-side illumination is foreseen using a TL-illumination set at one side of the tank and for higher efficiency a diffuse reflecting polystyrene foam-layer (not shown) at the other side.

In the above indicated way one has a proper irradiance control, although some possible sources of error must be kept in mind:
The position of the sensor within the flask is rather critical. To find a proper average irradiance value a well defined central position in the flask should be achieved. The ater in the incubator and flasks should be kept free of airbubbles which cause lightscattering in an unpredictable way.
Influence of ambient light should be controlled, especially in daylight situations. With a polystyrene foam-layer around the incubator an ambient light shield can be realized but each shield may influence the light situation as well, due to reflections. Long term stability of the TL33-light output is still unknown. Besides a reduction in the absolute
irradiance value also variation in the spatial irradiance distribution of the tubes has to be expected. The irradiance value and uniformity in the flasks' plane will change after some time. The fluorescent tubes should therefore be frequently replaced, preferably before the tube-ends are burned in, to maintain the hereafter described situation (section 5). Last but not least there are always some optical imperfections and tolerances in sensor calibration as well (see section 4).

Photo 3. The mini spherical irradiance sensor. A provision is made to position the sensor in a more or less fixed central position in the incubation flask.

NEUTRAL DENSITY FILTERCOATING

NEUTRAL density water-resistant filtercoatings or filtermaterials are rather rare. After some research a good result is achieved with epoxy-resin in which dark pigments are mixed in different ratios. Compared with commercially available filtermaterials spectral neutrality is in most cases better with the advantage that every density value needed can be made.
A proper transmission density sequence for P-I measurements is made in epoxy filterlayers only 0.8 mm thick. The layers with the different transmission densities are integrated on the front- and backside of each flask. The other sides are made totally opaque. The spectral transmittance of the epoxy filterlayers is measured using a spectroradiometersystem and a tungsten halogen radiant source with daylight filter.
The specified transmittance is actually the quotient of two spectral measurements, one with the filterlayer and another without the layer.
Fig.1 gives the relative spectral transmittance measured for the highest density filterlayer with a transmissionfactor of about 2%. This spectral transmittance can be considered as neutral as necessary for irradiance measurements with an arbitrary spectral response.
Additionally the measured relative spectral transmittance of a filterlayer with a 30% transmissionfactor is given.
The lowest line in Fig.1 is the relative spectral transmittance measured on a complete flask with filterlayers on each side with a transmissionfactor of 50% per layer. These layers are slightly polluted by the primer used for the opaque sides of the flask, resulting in a small increasing red transmittance.

Fig.1. >>

Experts to add figures

The thickness used for the epoxy-layers is rather small for a good control of the higher density values of these filterlayers; in fact the thickness forms a critical factor for these filters. Because the flasks have rims on both sides, it was most practical to use these rims to define the thickness of the epoxy-layers. After only a small correction both rims where brought to an equal height of about 0.8 mm. A thickness of for instance 3 mm would be far less critical. These filters should then be made separately in the proper dimensions to fit on the flasks afterwards. Four identical filter/flask series are made to be used in four identical incubators. The 4 times 12 flasks are numbered 1-0 to 4-11, with the first number for the series and the second number indicating the transmission-step. Increasing numbers correspond with increasing transmittance- and irradiance values. So -0 corresponds with a totally opaque flask, -11 with a clear flask (front and backside only). The weight of these two extreme flasks, -0 and -11, is about 4 g lower as the weight of the other coated flasks because of the lack of filterlayers.
To balance the turning wheel these flasks shoud be mounted in opposite position. Fig.2 is a plot of the aimed transmission sequence together with transmissionfactors measured inside the flasks of the four separate filter/flask series in the incubator. The mutual differences are mainly caused by the above mentioned effect concerning the critical thickness of the filterlayers. The transmittance holds for the diffuse lightcondition in the incubator. Due to scattering in the somewhat diffuse filterlayers the transmittance is somewhat lower than expected. Nevertheless, the aimed trend is fairly good achieved. Table 1 gives an overview of the attained transmissionfactors for the four filter/flask series.

Fig.2. >>

Warning:
With these filterlayers transmission density is achieved by absorption. Exposing them to high irradiance values may result in an increasing temperature and distortion of the material. However, in a (cooled) water tank this effect does not appear.

In general the mechanical resistance of these filterlayers will be no problem at all, even at low temperatures (storage at -6 degrees Celsius for weeks of a coated flask did no harm).

Fig.3 shows the relative spectral reflectance of the 18 mm thick polystyrene foam-layer, used for higher efficiency of the TL-illumination and measured to be sure that the spectral influence is negligible, which indeed is the fact.

Fig.3. >>

Three mini spherical irradiance sensors are made, consisting of a silicon photodetector in front of which a green filter is mounted and a spherical collecting element made of diffuse epoxy. A provision is made to position the sensor in a more or less fixed central position in the flask (see photo 3). Fig.4 gives the measured typical spectral sensitivity for this type of sensor, restricted within the TL33-spectrum and Fig.5 the typical spatial sensitivity measured in two planes in front of a TL-set: one radial plane
around the sensor, the other axial through the top of the sensor. In the axial plane the influence of the obstruction, caused by the connecting cable, is obvious.

Fig.4. >>

Fig.5. The typical spatial sensitivity of the sensor, measured by rotating the sensor in two planes in front of a TL-set: one radial plane around the sensor, the other axial through the top of the sensor.

For the absolute calibration of the sensors in Watt*m-2 and uEinst.*sec-1*m-2, the spectroradiometersystem is used again, consisting of a spherical collecting element as well, an optical fiber, a Jarrell Ash gratingmonochromator and a silicon photodetector. Furthermore a standard tungsten striplamp as a wellknown spectral radiance source. This source is used for calibration of the radiometersystem first. Immersed in water the mini sensors are intercalibrated for the TL33-spectrum using the calibrated spectroradiometersystem and a TL-illuminationset at 10 cm distance.

Fig.6 is the absolute spectral irradiance distribution of the TL33-set together with the integrated irradiance value measured at the calibration distance of 10 cm. From this integrated irradiance value the calibrationfactor or "multiplier" for the mini sensor results, as indicated on the labels of these sensors.
Table 2 specifies the same multipliers as indicated on the labels for the three different mini irradiance sensors delivered. These multipliers have to be set in the photocurrentmeter used. There is a difference in relative sensitivity especially for the first sensor compared with the other two. With the appropriate multiplier the absolute sensitivity of the three sensors agrees within 2%.

Fig.6. >>

The measured spectral distribution of the TL33-set presented in fig.6 corresponds with manufacturers data. The spectral spikes on the curve result from the mercury discharge inside the tube.

TOLERANCES AND IMPERFECTIONS

Only an estimated maximum overall sensor calibration inaccuracy of +/- 15% can be mentioned, which is not an extreme tolerance in radiometric calibration (the overall inaccuracy can be roughly differentiated in: a 5% inaccuracy for the emission of the standard radiance source, 5% for the calibration of the radiometersystem and 5% for the sensor intercalibration). On the other hand, based on comparison with other calibrated irradiance sensors and a different radiometric system, the experience is that mostly the agreement turned out to be better than presumed by the above mentioned inaccuracies. So, the general experience is that the reliability of the present irradiance measurement is fairly good.
Because of aging effects or, in general, long term sensor instability, it is advisable to recalibrate sensors after at least a period of some years, depending on the aimed accuracy and general state of the

sensor. Calibration is carried out at a temperature of 18 degrees Celcius. These sensors have a small negative temperature coefficient of about 0.17%/degr. for the selected wavelength, caused by the silicon detector incorporated. Because of the rather small temperature range in the incubator this effect can be ignored in most cases.

To be sure of a good linear response, these sensors should be used in combination with a low input impedance photocurrentmeter such as for instance a LI-COR LI-1000 or LI-189.

From the sensor configuration and the measured spatial sensitivity, it is clear that this sensortype is affected with an obstructed, incomplete field of view, caused in fact by the electric output connection. The sensor is positioned (in the flask) in such a way that this less sensitive direction is orientated in the dark neck of the flask to minimize the influence of this imperfection. By this the influence is negligible, although the restriction remains.

The sensors should be kept in good condition by storing them always mounted in an empty flask so that the sensors are kept free from dirt, scratches
and/or other damages.

With respect to the neutral density filterlayers the only requirement is in fact the spectral neutrality which is, as stated before, sufficient for reliable irradiance control with the spectral response of fig.4. The only imperfection in the application in the ICES incubator is actually the absorption due to which part of the available light is lost (in general, local light attenuation may be achieved more efficiently by light scattering instead of absorption, see discussion).

Changing the filterflasks partly by clear ones will influence the average irradiance more or less, which holds for changing every absorbing and/or reflecting element in or even around the incubator.

For completeness, a general error resulting from spectral differences between the incubator lightsource and the actual daylight (underwater) spectrum, actually the radiation effectiveness for phytoplankton primary production, should be mentioned. The TL33-spectrum is expected to be less effective compared with most natural spectral situations. The choise of the TL33-type fluorescent tubes is therefore questionable. The available daylight types of fluorescent tubes will be generally more effective.

IRRADIANCE MEASUREMENTS IN FOUR INCUBATORS

Four complete incubators were compared with respect to the irradiance in the filter/flask series during revolution in front of the TL- illuminationsets. For convenience the measurements were carried out in one incubator, using four separate TL-illuminationsets and four separate filter/flask series. First a registration was made of the (spatial)
irradiance distribution during one revolution for different illumination conditions. The irradiance is measured in absolute values (expressed in uEinst.*sec-1*m-2) with the mini sensor positioned in the centre of the filterflask 1-11 and all the other filterflasks in position, each one filled with 55 ml water. The same measurement for one illumination condition was carried out with all the twelve flasks clear (uncoated).

Top-position of the flask containing the sensor, during revolution, is indicated as zero-position and clockwise increasing to 360 degr. which is top-position again.

At zero-position the transmission sequence of the four filter/flask series was measured, and the result plotted in fig.2.

Fig.7 is the registration of the irradiance distribution with the coated flasks and the polystyrene foam-layer with respectivily 2,4,6,8 and 10 fluorescent tubes switched on in a pattern as indicated below:

Fluorescent tubes switched on (O):

2 ----------------------- : XXOXXXXOXX
4 ----------------------- : XOXOXXOXOX
6 ----------------------- : XOXOOOOXOX
8 ----------------------- : XOOOOOOOOX
10 ----------------------- : OOOOOOOOOO

Maximum irradiance is reached when the flask passes the middle of the fluorescent tube(s) where the illuminance of the tube is maximum. This characteristic holds for most of the the curves measured. The pattern for six fluorescent tubes switched on results in the most uniform irradiance distribution. It was found that the small spikes on the maxima of the curves are caused by reflection from the coolingpipe situated in the corners of the water tank on the opposite side of the illuminationset.

Fig.7. >>

Figs.8 and 9 show the comparison of the four illuminationsets with (fig.8) and without (fig.9) the polystyrene foam-layer used and with coated flasks.
No big difference exists in the output and spatial distribution of the four different sets. The obvious effectiveness of the polystyrene foam-layer is demonstrated, although uniformity of the distribution certainly is not improved as one would expect at first.

Fig.8. >>
Fig.9. >>

Fig.10 shows the irradiance distribution when dual-side illumination is used.
With the tube direction of both sets in crossed position the irradiance becomes 50% higher, with the advantage of better uniformity, compared with the single-side illumination with the polystyrene foam-layer in position.

Fig.10. >>

Fig.11 demonstrates a 50% irradiance gain when (12) clear flasks are used instead of (12) coated flasks (compare fig.11 with figs.8 and 9).
The effectiveness of the foam-layer is obvious again. The maximum irradiance is only 10% lower in comparison with dual-side illumination with parallel tube direction (compare fig.11 with fig.10).

In table 3, the statistics for the measured irradiance distributions have been listed; all statistical values refer to one complete revolution comprising 360 samples. Table 4, is a specification of the average absolute irradiance in the four different filter/flask series. These values result from the averages specified in table 3, multiplied by the easured relative transmission factors, determined in the flask zero-position for the different filter/flask series (see fig.2 and table 1).

Fig.11. >>

DISCUSSION AND RECOMMENDATION

Within certain limits the aimed light differentiation and control is achieved. A general restriction concerning the lightlevel remains. When only one illuminationset is used the maximum averaged absolute irradiance is rather low, even with the polystyrene foam-layer in position. The coated flasks absorb part of the available light and due to the 0paque sides there is also a spatial limitation of the light-input in the flasks (see table 3, influence of flask-coating). However, the opaque sides were necessary for a good control of the transmissionfactors of the transmission sequence in the filter/flask series. In this way the transmission is well defined by the absorbing front- and backside filterlayers only. For practical reasons the thickness of the filterlayers should be increased. These filters are not yet standard available. Probably a more efficient use of the available light is possible by coating the flasks with a neutral diffuse white epoxy-coating. Attenuation then is mainly achieved by diffuse scattering/reflection instead of absorption. As a consequence the transmissionfactor then is difficult to predict and to control, whereas a well defined transmission sequence then is hardly to achieve. Moreover, the now available neutral (diffuse) white epoxy is less neutral than the NEUTRAL black epoxy which is used now. Possibly also a well protected metallic coating could be used as a more efficient one.

Another point of view is to leave all the flasks clear with the advantage that one can more easily dispose them. It is recommended (in an eventually revised model of the incubator with a transmission/irradiance sequence) to mount the filters on two filterwheels on both sides of the clear flasks. Furthermore, more compactly mounted circular fluorescent tubes could be used dual-side for more effective and uniform illumination.

Even more than one concentric circular tube (or spiral tubes) could be effectively used.

In general, an optimal spectral characteristic of the fluorescent tube(s) with respect to the effectiveness for primary production is recommended.

The available daylight types of fluorescent tubes are expected to be more effective than the TL33-type.

J. de Keijzer.

SUMMARY

As a result of some special developments, irradiance differentiation and control in the ICES incubator, needed in order to be able to accomplish reliable P-I relation measurements with respect to phytoplankton primary production studies, is achieved. Due to these developments neutral density epoxy filterlayers could be made on four identical filter/flask series.

Besides that, it was possible to construct some mini spherical irradiance sensors, small enough to measure the irradiance inside the flasks. The filters and sensors proved to have good optical qualifications.

The maximum averaged absolute irradiance is rather low when single-side illumination is used. Therefore, when an incubator with an irradiance sequence is needed for P-I relation measurements, a more effective and uniform dual-side circular TL-illumination is proposed with the flasks between two filterwheels.

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REFERENCE

Colijn, F., G.W. Kraay, R.N.M. Duin & M.J.W. Veldhuis, 1993. Design and tests of a novel Pmax incubator to be used for measuring the phytoplankton primary production in ICES monitoring studies. Annex 5 from Report of the Working Group on phytoplankton and the management of their effects. ICES C.M.1993/ENV:7 Ref.:L.

Tables 1, 2, 3 and 4 >>>>>.

Experts to add tables

ANNEX C-6: GUIDELINES CONCERNING PHYTOPLANKTON SPECIES COMPOSITION, ABUNDANCE AND BIOMASS

1. Introduction
2. Sampling
2.1 Preservation and storage of samples
2.2 Qualitative determinations
2.3 Quantitative determinations (phytoplankton counting)
3. Biomass determinations
3.1 Introduction
3.2 Biovolume calculation
3.3 Carbon content calculation
4. Semi-quantitative analysis of phytoplankton samples
4.1 Introduction
4.2 Counting procedure
5. References

1. INTRODUCTION

Phytoplankton serves as an indicator of the effects of eutrophication. Nutrient enrichment may give rise to shifts in phytoplankton species composition and biomass. Furthermore, an increase in the frequency, magnitude and duration of harmful phytoplankton blooms may occur.

Analysis of phytoplankton species composition, abundance and biomass is carried out for the following purposes:

- to describe temporal trends in phytoplankton species composition, their abundance, biomass and intensity of abundance of blooms
- to describe spatial distribution of phytoplankton species, their abundance, biomass and blooms
- to identify key phytoplankton species (e.g. dominating, harmful and indicator species)

2. SAMPLING

Phytoplankton species composition, their abundance and biomass in the euphotic zone form the basis for the determination of temporal trends of phytoplankton. High frequency sampling at a number of stations covering all basins in the Baltic Sea area is needed to reveal trends. As phytoplankton shows a substantial seasonal variation, sampling needs to cover the entire growth season, which in parts of the Baltic Sea extends over the entire year. In addition to the sampling at fixed sampling stations, ships-of-opportunity transects, satellite images and aerial surveillance help to identify variability in temporal and spatial extent of phytoplankton. Synoptic surveys are necessary for the study of the extension of phytoplankton blooms.

For the purpose of quantitative studies in the open sea, the minimum requirement is to take an integrated sample from 0-10 m depth using a hose (Lindahl, 1986). In Coastal Monitoring Programme (CMP), the sample from 0-1 m or an integrated sample (0-10 m) could be analysed.

The same integrated sample should be used for chlorophyll determination and, if desired, primary production.

An additional sample, 10 – 20 m, is recommended.

The 0 – 10 m integrated sample may be approximated by pooling equal amounts of water from the depths 0 – 1 m, 2.5 m, 5 m, 7.5 m and 10 m.

The integrated sample should be thoroughly mixed in a bucket. One subsample of 200 cm^3 is drawn from the well-mixed sample for quantitative phytoplankton counts.

For ship-of-opportunity and helicopter sampling a single sample from the mixed surface layer can be taken.

If there is a subsurface chlorophyll a maximum an additional sample may be taken at this depth, using a water sampler. 200 cm^3 of this sample is drawn for at least qualitative analysis of phytoplankton species composition. Another subsample is taken for chlorophyll a determination.

It is recommended to take a net sample from the 0 – 20 m water column in order to obtain a concentrated plankton sample. This sample serves as a support for species identification. Observing living material facilitates identification. A plankton net with a 10 µm mesh-size is recommended. In case of higher concentration it is advisable to use a net with 25 µm mesh-size.

2.1 PRESERVATION AND STORAGE OF SAMPLES

2.1.1 PRESERVATIVES

Acid Lugol's solution (Willén 1962):

200 cm^3	distilled or deionized water
20 g	potassium iodide (KI)
10 g	resublimated iodine (I_2)
20 cm^3	glacial acetic acid (conc. CH_3COOH)

Mix in the order listed. Make sure the previous ingredient has dissolved completely before adding the next. Store in a tightly locked glass bottle cooled.

Alkaline Lugol's solution (modified after Utermöhl 1958):

Replace the acetic acid of the acid solution by 50 g sodium acetate (CH_3COONa). Use a small part of the water to dissolve the acetate.

Neutralized formaldehyde gives incomparable results to Lugol's solution and should not be used, except at a few coastal stations where long time series are already established using formaldehyde.

2.1.2 Preservation

Net samples to be studied alive can be kept fresh for a few hours in an open container in a refrigerator.

For preservation of water samples, 0.25 – 0.5 cm^3/ 100 cm^3 sample of acid Lugol's solution have to be added immediately. If coccolithophorids need to be preserved with the coccoliths intact, a parallel subsample should be fixed with 0.25 – 0.5 cm^3 alkaline Lugol's solution / 100cm^3 sample. Clear, colourless iodine-proof bottles with tightly fitting screw caps should be used for iodine-preserved material. With such bottles it is easy to see when the iodine becomes depleted and more preservative needs to be added.

They should be stored dark and cool and counted as soon as possible, and within a year. Samples stored for more than one year are of little use.

2.2 QUALITATIVE DETERMINATIONS

Net samples are recommended to be studied for the identification of sparsely occurring microplankton species with a standard research microscope. The advantages include potentially higher resolution, thinner preparations and the possibility to turn the cells around by tapping the cover glass. This is especially helpful when examining the plate structure of dinoflagellates. Dinoflagellate plates are also well studied using the epifluorescence method with Calcofluor (Andersen & Throndsen, 2003).

2.3 QUANTITATIVE DETERMINATIONS (PHYTOPLANKTON COUNTING)

The recommendation is based on the counting technique with an inverted microscope as described by Utermöhl (1958).

2.3.1 SETTLING PROCEDURE

Before sedimentation the sample should be adapted to room temperature to avoid excessive formation of gas bubbles in the sedimentation chambers. Gas bubbles will adversely affect sedimentation, the distribution of cells in the bottom-plate chamber, and microscopy.

Immediately before the sample is poured into the sedimentation chamber, the bottles should be shaken firmly but gently in irregular jerks to homogenize the contents. Too violent shaking will produce a lot of small bubbles, which may be difficult to eliminate. A rule of thumb is to gently turn the bottle upside-down at least 50 times. If the sample must be shaken vigorously in order to disperse tenacious clumps, this should not be done later than one hour before starting sedimentation.

The chambers should be placed on a horizontal surface and should not be exposed to temperature changes or direct sunlight. Covering the settling chamber(s) with an overturned plastic box will provide a fairly safe and uniform environment for sedimentation. If moistened tissue paper is included under the hood, problems caused by evaporation will be reduced considerably. It is essential that the supporting surface is vibration free, since vibrations will cause the cells to collect in ridges.

Settling time is dependent on the height of the chamber and the preservative (e.g. Hasle, 1978 and Rott, 1981). The times given in Table 1 are recommended as minimum. If vibration is a problem, the minimum time should not be significantly exceeded. Otherwise it is suggested that counting be performed within four days. Sedimented samples not counted within a week should be discarded. Separated bottom chambers not counted immediately should be kept in an atmosphere saturated with humidity.

- **Table 1:** Settling time in phytoplankton samples, preserved with Lugol solution, in dependence of the volume of the sedimentation chamber

Volume of chamber (cm3)	Height of chamber (cm)	Settling time (h)
2	1	3
10	2	8
25	5	18
50	10	24

Sedimentation chambers of 100 cm^3 (height 20 cm and settling time 48 h for Lugol) should be used with caution since convection currents are reported to interfere with the settling of plankton in chambers taller than five times their diameter (Nauwerck, 1963 and Hasle, 1978). Such chambers can be used only when phytoplankton is very sparse, as in late autumn and winter. For such samples it is recommended scanning the whole chamber bottom.

If the cells are too strongly stained by iodine for comfortable identification, surplus iodine can be chemically reduced to iodide by dissolving a small amount of sodium thiosulphate ($Na_2S_20_3 . 5 H_20$) in the aliquot to be sedimented.

2.3.2 COUNTING PROCEDURE

In order to save time and to achieve a reasonable accuracy in counting, the sedimented sample should first be examined for general distribution of cells on the chamber bottom, and the abundance and size distribution of the organisms. The settled sample should be discarded if the distribution is visually uneven, one-sided or in ridges, indicating convection or vibration, respectively. If this occurs consistently, measures should be taken to eliminate the sources of disturbance.

Counting begins at the lowest magnification, followed by analysis at successively higher magnification. For the sake of adequate comparison between samples, regions and seasons, it is important to always count the specific species at the same magnification. In special situations, such as bloom conditions, however, this may not be possible. Large species, easy to identify (e.g. *Ceratium* spp.), which are usually also relatively sparse are counted at the lowest magnification and over the entire chamber bottom. Smaller species are counted at higher magnification and possibly on only a part of the chamber bottom.

Small microplankton species can preferably be counted together with the nanoplankton when they occur in abundance, or they can be counted using an objective with intermediate magnification, 20 – 25x. A grid of 5 x 5 squares in one of the oculars is very helpful when counting dense fields of small cells.

The recommended magnifications for different phytoplankton sizes are listed in Table 2.

- **Table 2:** Recommended magnification for counting of different size classes of phytoplankton (From Edler and Elbrächter, in prep.).

Size class	Magnification
0.2 – 2 µm (picoplankton)*	1000 x
2 – 20 µm (nanoplankton)	100 – 400 x
>20 µm (microplankton)	100 x

* picoplankton cannot be properly analysed using the Utermöhl method

Counting the whole chamber bottom is done by traversing back and forth across the chamber bottom. The parallel eyepiece threads delimit the transect, where the phytoplankton are counted (Fig. 1.). Phytoplankton cells crossing the upper thread are counted, but not those crossing the lower thread.

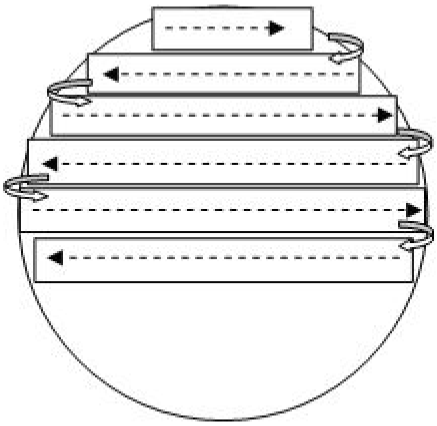

Figure 1. Traversing the whole chamber bottom with the parallel eyepiece threads to indicate the counted area. (From Edler and Elbrächter, in prep.)

Counting part of the chamber bottom can be done in different ways. If half the chamber bottom shall be analysed every second transect of the whole chamber counting method is counted. If a smaller part shall be analysed, one, two, three or more diameter transects are counted. After each transect is counted the chamber is rotated 30-45°.

How much of the chamber area should be counted and the magnification to be used is dependent on the size of the organisms and their abundance, and on the kind of counting units used. The common counting unit is the cell. This applies also to colonies with irregular numbers of cells. Estimation of cell numbers in small-celled and densely-packed colonies may be realised by visual dividing of the colony into smaller areas, counting cell numbers in one area and multiplying with the number of "small areas".

Colonial algae which occur regularly as groups of four cells or a multiple are most conveniently counted and reported as colonies, e.g.:

Choricystis

Crucigenia

Crucigeniella

Desmodesmus

Dictyosphaerium

Elekatothrix

Gonium

Merismopedia

Pandorina

Pediastrum

Scenedesmus

Tetrastrum

Willea

Filamentous cyanobacteria are to be counted in lengths of 100 μm. Numbers of 100 μm pieces per litre are reported. Diatom with any plasma inside the cell should be counted as a living cell.

While counting, the species/individuals have to be allocated to size classes according to the scheme of Olenina et al. (2006). This is important for a reliable biovolume calculation.

At least 50 counting units of each dominating taxon should be counted, and the total count should exceed 500. All cells should be counted and reported even if fewer counted units progressively will decrease the precision of the count and increase the statistical error of the population estimate. The approximate 95 % confidence limits of a selected number of counted units are given in Table 3. They have been calculated according to the formula:

$$95\%\ \mathrm{C.L.} = n \pm 2 \times (100/\sqrt{n})\%$$

where n is the number of units counted. Actually the error is not symmetrical, but increasingly asymmetrical with lower counts. Thus, for four units counted the theoretical limits are -73 to +156 % (Lund *et al*., 1958, Kozova and Melnik, 1978).

- **Table 3:** The approximate 95 % confidence limits of a selected number of counted units.

Count	95 % C.L. (%)
4	100
5	89
7	76
10	63
15	52
20	45
25	40
40	32
50	28
75	23

100	20
200	14
400	10
500	8.9
700	7.6
1000	6.3
2000	4.5
5000	2.8
10000	2

It should be recognized that these are not maximum errors. The statistics assume perfectly random-distribution of cells on the bottom of the sedimentation chamber, a condition which is probably never realized. The several subsampling steps involved also tend to increase the variance (cf. Venrick, 1978a; Venrick, 1978b). In order to check the precision of the method it is recommended to count one dominating species in low and one in high magnification in a new subsample in every 20th sample.

With species for which the counting unit is smaller than the individual, e.g. some colonial forms, chain forming diatoms, and filamentous species with average filament length in excess of 100 µm, the distribution of the counting units will be aggregated even in perfectly sedimented samples. The variance will be higher, and the precision accordingly lower. If it is necessary to keep the error within the same limits as for "randomly" distributed units, the number of counted units should be increased in the ratio average size of individual/size of counting unit.

The number of counting units per dm^3 sea water is calculated by multiplying the number of units counted with the coefficient C, which is obtained from the following formulas:

$$C(\mathrm{dm}^{-3}) = \frac{A \times 1000}{N \times a_1 \times V} \qquad \text{or } C(\mathrm{dm}^{-3}) = \underline{A \times 1000}$$

where:

A = cross-section area of the top cylinder of the combined sedimentation chamber

the usual inner diameter is 25.0 mm, giving A = 491 mm^2 (the inner diameter of the bottom-plate being irrelevant)

N = number of counted fields or transects

a_1 = area of single field or transect

a_2 = total counted area

V = volume (cm^3) of sedimented aliquot

Reliable quantitative counting of the picoplankton fraction requires fluorescence microscopy.

When counting phytoplankton in a sedimentation chamber, it is suitable to count protozooplankton (e.g. ciliates and colourless flagellates). This recommendation is also valid for these forms. However, it must be stressed that the protozooplankton are a separate group and must not be mixed with the phytoplankton. Thus, they must not be included in abundance or biomass values of phytoplankton. The exceptions are the autotrophic ciliates *Mesodinium rubrum* and the genus *Laboea* that should be counted and included in abundance and biomass values of phytoplankton.

While counting, the species/individuals have to be allocated to size classes according to the scheme of Olenina et al. (2006) and the latest update of its appendix.

2.3.3 CLEANING OF THE SEDIMENTATION CHAMBERS

After use no part of the combined sedimentation chamber should be allowed to dry out before it is carefully cleaned. Dried phytoplankton or formalin preservative may be quite difficult to remove. The separate parts are first rinsed under running tap water, and then soaked for a few minutes in Lukewarm water with some nonabrasive detergent added, thereafter cleaned with a soft brush or soft tissue paper, and rinsed with tap water. The sedimentation chamber may also be cleaned with 95% ethanol. Finally, they are given a rinse with deionised or distilled water, and are put away to dry. Special care should be taken not to scratch either end of the top cylinder and the entire upper surface of the bottom plate.

3. BIOMASS DETERMINATIONS

3.1 INTRODUCTION

Biomass data are a much better descriptor of phytoplankton than abundance, especially because the latter is strongly influenced by the highly abundant picoplankton and small nanoplankton, which can be analysed only with limited certainty. Thus, biomass data are preferred for characterising spatial and temporal phytoplankton patterns and modelling. Depending on the purpose of the investigation, phytoplankton biomass can be expressed as cell volume (or weight) or carbon. The transformations to cell volume are based on measurements of the size of the species and the adaptation of the shapes to geometrical figures. The mandatory geometric formulas, size groups and the resulting biovolumes per counting unit are compiled in the paper of Olenina et al. (2006)and its updated appendix. In a further step, the carbon content should be calculated because organic carbon is the universal component of organisms and is the energy source transported along the food chain.

3.2 BIOVOLUME CALCULATION

As specified in Section C.4.3. 2.4, the species/individuals have to be allocated to size classes according to the scheme of Olenina et al. (2006) during the counting process. The individual biovolumes of the different counting units have to be multiplied with their abundance to get the biovolume per dm^3.

Biovolume $_{taxon}$ [mm^3 dm^{-3}] = abundance [dm^{-3}] x VCU x 10^{-9}

VCU = volume of counting unit (in µm^3)

From the bio volume data, the biomass (wet weight) can simply be derived by a rough assumption of a plasma density of 1 g cm^{-3}.

3.3 Carbon content calculation

In the previous guidelines (HELCOM 1988) it was recommended to calculate the carbon content from the plasma volume by a constant factor. Since the calculation of the plasma volume of diatoms bears a lot of uncertainties and, moreover, the conversion factor is not constant in reality, the calculation of carbon has been suspended for some years.

New formulas by Menden-Deuer and Lessard (2000) take the decrease in specific carbon content with cell size into account and calculate the carbon content of diatoms directly from the cellular biovolume without plasmavolume calculation.

The **general formula** for phytoplankton is:

$$\text{Carbon [pg C cell}^{-1}] = 0.216 \times CV^{0.939}$$

Diatoms require a particular formula because of their lower specific carbon content:

$$\text{Carbon [pg C cell}^{-1}] = 0.288 \times CV^{0.811}$$

If cell aggregates are the counting unit (CU), their carbon content has to be calculated via the cells carbon by the following formulas. It has to be differentiated between counting of cell packages (e.g. 100 cells of *Microcystis* as a CU) and filaments (e.g. 100 µm of*Nodularia* as a CU). In filaments, the cell length has to be known.

For **multi-cell colonies**:

$$\text{Carbon [pg C CU}^{-1}] = 0.216 \times CPU \times (VCU/CPU)^{0.939}$$

For filaments:

$$\text{Carbon [pg C CU}^{-1}] = 0.216 \times LCU/CL \times (VCU*CL/LCU)^{0.939}$$

CU = counting unit

VCU = volume of counting unit (in µm^3)

CPU = number of cells per counting unit

CL = cell length (in µm)

LCU = length of counting unit (mostly 100 µm)

The calculation of the carbon content is non-obligatory, but if executed it has to be done according to the given formulas.

4. SEMI-QUANTITATIVE ANALYSIS OF PHYTOPLANKTON SAMPLES

4.1 INTRODUCTION

Microscopic determination is the only method to get information on the species composition of phytoplankton samples. This information is needed in order to reveal changes in the phytoplankton communities in time and space and e.g. to estimate the potential toxicity of a bloom. The counting of cell number is time consuming, and when, mainly information on phytoplankton species composition is needed (ship-of-opportunity transects, additional vertical samples), a semi quantitative counting method can be used instead of the quantitative one. In this method, all the taxa will be identified and listed, but their abundance is estimated using a semi-quantitative ranking (Leppänen *et al*., 1995).

4.2 COUNTING PROCEDURE

For the analysis, the inverted microscope technique is used. At least half of the chamber bottom should be analysed using small magnification (10x-objective) and two bottom transects with larger magnification (40x-objective). All the species found should be listed using the HELCOM counting software with the net option, so when recorded they get the smallest ranking 1 automatically. The semi quantitative ranking should be done using a scale from one to five. The ranking is sample specific, and several species can also get the same ranking, even the highest one.

1. ***very sparse***, one or a few (less than five of the >20 µm fraction) cells or units in the analysed area = in the sedimented sample
2. ***sparse***, slightly more cells or units in the analysed area
3. ***scattered***, irrespective of the magnification several cells or units in many fields of view
4. ***abundant***, irrespective of the magnification several cells or units in most the fields of view
5. ***dominant***, irrespective of the magnification many cells or units in every field of view

When the accurate abundance of a species (e.g. a potentially toxic one) should be counted, at least 20 fields (with 40 x objective), or one transect (with 10 x objective) should be analysed.

The sedimentation chambers etc. should be cleaned as for the quantitative analysis.

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Additionally valuable taxonomic information can be found in various scientific papers, Baltic Sea Phytoplankton Identification Sheets (Ann. Bot. Fennici) and ICES Identification Leaflets for Plankton.

For phytoplankton images validated by the HELCOM Phytoplankton Expert Group see the HELCOM PEG Gallery at www.nordicmicroalgae.org: http://nordicmicroalgae.org/galleries/helcom-peg

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ANNEX C-7 MESOZOOPLANKTON

1. Introduction
2. Purposes
3. Sampling
4. Preservation
5. Sub-sampling
6. Analysis
    6.1 Abundance
    6.2 Biomass
7. Data reporting
8. Quality Assurance
References

1. INTRODUCTION

The mesozooplankton (0.2-20 mm) constitutes an important part of the pelagic food web, since they form the link between primary producers and higher trophic levels. Changes in phytoplankton biomass and species/size composition change mesozooplankton community structure and productivity. Such changes potentially influence fish stock recruitment and sedimentation (i.e. oxygen concentration in the bottom water).

2. PURPOSES

The sampling of mesozooplankton serves, *inter alia*, the following purposes:

- to describe the species composition and the spatial distribution of mesozooplankton abundance and biomass;

- to describe temporal trends (i.e. over several years) in mesozooplankton biomass and community structure.

3. SAMPLING

Mesozooplankton should be sampled by means of vertical hauls using a WP-2 net of 100 µm mesh size. The WP-2 net should be hauled vertically with a speed of about 0.5 m/s.

The nets should always be equipped with flowmeters. They should be mounted at 1/4 of the diameter of the ring (UNESCO 1968). If the filtration capacity of the net is lower than 70% the sample should be discarded and a new sample taken after the rinsing of the net.

The weight to keep the wire vertical should be 25 kg (40 kg when the wire angle tends to exceed 25º, UNESCO, 1968).

The wire angle should always be reported. A correction table for sampling depth is given in Table C.7.1. If the wire angle exceeds 40º, the sample should be discarded. Records of wind speed should be kept.

For fractionated hauls the following intervals should be considered (Fig. C.7.1):

- bottom to halocline (included)
- top of halocline to thermocline (included)
- top of thermocline to surface

If there is no thermocline, a standard haul of 25-0 m should be made.

If there is no halocline, there should be a standard haul of 75 m to the thermocline (included) or to 25 m if there is no thermocline either.

If an anoxic bottom layer is present, sampling should be conducted above the anoxic zone.

No hauls shorter than 5 meters should be made.

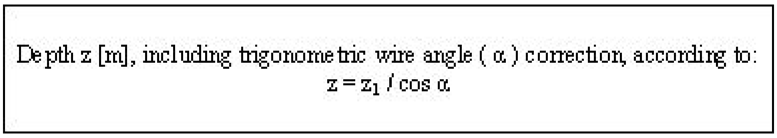
In the Kattegatt and Belt Sea a standard haul of 25-0m should be made.

After collection of each sample the net shall always be rinsed by use of a gentle flow from a hose. When fractionated hauls are taken, only the part below the strap should be rinsed. After emptying, the whole net shall be rinsed with the cod-end open.

After each cruise, the net shall be washed in warm freshwater with a detergent to secure optimum filtration capacity.

When jelly-fish appear in the sample, it is recommended to discard the sample and take a new. When it is impossible to avoid jelly-fish, they should be rinsed from other mesozooplankton and then discarded. When applicable, these procedures should be recorded.

Table C-7.1. Correction of depth from wire angle


Depth z [m], including trigonometric wire angle (α) correction, according to:

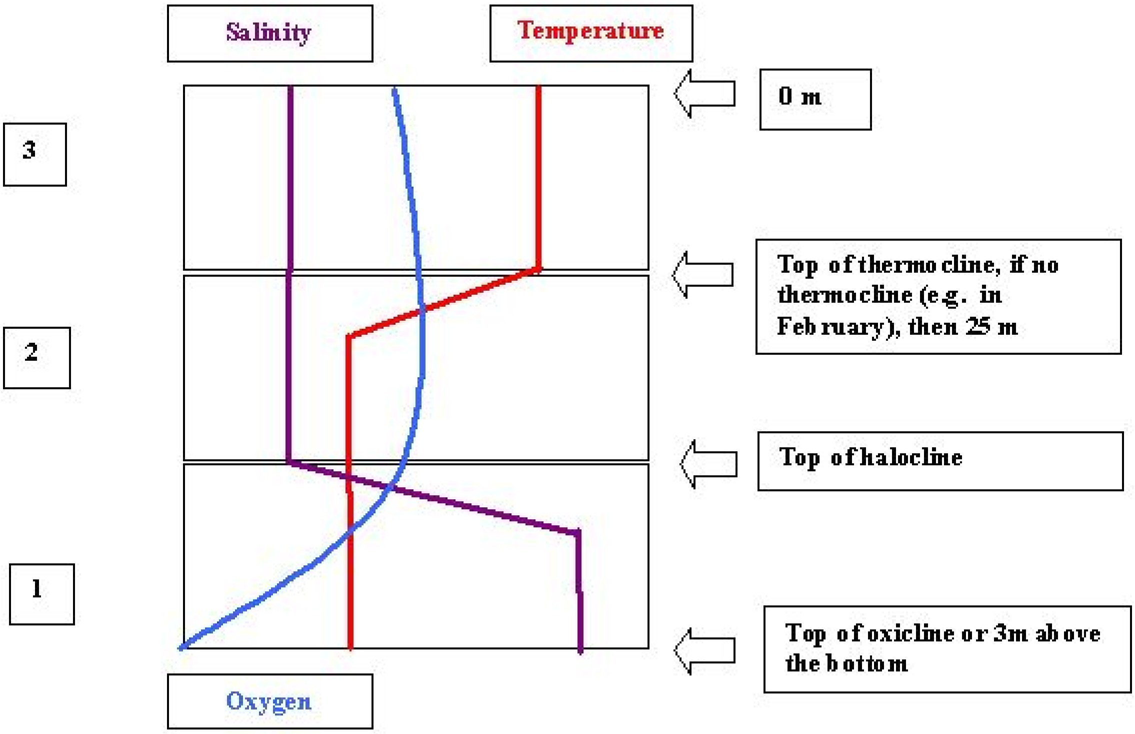
$$z = z_1 / \cos \alpha$$

depth z_1	wire angle					
(m)	**5°**	**10°**	**15°**	**20°**	**25°**	**30°**
5	5	5	5	5	6	6
10	10	10	10	11	11	12
15	15	15	16	16	17	17
20	20	20	21	21	22	23
25	25	25	26	27	28	29
30	30	30	31	32	33	35
35	35	36	36	37	39	40
40	40	41	41	43	44	46
45	45	46	47	48	50	52
50	50	51	52	53	55	58

55	55	56	57	59	61	64
60	60	61	62	64	66	69
65	65	66	67	69	72	75
70	70	71	72	74	77	81
75	75	76	78	80	83	87
80	80	81	83	85	88	92
85	85	86	88	90	94	98
90	90	91	93	96	99	104
95	95	96	98	101	105	110
100	100	102	104	106	110	115
110	110	112	114	117	121	127
120	120	122	124	128	132	139
130	130	132	135	138	143	150
140	141	142	145	149	154	162
150	151	152	155	160	166	173
160	161	162	166	170	177	185

170	171	173	176	181	188	196
180	181	183	186	192	199	208
190	191	193	197	202	210	219
200	202	203	207	213	221	231

Figure C-7.1.


Salinity
Temperature
0 m
3
Top of thermocline, if no thermocline (e.g. in February), then 25 m
2
Top of halocline
1
Top of oxicline or 3m above the bottom
Oxygen

e. g. haul 1: 5 – 61 m

haul 2: 61 – 30 m

haul 3: 30 – 0 m

4. PRESERVATION

The samples should be preserved in 4% formaldehyde solution (1 part 40% formaldehyde solution and 9 parts water). The formaldehyde has to be buffered to pH 8-8.2 with disodiumtetraborate (borax) ($Na_2B_40_3 . 10 H_20$). The samples should be stored until the subsequent assessment is completed.

5. SUB-SAMPLING

A calibrated Stempel-pipette or a Kott Splitter is recommended. The Kott Splitter is somewhat better in precision but is more time-consuming to handle (G. Behrends, A. Korshenko, pers.comm.).

For the work with Stempel-pipette the sample is concentrated by sieving or diluted with tap water as necessary. The volume of the sample is measured in a graduated glass/plastic ware.

A few drops of a detergent should be added to allow the cladocerans to mix in the sample

The sample should be mixed intensively until all organisms are distributed randomly in the sample volume. Non-random distribution in the sample during sub-sampling is the most important source of errors. Aggregations of organisms should be taken out of the sample and the organisms counted.

6. ANALYSIS

The microscopes used should have magnifications to at least 125 X.

6.1. ABUNDANCE

All specimen should be identified and counted until one has reached 100 individuals of each of the three dominating taxonomic groups (excluding nauplii, rotifers and tintinnids). If this figure is not reached in one subsample, additional subsamples must be counted. The taxonomic group(-s) that reached 100 individuals in the previous subsamples, need not be counted in the next subsample(-s). The precision of calculated abundance for organisms of the first three groups, that will be counted up to 100 specimens, amounts to 20% (Tables C-7.1 and C-7.2). The estimation of abundance for other groups ("tail") will be less precise (Cassie 1971, HELCOM 1988).

The term "taxonomic groups" includes species, genera, families and different developmental stages of copepods.

The abundance of nauplii, rotifers, tintinnids and meroplankton larvae can be estimated semi-quantitatively from the first subsample. The presence of macrozooplankton organisms and rare species can be noted after an overview of the whole sample.

Although macrozooplankton, nauplii, rotifers and tintinnids fall outside the size range of mesozooplankton, as do many of the meroplankton, there is a considerable amount of historic data on these groups. Thus they could be reported.

Table C-7.2. Lower and upper 95% confidence limits (in units and as a percentage) for number of counting specimens lower than 17 [4]

N ind counted	Lower limit	Upper limit	Lower limit (%)	Upper limit (%)
0	0	3.7	!!!	!!!
1	0.03	5.6	97	460
2	0.2	7.2	90	260
3	0.6	8.7	80	190
4	1.1	10.2	72.5	155
5	1.6	11.8	68	136
6	2.2	13	63.3	116.7
7	2.8	14.4	60	105.7
8	3.4	15.7	57.5	96.3
9	4.1	17	54.4	88.9
10	4.8	18.3	52	83
11	5.5	19.6	50	78.2
12	6.2	21	48.3	75

13	6.9	22.2	46.9	70.8
14	7.6	23	45.7	64.3
15	8.4	24.7	44	64.7
16	9.1	25.3	43.1	58.1

Table C-7.3. Lower and upper 95% confidence limits (in units and as a percentage) for number of counting specimens more than 17 (see also [2, 3, 4])

N ind counted	Lower limit	Upper limit	Lower limit (%)	Upper limit (%)
17	8.9	25.1	47.5	47.5
18	9.7	26.3	46.2	46.2
19	10.5	27.5	45	45
20	11.2	28.8	43.8	43.8
25	15.2	34.8	39.2	39.2
30	19.3	40.7	35.8	35.8
35	23.4	46.6	33.1	33.1
40	27.6	52.4	31	31
45	31.9	58.1	29.2	29.2

50	36.1	63.9	27.7	27.7
60	44.8	75.2	25.3	25.3
70	53.6	86.4	23.4	23.4
80	62.5	97.5	21.9	21.9
90	71.4	108.6	20.7	20.7
100	80.4	119.6	19.6	19.6
110	89.4	130.6	18.7	18.7
120	98.5	141.5	17.9	17.9
130	107.7	152.3	17.2	17.2
140	116.8	163.2	16.6	16.6
150	126	174	16	16
275	242.5	307.5	11.8	11.8
300	266.1	333.9	11.3	11.3
350	313.3	386.7	10.5	10.5
400	360.8	439.2	9.8	9.8
450	408.4	491.6	9.2	9.2

500	456.2	543.8	8.8	8.8
600	552	648	8	8
700	648.1	751.9	7.4	7.4
800	744.6	855.4	6.9	6.9
900	841.2	958.8	6.5	6.5
1000	938	1062	6.2	6.2
1500	1424.1	1575.9	5.1	5.1

6.2 BIOMASS

The biomass factors for the different taxonomic groups and developmental stages should be used (Hernroth, 1985). The method of Standard Size Classes (Witek Z., G. Breuel, M. Wolska-Pyś, P. Gruszka, A. Krajewska-Sołtys, L. Ejsymont, D. Sujak 1996. Comparison of different methods of Baltic zooplankton biomass estimations. Proceedings of the XII BMB Sympozjum, Institute of Aquatic Ecology, University of Latvia: 87-92)** should be used if appropriate factor is missing. The improvement of present factors taking into account the seasonal and geographical differences in individual volume is an urgent QA task.

Direct measurements of ash free dry weight (AFDW) of ½ sample should be used. Samples, which have been deep frozen (- 18 C) on pre-weighted glass fibre filters (Whatman GF / C, d = 47 mm), should be dried at 60 C in an oven (Lovegrove, 1962, 1966) and ashed at 500 C.

**Reference to Standard Size Classess has been added instead of reference to table C.7.2. which is not available.

7. DATA REPORTING

Data should be reported according to ICES data reporting formats.

8. QUALITY ASSURANCE

[The quality assurance instructions should be according to the recommendations given in the SGQAB Report of 1998, section 10, part B. This work should be done by the BMB Zooplankton Working Group.

Additional items that should be included:

- participation in ring - tests;
- intercalibration of equipment;
- participation in scientific workshops when e.g. recent taxonomical changes can be implemented.]

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ANNEX C-8 SOFT BOTTOM MACROZOOBENTHOS

1. Introduction
2. Purpose
3. Sampling strategy
4. Ship-board routines
   4.1 Sampling
   4.2 Sieving
   4.3 Other sampling methods
   4.4 Fixation
5. Laboratory routines
   5.1 Staining
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6. Recommendations for Quality Assurance
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   6.3 Taxonomy
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References

1. INTRODUCTION

The species composition of benthic communities generally depends on the substrate, depth, wave exposure, oxygen availability and salinity, etc.

Macrobenthic communities are an appropriate target for monitoring since:

1. an important component of benthic communities is formed by species which are long-lived and which therefore integrate environmental change over long periods of time;

2. they are relatively easy to sample quantitatively;
3. they are well-studied scientifically, compared with other sediment-dwelling components (e.g. meiofauna and microfauna) and taxonomic keys are available for most groups;
4. community structure responds in a predictable manner to a number of anthropogenic influences (thus, the results of changes can be interpreted with a degree of confidence);
5. there may be direct links with commercially valued resources, e.g. fish and for wintering birds (via feeding).

The so-called positive effects of nutrient enrichment/eutrophication may increase the food supply to the benthos and, therefore, may give rise to changes in species composition and numbers, increased biomass, shifts in functional groups and changes in community structure. Evident negative effects of eutrophication, as wide range anoxia, leads to impoverished benthic communities or even bottoms depleted with macrofauna.

This Manual is based on the previous HELCOM guideline (HELCOM, 1988) as well as on the OSPARCOM Guidelines for JAMP (OSPARCOM 1997). Much information exists on methodology for benthos investigations. The most relevant reports are those by Rumohr (1990) - available now in www.ices.dk/pubs/times/times08.doc - , which deals largely with methodology for the collection and treatment of samples of the soft-bottom macrofauna, and by Rees *et al*. (1991), which focuses on the monitoring of benthic communities around point-source discharges. The latter also deals more generally with the role of benthos studies in investigations of human impact and includes guidance on approaches for the sampling of different substrate types.

Both reports refer to a range of earlier documents which are of value in the planning and conduct of marine benthos sampling programmes. The most valuable of these is that by Holme and McIntyre (1984) which is a standard reference for anyone working in this field. Guidelines which have been published since the two sets of ICES guidelines (i.e. Rumohr 1990 and Rees *et al* 1991) include Gray*et al.* (1992) concerning approaches to marine pollution assessment and which provides practical examples of applying the PRIMER ('Plymouth Routines in Multivariate Ecological Research') package for univariate, graphical and multivariate data analyses.

2. PURPOSE

The monitoring of benthic communities is carried out for, *inter alia*, the following purposes:

1. to monitor the spatial variability in species composition, abundance and biomass within the maritime area resulting from anthropogenic nutrient inputs;
2. to monitor temporal trends in species composition, abundance and biomass within the maritime area (at a timescale of years) in order to assess whether changes can be related to temporal trends in nutrient inputs;
3. to support the development and implementation of a common procedure for the identification of the status of the benthic communities;

4. to understand the relationship between nutrient concentrations and temporal trends in species/community characteristics.

3. SAMPLING STRATEGY

Sample sites should be representative of the whole monitoring area and therefore, characteristic habitat structures and substrates must be sampled. Prior to temporal trend analysis, checks must be made to ensure that sample sites are inhabited by a homogenous benthic community rather than non-comparable, heterogeneous benthic communities.

Establishment of the baseline community structure and variability at the site under consideration is important. Sample points must be spread out over the extent of the habitat studied to ensure an adequate consideration of spatial variation. It cannot be assumed that one point is representative of the habitat as a whole. When measuring anthropogenically induced change a control reference site is required for each test site. It is important that similar habitats are selected for comparison.

Guidance on the design and implementation of field sampling programmes around waste discharges (Rees *et al*., 1991) may usefully be applied to eutrophication-related studies. The strategy comprises five stages, as follows:

Stage 1: desk study;
Stage 2: planning a sampling programme;
Stage 3: analysis and interpretation of data;
Stage 4: rationalisation of sampling design for regular monitoring;
Stage 5: establishment of routine.

4. SHIP-BOARD ROUTINES

4.1 SAMPLING

Sampling on shallow stations (70 m or less) is recommended to be conducted during daytime, since some benthic species have semipelagic activity during the night.

The following information should be recorded in the field:

- type of positioning system and its accuracy;
- whether or not a buoy was used;
- whether or not the ship was anchored;
- the time of day;
- the weather conditions and state of the sea during sampling;
- the depth from which the sample was taken;

- a description of the sediment, including:

1. surface colour and colour change with depth (as a possible indicator of redox state);
2. depth of the oxygenated surface layer
3. smell (H_2S);
4. a description of sediment type (e.g. caly, sand, mud, etc), including important notes, e.g., the occurrence of concretions, loose algae, etc.

- the type and specification of the sampler.

Near-bottom temperature, salinity and oxygen have to be measured. If more than one sample is taken at a station, the depth range of samples should be recorded. An estimate of the volume of sediment retained should be made for each sample taken, as a measure of sampler efficiency. Criteria for rejection of samples collected by grabs are given by Rees *et al.* (1991) and in the QA part of this guideline.

The widely applied 0.1 m^2 Van Veen grab should be used as the standard gear for benthic macrofauna sampling in the Baltic Sea, because of its very good reliability and simplicity of handling at sea. The emptied grab should weigh about 25-35 kg when used for fine grain size and up to 80 kg in sandy bottoms. In order to reduce the shock wave caused by lowering the grab, the windows on the upper side shall cover an area as large as possible, in practice around 60% of its upper surface. The windows shall be covered with metal gauze of 0.5 x 0.5 mm mesh size.

There may be cases where the use of other gear with smaller sampling area is advisable, e.g. if the fauna is very dense and uniform. When other gears than the standard grab are employed, intercalibrations have to be done on a regional basis and on specific sediments on which these samplers will be used. When a change of gear is intended, it is recommended to sample parallel with both gears for a period of 3-5 years.

Precautions that must be taken when using the grab:

- The settling down and the closing of the grab must be done as gently as possible. Winch operation should be standardized (complete stop and slow lowering (< 0.5 m/s) for the last few meters). This will reduce the shock wave and the risk of sediment loss as a result of lifting the grab before completed closure;
- The wire angle must be kept as small as possible to ensure that the grab is set down and lifted up vertically.

If, as often happens on sandy bottom or erosion sediments, less than 5 l of sediment is collected, the sample should be regarded as not quantitative, and a new sample should be taken after loading the grab with an extra weight. This may as much as double the effective sampling depth of the grab. If less than 5 l of sediment is still collected, the sample may be used, but the low sample volume should be stressed when results are given (Dybern *et al*. 1976). The evidence of this problem may be different in different parts of the Baltic Sea, depending on, e.g., how deep in the sediment the species live.

The choice of sample size and number of samples is always a compromise between the need for statistical accuracy and the effort which can be put into the study. One way to do this is to calculate an index of precision. The ratio of standard error to arithmetic mean may be used (Elliott, 1983), i.e. (x = arithmetic mean, s = standard deviation, n = number of samples). A reasonable error would probably be 0.2 ± 20%.

On the representative stations, at least 3 to 5 samples should be taken, depending on area and species composition, to enable the investigator to reach a certain level of precision by sorting as many samples as necessary. The same procedure is strongly recommended for all other benthos stations unless another sampling strategy (area sampling) is employed in national/coastal monitoring programmes.

Each laboratory shall carefully check the exact sampling area of its grab at several occasions in order to make possible a correct calculation of the number of individuals per square metre. (The area of the grab has a tendency to increase, especially when sampling in stiff clayey sediments.)

4.2 SIEVING

The standard sieve for the Baltic Monitoring Programme shall be of metal gauze (stainless steel, brass or bronze) and have a mesh size of 1.0 x 1.0 mm. In order to collect quantitatively developmental stages of the macrofauna and abundant smaller species it is, however, recommended to use an additional sieve with mesh size of 0.5 x 0.5 mm. This sieve must have the same material as the 1 mm sieve. The mesh size of the sieves has to be checked from time to time for damage and wear. The kind of mesh should be stated.

Attention must be paid to the following points:

- Each sample must be sieved, stored and documented separately;
- The volume of each unsieved sample must be measured. This can be done by grading the container or by using a ruler;
- The grab has to be emptied into a container and should be brought portion by portion onto the sieve as a sediment-water suspension. The use of sprinklers and hand-operated douches to suspend the sample is recommended. Very stiff clay can be gently fragmented by hand. Between the pourings the sieve must be cleaned to avoid clogging and thus to ensure an equal mesh size during the whole sieving procedure;
- The sieving of the sample has to be done carefully in order to avoid damage of fragile animals. Therefore, a direct jet of water against the sieve should be avoided;
- Visible fragile animals, e.g. some polychaetes, shall be hand-picked during the sieving; stones and big shells should be picked out to avoid the grinding effect;
- All residues retained on the sieves should be carefully flushed off the sieves with water from below. Spoons and other tools for sample transfer should be applied carefully. The minor residues in the sieve should be transferred with water;
- When the 0.5 x 0.5 mm sieve is used, the 0.5 and 1.0 mm sieve fractions must be kept separate throughout all further processing;
- Fixed samples should never be sieved.

4.3 OTHER SAMPLING METHODS

Dredge hauls are a valuable complement to grab samples, since mobile as well as large but comparatively rare species are more easily caught by dredging. Dredging is not a quantitative sampling method, but can be useful for qualitative sampling with a five-point scale of abundance. Standardized dredging should always be used when van Veen grabs are likely without fauna, if not done on a routine basis at every sampling. When done so, a visual documentation by video or photography is recommended. Video control and track plotting of dredging actions is recommended as that is the only suitable way of estimating the dredge effort. Descriptions of suitable dredges can be found in Holme & McIntyre (1984) and Bergman & van Santbrink (1994).

In areas where the burrowing depth of the fauna are beyond the penetration depth of the grabs (or that type of gear cannot be used), core samplers may be advisable to use, provided that their efficiency has been satisfactorily proven by intercalibrations to the standard grab.

Photographic and video and records are recommended as a complement to traditional sampling methods. Sediment profile imaging (see e.g. Rhoads and Germano (1982), Rumohr (1995)) may provide a useful means for rapid surveys and classification of sediment structure and bioturbation depth. Side-scan sonar images will provide information on bottom topography and substrate type, which can be useful in the planning of benthos monitoring programmes or in the interpretation of the data. Images should be complemented with 'ground-truth' measurements by underwater video recording and/or grab sampling of sediments.

4.4 FIXATION

The hand-picked animals and the sieving residue shall be fixed in buffered 4% formaldehyde solution (1 part 40% formaldehyde solution and 9 parts water). All necessary measures should be taken to avoid health damage by formalin. For buffering, 100 g of hexamethylenetetramine (Hexamine = Urotropin) shall be used per 1 dm^3 of 40% formaldehyde. Sodiumtetraborate (= Borax) in excess may also be used.

5. LABORATORY ROUTINES

5.1 STAINING

In special cases, i.e. samples from sandy bottoms, it may be advisable to stain the 1 mm sieve samples to facilitate the sorting process. However, in some cases staining may cause problems with species determination.

The staining shall be done before sorting by:

- wash the sample free from the preservation fluid by using a sieve with a mesh size smaller than 0.5 X 0.5 mm;
- allowing the sieve to stand in Rose Bengal stain (1 g/dm^3 of tap water + 5 g of phenol for adjustments to pH 4-5) for 20 minutes with the sample well covered.

However, Rose Bengal (1 g/dm^3 of 40% formaldehyde) may be added already to the fixation fluid.

5.2 SPLITTING OF SAMPLES

Splitting and pooling of samples should be avoided. Instead of splitting use a smaller core sample obtained using a standardized method, which should be fully documented.

5.3 SORTING

Small portions of the unsorted material shall be put on a 0.5 mm mesh size sieve and washed with tap water, so that sorters are not exposed to formalin vapour. Sorting should always be done using magnification aid (magnification lamp, stereo-microscope). Any finer fraction (< 1 mm) should always be sorted under a stereo-microscope.

Broken animals shall only be counted as individuals by their heads (e.g. polychaetes) or hinges of bivalves with adhering pieces of tissue.

5.4 BIOMASS DETERMINATION

Samples should be stored for at least three months before weighing. The biomass shall be determined as dry weight and ash-free dry weight.

The biomass determination shall be carried out for each taxon separately.
All polychaetes should be removed from the tubes, other methods have to be explicitly stated (e.g. for large numbers of polychaetes).
The dry weight shall be estimated after drying the formalin material at 60°C to constant weight (for 12-24 hours, or an even longer time, depending on the thickness of the material).

5.5 SUPPORTING MEASUREMENTS

The use of ash-free dry weight is recommended in routine programmes, because it is the most accurate measure of biomass (Rumohr et al., 1987; Duineveld and Witte, 1987). Ash-free dry weight should be determined after measuring dry weight. It is measured after incineration at 500-520° C in an oven until weight constancy is reached, depending on sample and object size. The temperature of the oven should be checked with a calibrated thermometer, because there may be considerable temperature gradients (up to 50° C) in a muffle furnace. Caution is advised to avoid exceeding a certain temperature (> 550°), at which a sudden loss of weight may occur owing to the formation of CaO from the skeletal material of many invertebrates ($CaCO_3$). This can reduce the weight of the mineral fraction by 44%. Such decomposition occurs very abruptly and within a small temperature interval (Winberg, 1971).

Before weighing, the samples must be kept in a desiccator, while cooling down to room temperature after oven drying or removal from the muffle furnace.

As a simple measure of grain size distribution for the upper 5cm the following sieves should be used: 63µm, 125 µm, 250 µm, 500 µm, 1000 µm and 2000 µm together with weight loss on ignition (500° C - 520° C), total organic carbon and pigments (recommended).

6. RECOMMENDATIONS FOR QUALITY ASSURANCE

Most of the recommendation given here are based on the outcome of two ICES/HELCOM workshops on Quality Assurance of Biological Measurements in the Baltic Sea (ICES, 1994; ICES, 1996).

6.1 GENERAL REMARKS

Experienced and well-trained personnel is a prime basis for maintaining quality standards on a high level. Allocation of resources for proper training and education of field and laboratory personnel is important.

Ring tests and intercalibration exercises at least on a regional basis should be undertaken regularly basis and be obligatory for institutions delivering data to HELCOM. They should be open to all institutions including private industry. Technicians who carry out the actual procedures rather than managing scientists should take part in the exercises.

Exact positioning and correct depths when sampling should be noted in the protocols to avoid comparisons between samples taken at different localities (although noted as the same station in the protocols). If exact positioning due to weather or technical problems is impossible, then fix station work to the correct depth.

Track-plotting during sampling (especially when dredging) is highly recommended, since it both gives information on the size of the area sampled and, if the track-plots are saved, after some time they can provide a detailed depth map of the station.

The number of steps in the sampling and sieving procedures must be kept as small as possible.

Decks hoses are not suited for washing subtle benthos samples. Washing samples on sieves by hand in water-filled containers is recommended as the most gentle way of washing samples.

The use of large sieves is encouraged because:

- the risk of clogging is kept low;
- on sandy bottoms so much sand might be collected that small sieves are filled or even overfilled;
- they reduce the risk of spilling when transferring samples from containers/buckets to the sieves.

Only suspended matter must reach the sieves. The use of water jets directly onto the sieving nets is forbidden.

Rejection criteria for samples are that the samples should be rejected if:

- less than 5 litres is obtained (van Veen, for Haps less than 15 cm penetration);
- incomplete closure is noted;
- obvious uneven bite is noted;

- spillage during transferring of samples is observed;
- samples clearly deviate from the other samples, if noted during sampling, (they should be kept though, but another sample should be taken to replace this in calculating the mean for the station).

If samples are sorted alive, care should be taken to avoid predation within the sample.

It is advisable to stain the samples to facilitate sorting, if this does not hamper species identification.

6.2 CONTROL ROUTINES

To check whether animals are lost when sieving because of using too high water pressure, an extra 1 mm sieve should be placed below the main sieve which should be checked for the number of animals found there after sieving the sample.

The sorting efficiency of the personnel sorting the samples should be checked by an experienced technician. At least 5 % of the samples should be checked for sorting efficiency. Also the species determination should be checked in the same way.

All data lists must be proof-read after input to the computer, before usage. Any spread sheet can be proof-read by the computer with a Sound Card, so you do not need two persons to do it.

One way to check the quality of numbers in the database is to compare individual mean weights. If they are abnormally high or low, the figures need verification.

6.3 TAXONOMY

Lists of taxonomic literature in use should be reported with the data.

Regional taxonomical workshops should be held on a regular basis and be attended by every laboratory.

A checklist of species in the area should be developed, distributed to the participating laboratories and updated regularly.

If the dry weight and ash-free dry weight are determined by drying-burning, an extra sample should be taken and kept preserved unsorted for some years as a reference, in order, for example, to be able to go back to check for the presence of new species.

It is advisable, even with routine samplings, to place some specimens of each taxon under museum curatorship to make later taxonomic checks possible.

6.4 IN-HOUSE QUALITY ASSURANCE

All laboratories should develop programmes for in-house QA, including the appliance of a quality assurance manual.

Signed protocols should be obligatory for all steps in the analyses.

Taxonomic certification of the persons responsible at the laboratories is recommended.

7. REPORTING REQUIREMENTS

Reporting should be in accordance with HELCOM/ICES Biological Data Reporting Formats (http://www.ices.dk/env/index.htm)

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ANNEX C-9 GUIDELINES FOR MONITORING OF PHYTOBENTHIC PLANT AND ANIMAL COMMUNITIES IN THE BALTIC SEA

Open the Guidelines for monitoring of phytobenthic plant and animal communities in the Baltic Sea

ANNEX C-10 GUIDELINES FOR FISH MONITORING SAMPLING METHODS OF HELCOM

- Background
  - Monitoring strategy
  - Programme design
- Monitoring methods
  - General
  - Choice of gear
  - Description of the gear
    - Passive gear
  - Sampling strategy and localities
  - Fishing performance
    - Passive gear
- Data collection
    - Passive gear
- References

BACKGROUND

Fish are to an increasing extent studied in environmental science. Standardized techniques for long-term monitoring and predictions of the size and productive capacity of fish populations, as well as continuous control of their health in a wide context are thus required. Since the guidelines for the HELCOM coastal fish monitoring programme was last updated in 1999 (Neuman *et al.* 1999) and some additional gear has been included in monitoring programmes, this document was produced to describe new guidelines for the fishing methods used, the use of the gear and the variables monitored to study coastal fish populations in the Baltic Sea and Kattegat. Country-wise information about this are listed in tables. The guidelines of this document could also serve as a platform for an integration of biochemical/physiological data and contaminant concentrations with basic ecological data.

MONITORING STRATEGY

The monitoring strategy is designed to prove the impact of exposure to eutrophication, habitat alteration, climate change, toxic substances and overexploitation. There are well-developed models predicting the reactions of the Baltic coastal freshwater fish community to these factors (Hartmann 1977, Neuman and Sandström 1996), but for marine fish, however, there is still a considerable lack of understanding.

When monitoring the impact of toxic substances, an integrated approach has been suggested by many reviewers (Owens 1991, Sprague 1991, Munkittrick 1992). Species should be selected according to well specified criteria, and monitoring should be performed on different levels indicating the health of the population. A tiered strategy is often recommended for the analysis of observed deviations, including biochemical endpoints to support interpretations of possible toxic influence (Neuman and Sandström 1996).

Two fish species have been approved for Baltic coastal monitoring: perch (*Perca fluviatilis*) and eel-pout (*Zoarces viviparous*). Both species are stationary, large enough to allow for biochemical and chemical sampling, generally abundant in their respective habitats, and with a biology allowing far-reaching analyses of reproductive impacts. Perch may be monitored in most sheltered habitats in the Baltic, while the eel-pout more represents the open coasts of the Baltic and the Kattegat. Flounder (*Platichthys flesus*) could potentially be used as an alternative to eelpout and perch as a sentinel indicator species. Flounder is, for example, the preferred indicator species for contaminant analyses and time trends of POP's and metals in Danish monitoring programmes.

PROGRAMME DESIGN

Responses at the community level are monitored by analysing changes in the abundances of populations, where cyprinids are generally acting as sensitive indicators of coastal eutrophication in the Baltic. In monitoring population characteristics like age structure, growth and reproduction is also added to the abundance studies.

The programme is designed for truly coastal species, which are mainly demersal. Most Baltic coastal areas are dominated by warm water adapted freshwater species. In the Kattegat, the eel-pout is one of few typical non-migratory coastal species available for monitoring of the whole life-cycle. Pelagic coldwater species like herring *(Clupea harengus)* often occur in coastal areas, but they are mainly migratory members of open sea communities and should therefore not be included in the present monitoring programme.

Established and standardized methods for fishing with gill nets and fyke nets are used for population monitoring as well as for sampling of fish for analytical purposes. A detailed description of the principles behind the system is given by Neuman (1985).

Other measurements on the individual level, being outside the scope of the basic programme described here, such as contaminant analyses, biomarkers, physiology, pathology etc, can easily be included (see Neuman 1985, Larsson 2006). The basic programme can be (and in some countries already is) applied both in reference areas (i.e. areas without local anthropogenic influence), and in hot-spot monitoring. Moreover, monitoring of fish diseases could also be included in the basic monitoring programme (Thulin *et al.* 1989).

MONITORING METHODS

GENERAL

An important objective in fisheries management and nature conservation is to retain a natural abundance and species composition of the fish community. Most methods for monitoring changes in fish abundance provide catches of several species and information on changes in the species composition of the community could thus also be extracted. In abundance studies the absolute density of a species can not usually be measured. Instead, studies are made of the changes in the relative measure catch per effort and in the species composition.

Abiotic ambient factors play an important role in the behaviour and metabolism in fish. Activity, for example, normally increases with increasing temperature, something that could potentially influence the catches in passive nets. Activity may also be influenced by changes in the wind conditions, currents, salinity and water transparency. Moreover, since fish are poikilotherm organisms, their metabolism, and thus growth and survival, is strongly influenced by temperature. Growth capacity has for example a strong positive temperature dependency up to an optimum temperature depending on the species and size. Furthermore, survival during the first year of life is both directly and indirectly, via food uptake and growth, linked to temperature. Consequently, when analysing data from fish monitoring, it is essential to include temperature. Variation in other important abiotic factors should also be registered since they are of importance for the interpretation of the catch data.

CHOICE OF GEAR

The choice of nets is governed by the species composition in the community to be studied and the desire to obtain reasonably accurate estimates of size- and age-distributions. Net sets have been extensively used by fishery biologists to study fish communities. The basic unit in the recommended programme is a link of four nets with different mesh sizes, set in a locality – ”station”– with uniform (hydrographical) conditions. In the northern Baltic, however, depths and substrates often show a considerably small-scale variation, making it difficult to find uniform areas large enough for a representative use of four nets. A multi mesh-size coastal survey net was developed to allow a representative sample of fish to be collected in such variable conditions. Coastal survey nets are consequently widely used in the Gulf of Bothnia and along the Finnish coast of the Gulf of Finland. In all other parts of the Baltic, net sets should be used. The Nordic coastal multi-mesh gillnets is a more recently developed multi mesh-size net, which since 2001 is used in Sweden, Åland and Finland (Appelberg *et al*. 2003, Söderberg *et al*. 2004, Söderberg 2006). In Denmark, a modified version of the Nordic coastal multi-mesh gillnets in combination with fyke nets are used in the monitoring of coastal fish populations (see tables below for further description).

DESCRIPTION OF THE GEAR

PASSIVE GEAR

The coastal survey net consists of 3 m (10 feet) deep bottom gill nets. The height in the water is about 2.5 m and the length is 35 m. The lower net-rope (main line) is 10 % longer than the upper net-rope (=38.5 m). The nets are made up of five parts, each 7 m long. These have different mesh sizes and are placed in the following order: 17, 22, 25, 33 and 50 mm (mesh bar). The nets are made of green monofilament nylon of 0.20 mm diameter in the two largest mesh sizes and 0.17 mm in the others. The upper net-rope for coastal survey nets is net-rope and the lower is plastic net-rope (weight = 3.2 kg/100 m).

Table 1: The coastal survey net

Country	Gear used as described	Gear not used	Gear used with following modifications
Finland	x (-2004)	x	
Åland	x		
Estonia		x	
Latvia		x	
Lithuania		x	
Poland		x*	
Sweden	x		
Denmark		x	

* Pilot studies with the use of coastal survey nets (mesh size 6-48 mm) were made in Vistula Lagoon in 2007 (June, August and October) to collect ichthyologic material from very shallow waters.

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- ***The set of nets used in July-August*** consists of bottom set gill nets which are 1.8 m (6 feet) deep and made of spun green nylon. A net consists of a 60 m long stretched net bundle which is attached to a 27 m net-rope (35 cm between floats, buoyancy 6 g/m) and a 33 m lower net-rope (weight 2.2 kg/100 m). A set of nets is composed of four nets with mesh sizes 17, 21.5, 25 and 30 mm. Yarn thickness is no. 110/2 for all mesh sizes, according to the Tex-system (e.g., 110/ 2 means 2 filaments each weighing 110 g per 10 000 m).
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Table 2: The set of nets used in July-August

Country	Gear used as described	Gear not used	Gear used with following modifications
Finland		x	
Åland		x	
Estonia			Addition: 14, 33, 38 mm mesh size, in some areas also additionally 42, 45, 50, 55, 60 mm monofilament gill net sets
Latvia			Addition: 14, 33, 38 mm mesh size, in some areas also additionally 42, 45, 50, 55, 60 mm monofilament gill net sets
Lithuania			Addition: 45, 70 mm monofilament gill net sets
Poland		x	
Sweden	x		
Denmark		x	

The set of nets used in October consists of bottom set gill nets which are 1.8 m (6 feet) deep and made of spun green nylon. A net consists of a 60 m long stretched net bundle which is attached to a 27 m net-rope (buoyancy 6 g/m) and a 33 m lower net-rope (weight 2.2 kg/100 m). A set of nets is composed of five nets with mesh sizes 21.5, 30, 38, 50 and 60 mm. Yarn thickness is no. 210/3 for mesh size 60 mm, no. 212/2 for 50–38 mm and no. 110/2 for the other sizes, according to the Tex-system (e.g., 110/ 2 means 2 filaments each weighing 110 g per 10 000 m).

Table 3: The set of nets used in October

Country	Gear used as described	Gear not used	Gear used with following modifications
Finland		x	
Åland		x	
Estonia	x		
Latvia		x	
Lithuania		x	
Poland		x	
Sweden	x		
Denmark		x	

-
- ***The Nordic coastal multi-mesh gillnets*** consists of 1.8 m (6 feet) deep bottom gill nets with a length of 45 m. The lower net-rope (main line) is 10% longer than the upper net-rope (=38.5 m). The nets are made up of nine parts, each 5 m long. These have different mesh sizes and are placed in the following order: 30, 15, 38, 10, 48, 12, 24, 60 and 19 mm (mesh bar). The nets are made of transparent monofilament nylon of 0.15 mm diameter in the seven smallest mesh sizes, 0.17 mm

in mesh size 48 mm and 0.20 in mesh size 60 mm. The upper net-rope has a buoyancy of 6 g/m and the lower net-rope weigh 22 g/m.

Table 4: The Nordic coastal multi-mesh gillnets

Country	Gear used as described	Gear not used	Gear used with following modifications
Finland	x		
Åland	x		
Estonia		x	
Latvia		x	
Lithuania		x	
Poland		x*	
Sweden	x		
Denmark			New Nordic Norm, Total length: 35m, depth: 1.5m, section length: 2.5m, mesh-sizes (thickness): 85(0.35), 68(0.28), 43(0.2), 19.5(0.15), 6.25(0.1), 10(0.13), 55(0.23), 8(0.1), 12.5(0.13), 24(0.16), 15.5(0.15), 5(0.1), 35(0.2), 29(0.16)

*Pilot studies with use of Nordic coastal multi-mesh gillnets (mesh size 10-60 mm) were conducted in Szczecin Lagoon in 2006 and 2007 (June, August, September and October). Main target was to compare data obtained with these gears with data obtained with bottom trawl.

-

- ***The fyke nets*** are 55 cm high with a semi-circular opening and a leader or wing that is 5 m long. They are made of 17 mm mesh in the arm and 10 mm in the crib of yarn quality no. 210/12 in twisted nylon.

Table 5: The fyke nets

Country	Gear used as described	Gear not used	Gear used with following modifications
Finland		x	
Åland		x	
Estonia	x		
Latvia		x	
Lithuania		x	
Poland		x	
Sweden	x		
Denmark	x		

SAMPLING STRATEGY AND LOCALITIES

Coastal nets and net series The smallest geographical unit is a *station* at which either a net set or two coastal survey nets are placed. A group of neighbouring stations with similar conditions (depth, exposure, etc.) and similar influence of environmental disturbance forms a *section*. An *area* is a denominated geographical area within which there may be one or more sections. The recommended number of stations and the number of visits per station may vary depending upon the morphometric characters of the area and the abundance of fish. To select stations for trend monitoring a predesign study has to be made. A large number of stations (>20) are visited once to provide a mapping of spatial variability. About 10 stations are then selected for a continued three year evaluation period. Based on these experiences, the number of stations may be further reduced after performing statistical tests of homogeneity. Monitoring of abundance trends, using net sets or survey nets, is generally possible by sampling a minimum of six stations per area.

Exceptions

Estonia uses fixed stations only in Hiiumaa and in the cold water (October) monitoring of the area Küdema. In all other areas random sampling inside the section(s) are conducted. The number of stations in most areas is at least 20. In Matsalu the number of stations has been at least 40. Near the tiny island Vaindloo, which is in the central part of Gulf of Finland 26 km from mainland, only 5 stations were monitored. But the abundance of fish has been very high there.

Nordic coastal multi-mesh gillnets The sampling strategy is based on depth-stratified random sampling using approximately 45 net stations distributed in different depth intervals (Söderberg *et al.* 2004). The smallest geographical unit is a *station* at which one Nordic coastal net are placed. A group of stations within the same depth interval (0-3 m, 3-6 m, 6-10 m or 10-20 m), forms a *section*. An *area*is a denominated geographical area within which there are a number of sections (depth intervals). The recommended number of stations is at least 45 but it may vary depending upon the morphometric characters of the area and the abundance of fish.

Fyke nets The smallest geographical unit is a *station* at which two fyke nets joined leader to crib are placed. A group of neighbouring stations with similar conditions (depth, exposure, etc.) and exposed to the same influence of environmental disturbances, forms a*section*. Within a section the bottom depth at the nets must not differ more than 2 metres between stations. An *area* is a named geographical area within which there may be one or more sections. The recommended number of stations and the number of visits per station may vary depending upon the morphometric characters of the area and the abundance of fish. To select monitoring stations a predesign study has to be made. A large number of stations are visited once to provide a mapping of spatial variability. About 20 stations are then selected for a continued three year evaluation period according to the routines described above. Based on these experiences, the number of stations may be further reduced after performing statistical tests of homogeneity.

FISHING PERFORMANCE

PASSIVE GEAR

Fishing techniques

Coastal nets and net series (July-August) are set lightly stretched from an anchored buoy kept at a fixed position during the fishing period. The direction of the net (the set) should be constant when fishing in shallow water. A main rule is that the nets are set parallel to the shore. Before the fishing is started each station must be carefully documented with regard to the type of bottom and position (longitude, latitude). Occasional broken meshes are tolerated. Checks must be made on every occasion when the nets are emptied.

Coastal nets and net series (October) are set lightly stretched from an anchored buoy kept at a fixed position during the fishing period. Before the fishing is started each station must be carefully documented with regard to the type of bottom and position (longitude, latitude). Occasional broken meshes are tolerated. Checks must be made on every occasion when the nets are emptied.

Nordic coastal multi-mesh gillnets are set lightly stretched. The direction of the net should be constant between years when fishing in shallow water. Before the fishing is started each station must be carefully documented with regard to the depth and position (longitude, latitude). Occasional broken meshes are tolerated. Checks must be made on every occasion when the nets are emptied.

Fyke nets are set tightly stretched at right angles to the shore. The fyke nets are placed in pairs with leader to crib. Stones with buoys are attached with short lines to the inner leader and the outer crib. Before the fishing is started each station must be carefully documented with regard to the type of bottom and position (longitude, latitude). Occasional broken meshes are not tolerated in fyke nets. Checks must be made on every occasion when the nets are emptied. Before the fyke nets are used, they must be checked on land to ensure that during stretching all parts should be extended.

Exposure

The nets are set between 14 and 16 hrs. They are collected on the following day between 7 and 10 hrs. Within each area the times for setting and lifting should vary as little as possible between fishing efforts. The time when the nets are set and collected during October could differ from the time in August due to shorter day-length in October.

Exceptions

In Estonia the nets are set between 18 and 21 hrs and collected between 8 and 11 hrs.

Fyke nets are emptied daily between 7 and 10. They are replaced immediately after being emptied.

Fishing period

The nets Fishing is done during the period July 25 – August 15, if possible within a 14-day period. Areas to be compared should be fished with as short time difference as possible.

Exceptions

In Estonia some areas (Kihnu, Vilsandi, Kõiguste) are fished during the first half of July and areas in Gulf of Finland in the second half of August. In Sweden, some test fishing with nets is conducted later than August 15, but not later than August 31. In Denmark the period between 15 July and 15 September is recommended for the monitoring of coastal fish populations. The recommended period for contaminant monitoring in flounder and biological effects monitoring in eelpout is October – November in Denmark.

Fyke nets Fishing is done during the period October 15–November 15, if possible within a 14-day period. Areas to be compared should be fished with as short time difference as possible.

Exceptions

In Estonia fyke nets are used parallel to nets during the summer monitoring to collect data about the eel (*Anguilla anguilla*). In Denmark the period between 15 July and 15 September is recommended for the monitoring of coastal fish populations. The recommended period for contaminant monitoring in flounder and biological effects monitoring in eelpout is October – November in Denmark.

Frequency

Coastal nets and net series At least six fishing efforts are conducted at each station yearly. All stations within a section are fished on the same day. If all sections cannot be fished on the same day, the fishing is continued in the remaining sections before returning to the first section.

Exceptions

In Estonia except in Hiiumaa and cold water fishing in Küdema (October), fishing is conducted in 5 to 40 random stations.

In Sweden and Åland the fishing effort are reduced from six nights to three nights from year 2006.

Nordic coastal multi-mesh gillnets One fishing efforts are done at each station each year. In Denmark the frequency for monitoring coastal fish populations is once every sixth year per station. For contaminant monitoring in flounder and biological effects monitoring in eelpout, however, the frequency is once per year.

Fyke nets At least six fishing efforts are conducted at each station. All stations within a section are fished on the same day. If all sections cannot be fished on the same day, the fishing is continued in the remaining sections before returning to the first section. In Denmark the frequency for monitoring coastal fish populations is once every sixth year per station. For contaminant monitoring in flounder and biological effects monitoring in eelpout, however, the frequency is once per year.

DATA COLLECTION

In the following tables every measured parameter (data about the station, ambient data and catch data) are marked with a cross (x) if measured.

PASSIVE GEAR

Table 6: Coastal net, net series and Nordic coastal multi-mesh gillnets (x = measured)

Parameter		Finland	Åland	Estonia	Latvia	Lithuania	Sweden	Denmark
Station	Latitude and longitude	x	x	x (Only in Hiiumaa	x	x	x	x

				and Küdema)				
	Water depth	x	x	x	x	x	x	x
	Bottom type							x
	Disturbance					x		
Ambient data	Water depth	x	x	x	x	x	x	x
	Water temperature, surface	x	x	x	x	x	x	x
	Water temperature, bottom	x	x	x	x	x		
	Wind direction	x	x	x	x	x	x	x
	Wind force	x	x	x	x	x	x	x
	Water current direction							x
	Salinity				x			x
	Visibility (Secchi depth)	x	x	x		x	x	

	Air pressure							
	Oxygen concentration				x			
Catch	Species	x	x	x	x	x	x	x
	Length, 1 mm			x	x		x	
	Length, 1 cm	x 2001-)	• x (2001-)			x (2001-)		x (0.5 cm)
	Length, 2.5 cm	x (2001-)	x (2001-)			x (2001-)		
	Weight			x	x		x	x
	Diseases	x	x	x	x	x	x	

This type of gear is not used in following countries: Poland

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- *Table 7: Fyke nets (x = measured)*
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Parameter		**Sweden**	**Denmark**
Station	Latitude and longitude	x	x
	Water depth	x	x

	Bottom type		x
	Disturbance	x	
Ambient data	Water depth	x	x
	Water temperature, surface	x	x
	Water temperature, bottom	x	
	Wind direction	x	x
	Wind force	x	x
	Water current direction		x
	Salinity		x
	Visibility (Secchi depth)	x	
	Air pressure		
	Oxygen concentration		

Catch	Species	x	x
	Length, 1 mm		
	Length, 1 cm	x (2001-)	x (0.5 cm)
	Length, 2.5 cm	x (2000-)	
	Weight		x
	Diseases	x	

This type of gear is not used in following countries: Finland, Åland, Estonia, Latvia, Lithuania and Poland

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ANNEX C-11: GUIDELINES CONCERNING BACTERIOPLANKTON GROWTH DETERMINATION

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1. INTRODUCTION

Bacterioplankton growth rate is an indicator of the nutrient status in aquatic environments. It is an estimate of the consumption of organic carbon in the ecosystem and therefore closely related to the biochemical oxygen demand *in situ* (cf. BOD_7). Bacterioplankton growth rate thereby indicates the rate of oxygen consumption that may lead to oxygen deficiency in the water column when exceeding oxygen supply. The growth rate indicator may be used in all aquatic environments.

The original method is published in international scientific journals and has been used in many marine research studies since the beginning of the 1980´s. The method has been part of the Helsinki commission guidelines for a longer period of time (Baltic Sea Environment Proceedings No. 27D). The current protocol is an adaptation from Smith and Azam [1].

Bacterial growth rate is a relatively unambiguous indicator of the flux of organic matter through the pelagic ecosystem [2]. Even if the relationship between the factors specific growth rate and abundance may vary, their product representing growth rate reflects the substrate supply of organic matter to the bacterioplankton community. Density limitation (i.e. competition) or other limiting factors (i.e. inorganic nutrients, temperature) do not therefore directly appear to control the bacterioplankton community growth rate at typical environmental conditions. This agrees with empirical observations that bacterial growth rate over larger scales correlates with trophic status of a system [2, 3], and at smaller scales between water layers and seasons [4]. This is true for a growth rate range covering several orders of magnitude.

Bacterioplankton growth rate may be complemented by bacterial abundance and biovolume estimate providing better precision in the biomass production values. This also allows a deeper understanding of whether specific growth rate or bacterial abundance explains changes in community growth rate.

1.1 BACKGROUND

Bacterioplankton are osmotrophs feeding on dissolved organic carbon and dissolved mineral nutrients. They often live like solitary cells free floating, but may also grow attached to particle surfaces. Bacterioplankton typically divide by binary fission, are rod shaped, spherical or c-shaped with an average dimension of 0.6 µm. Small heterotrophic flagellates are their main predators.

The rate of bacterial biomass production was suggested as an indicator of the consumption of organic carbon in an ecosystem by Billen et al. [2]. A positive relationship between nutrient status and bacterial growth, as well as biomass, across different ecosystems has been demonstrated in independent studies [2, 3]. Increased organic production is detected by the variable, whether due to increased phytoplankton growth or import of organic matter from river and waste water discharge.

Bacterial biomass production is closely linked to biological oxygen demand in an environment, and bacterioplankton accounts for about 50% of the oxygen demand in aquatic environments [5]. This is due to oxygen constituting the major electron acceptors in aerobic environments and that bacteria channel a large part of the carbon flux in aquatic ecosystems. Bacterioplankton respiration is therefore an important cause of oxygen depletion when eutrophication prevails, as more than 80% of the marine secondary production occurs in the pelagic environment [6, 7].

1.2 PRINCIPLE

Bacterial growth rate is estimated by uptake of the DNA base thymidine that is radioactively labelled. Thymidine has been shown to be almost exclusively taken up by heterotrophic bacteria in natural samples [1, 8]. Uptake by photosynthetic plankton does not seem to interfere significantly. The synthesis of DNA in a cell is coupled to cell division. Before a cell may divide, the DNA should have doubled to provide all genetic information required for the cell. Thereby twice as much thymidine should have been incorporated when the cell is ready for division.

Thymidine labelled with tritium (^3H) in the methyl group is used. The amount of thymidin taken up is thereby proportional to the amount of radioactivity taken up.

The amount of thymidine taken up is transformed to the number of cells produced by empirical knowledge of the amount of thymidine per cell on average. The theoretical conversion factor correlates relatively well with the empirically derived, but is typically slightly below the latter. The reason is that thymidine pools within the cell dilute the added radioactive thymidine, leading to some underestimation of the true thymidine incorporation by theoretical factors. The fact that some bacteria do not assimilate thymidine, and that some predation on bacteria occur during incubation, also leads to somewhat higher empirical conversion factors.

Bacterial cell growth may be transformed to biomass production by knowing the carbon content per cell. Further transformation to oxygen consumption can be used by using literature values of growth efficiency and respiration quotient (RQ).

1.3 EXTENT

Bacterial growth rate may be applied in freshwater as well as oceanic salinity (0-35). The requirement is that radioactive thymidin is added in sufficient excess to natural extra-cellular pools (e.g. 25 nmol dm^{-3} tritiated thymidine in brackish water). The applied conversion factor should also be valid for the studied environment.

1.4 DISTURBANCES

Avoid exposing the sample to markedly different temperature or light irradiance compared to *in situ* conditions. Ice-cold (0°C) TCA solutions and tubes are essential for the precipitation step. Be careful to pre-cool solutions and tubes prior to use.

Careful removal of the supernatant after centrifugation is a critical step in the procedure. The sample tubes should be kept at room temperature during this procedure to avoid formation of mist on the tube walls. Use a glass pipette (Pasteur) where the tip has been narrowed by heating over a gas burner. Remove all liquid to the bottom, following the side opposite to where the precipitate is expected. Also remove any drops under the lid.

Do not use latex protection gloves as they may create fluorescence.

1.5 CONTAMINATION RISK

Work antiseptically with sterile tips and tubes. Avoid especially contaminating the thymidine stock solution by carefully removing aliquots for each experiment to a sterile tube.

Keep the samples away from any biocide (e.g. formaldehyde, Lugol solution, Latex rubber, TCA etc.).

1.6 SAFETY

Isotope

Tritiated [metyl-3H] thymidine with a specific activity of typically 80 000 Ci mol^{-1} and concentration of 12.5 µmol dm^{-3} (1mCi/ml) is used. The isotope is a ß-emitter and has a range of 10 mm in water. Protect face and eyes from concentrated stock solution. Use a laboratory coat and protective gloves. Diluted working solution should be handled according to laboratory procedures.

TCA

TCA is corrosive on eyes, skin and mucous membrane. Vapour and dust may cause irritation and harm lungs. Use mouth protection, protective gloves and laboratory coat when weighting the substance. Work in a ventilated hood.

2. PREPARATIONS

2.1 CLEANING AND PURIFICATION

Use tubes well rinsed with Milli-Q water for sub-samples the sampling bottles, or taken directly from the bag. These tubes may be re-used following rinsing with Milli-Q water. All bottles and tubes should be clean and not have been in contact with biocides like TCA or formaldehyde.

Tips for e.g. automatic pipettes may be re-used following rinsing with Milli-Q water. Only use tips exclusively for each solution.

Rinse the tip with Milli-Q-water between each depth when dispensing water samples.

2.2 IDENTIFICATION OF SAMPLE

50 ml polypropylene tubes for sub-sampling should be labelled with variable, sample depth and replicate (if applied).

Micro-centrifuge tubes (1.5 ml) are labelled with cruise, station, depth and treatment on the lid with water resistant marker pen.

Place samples in proper order to simplify data treatment.

2.3 REAGENTS

Isotope

Tritiated [methyl-^{3}H] thymidine according to item p. 9 is used.

Trichloroacetic acid (TCA)

TCA contains a lot of crystal water. 100 % TCA is prepared by mixing 500 g TCA (e.g. Merck, ProAnalysi) with 227 l Milli-Q water. 5 % and 50 % TCA is prepared from the concentrated solution by dilution with Milli-Q water.

TCA is corrosive for eyes, skin and mucous membrane. Vapour and dust may be irritating and cause lung damage. Use mouth protection and laboratory coat. Work in a ventilated hood.

Scintillation liquid

Touluene- and Xylene free scintillation liquid is recommended (e.g. Optiphase HiSafe, Wallac OY). The scintillation liquid should be possible to mix with water.

Ice

Crushed ice may be used as cooling medium for TCA tubes.

2.4 BEFORE CRUISE/SAMPLING

Eppendorf tubes (1.5 ml) are placed in 5 ml scintillation vial without lid. The tubes are labelled with cruise, station, depth and treatment. Place the tubes in the order that results are wanted to appear in the scintillation file or print out.

At the beginning of the sampling day incubator for tubes and cold centrifuge are switched on for pre-cooling to the desired temperature. Label tubes for sub-sampling (i.e. 50 ml Falcon tubes) with station and depth, and place them in racks. Have two thermoses ready labelled "Above thermocline" and "Below thermocline", respectively. Have a glass pipette (Pasteur-type) with thin tip attached to a vacuum source (e.g. water tap vacuum device). Bench surfaces used with radioactive samples should be covered with protective paper.

2.5 PROTOCOL

A sampling protocol for logistic data according to ICES recommendation should be used.

3. SAMPLING

3.1 SAMPLING

3.1.1 SAMPLING STRATEGY

It is recommended to take at least 2 samples at different representative depths of the monitored layers of the water column. Surface layer and deepest layer are prioritised. Layers are defined by hydrographic profiles. Required power of the data and natural variability set the required number of samples.

A sampling frequency of 10 samples per year is required to get confident annual estimates (J. Wikner, unpubl. results). Samples should be distributed in the seasonal curve to provide a good coverage of different levels (more samples during the productive season).

An economic alternative is to allocate at least 2 samples to a representative month with limited inter-annual variation. This strategy, however, results in a lower power to detect trends and less ability to cover changes in seasonality. August is recommended based on current experience. Low frequency stations should preferably be evaluated together with high frequency stations located in the same sea area.

It is advocated that at least one high frequency station of 18 samples per year is monitored in each contracting country. This allows an analysis of intra-annual variation and for following changes in seasonal dynamics.

3.1.2 SAMPLING METHOD

Sampling may be performed with a rosette sampler or Niskin bottles attached to a wire.

Water samples are collected according to HELCOM guidelines.

A Milli-Q rinsed polypropylene tube is rinsed once with sample water before a sample of 50 ml is collected. Store the tube with a closed lid as close to *in situ* temperature as possible until start of the incubation according to item 4.4.1.

Fill the thermoses with water from the surface and deep water layer to be used for incubation of samples from depth with similar temperature.

3.2 PRESERVATION/PROCESSING

Processing is done within 1 hour from sampling according to item 3.3 and 4. At rough weather processing may wait up to 8 hours. Note delays exceeding 1 hour in the protocol.

3.3 STORAGE

Tubes with sub-samples are stored as close to in *situ* temperature as possible. Refrigerator or other incubators may be used.

Samples in micro-centrifuge tubes with 50% TCA added may be stored at 4°C for up to 7 days before processing.

Micro-centrifuge tubes with TCA precipitated material in scintillation liquid may be stored at room temperature and in the dark until analysis in a scintillation counter. Counting should be done within 5 days.

4. METHOD DESCRIPTION

4.1 REAGENTS

Make 50 % (w/v) TCA and 5 % (w/v) TCA in sufficient volume to last at least one cruise. Store working solutions of TCA in polypropylene tubes (e.g. Falcon®) submerged in ice slurry during the whole processing procedure.

Withdraw the volume of [methyl-^{3}H] thymidine that is required to run analysis at one station to a sterile (fresh) Micro-centrifuge tube.

4.2 CALIBRATION SOLUTIONS

None.

4.3 PROCESSING

4.3.1 PREPARATIONS

Switch on the cold centrifuge to pre-cool the rotor to +4°C. Centrifuges without cooling may be put in a refrigerator. If an incubator like micro-centrifuge tube ThermoStat plus is used, it is set to +2°C. If a cold plexiglass block is used, it should be tempered at least one hour before use at -20°C.

4.3.2 UPTAKE OF LABELLED THYMIDINE

- For every sample depth two Micro-centrifuge tubes are filled with 1 ml of sample. Additional replication per depth may be applied as appropriate. Replicates should be measured on at least one station per cruise for analytical quality assurance. The same tip may be used for all depths provided that rinsing with two sample volumes Milli-Q water is done in between
- Add 100 ml 50 % TCA to the background treatment samples, mix 3 s with a blender and incubate for 5 min. TCA stop cell activity in the samples
- Withdraw the amount of [^{3}H-methyl] thymidine that is required for one station from the stock solution to a clean micro-centrifuge tube. Add 2 µl of thymidine to samples and then background samples. The same tip may be used for all tubes, by placing the drop of isotope on the wall above the water surface in the sample tube. Mix the tubes 3 s in a blender.
- Place the tubes in the thermos with closest temperature to the water depth of the sample. Note time of incubation start, specific activity and batch number in the protocol. Incubate for 1 hour. If a cooled plastic block is used, cool it at -20 °C in the mean time
- Stop the incubation by placing the micro-centrifuge tubes in the cooling device used at +2 °C for 5 min. Note the stop time in the protocol

4.3.3 PRECIPITATION OF BACTERIAL BIOMASS WITH TCA

- Add 100 ml 50 % TCA to the samples (*not* the background vials) and mix for 3 s. The TCA solutions should be ice-cold at this step. Incubate the samples at +2 °C or on ice for 5 min. If centrifugation can't be done directly, samples may be stored in this condition at +4 °C for up to 7 days
- Place the micro-centrifuge tubes in a cooled (+4 °C) centrifuge with the ”necks” facing outwards and towards the rotor. Samples should not be frozen at this stage. Centrifuge the micro-centrifuge tubes at 16000 x g (13000 rpm, see item 8 Equipment) for 10 min. If not all tubes fit in the rotor, store the remaining tubes in the refrigerator
- Place the micro-centrifuge tubes in a tube rack at room temperature. Remove the supernatant with a Pasteur pipette with a thin tip using a vacuum source. Note that the supernatant is radioactive. Be very careful to remove all liquid. Also remove all mist and droplets on the tube wall and under the lid. The typically invisible pellet is located in the tube bottom facing outward from the rotor. Some precipitate may however stick to the tube wall on the same side. Don't touch the pellet
- Wash the pellet and tube with 5 % TCA. Make sure that no air bubbles are left in the bottom of the tube so that the pellet is washed properly. Close the lid, mix the sample 5 s and turn it up-side down to also wash the inside of the lid

- Centrifuge the micro-centrifuge tubes with the ”necks” facing outward at 16000 x g (13000 rpm) for 10 min. Remove the supernatant as above
- Add 1 ml of scintillation liquid to each tube. Close the lid and mix 5 s on a blender. Hang thee micro-centrifuge tubes in the 5 ml scintillation vials. Store the samples as defined in item 3.3.

4.4 CALIBRATION

The scintillation should be calibrated with sealed standards, typically provided by the manufacturer. Record calibration date and result. Change standards before the expiration date.

Quench correction curve installed by the manufacturer is typically used.

4.5 ANALYSIS

Scintillation counting can be done in the 5 ml scintillation vials with micro-centrifuge tubes.

Run standards before the samples and follow the manual of the scintillation counter.

Record the results preferably in a computer file to minimize errors while entering values manually and save time. Data may also be printed.

The samples are counted in the [^{3}H]-window and settings generating disintegrations per minute (DPM) values from counts per minute (CPM) based on a quench curve installed by the manufacturer (see item 4.4).

The following settings have successfully been used in a Beckman LS6500 scintillation counter for counting tritium:

ID: 3H, 5MIN, DPM

User : 1 Comment:

Preset time : 5.00

Data calc : SL DPM H#: : Yes Sample repeats : 1 Printer: : STD
Count blank : No IC# : no Replicates : 1 RS232 : OFF
Two phase : No AQC : no Cycle repeats : 1
Scintillator : liquid Lumex : no low sample rej: 0
Low level : no Halflife correction date: none

Isotpe 1: 3h %error: 2.00 Factor: 1.000000 BKG.SUB:0

Background quench curve: Off Color quench correction: On

Quench Limits Low: 2.672 High: 316.80

5. CALCULATIONS

5.1 CALCULATION FUNCTIONS

5.1.1 TRANSFORMATION OF DPM TO CELL GROWTH

The amount (mol) of incorporated ^{3}H-thymidine ml^{-1} h^{-1} (n_{ty}) is calculated as:

$$\Delta n_{ty} = \frac{(dpm_s - dpm_b) \times 4.5 \times 10^{-13}}{v \times \Delta t \times SA} \quad (1)$$

where

dpm_s=disintegration per minute in the sample (average of replicates if present)

dpm_b= disintegration per minute in the background (average of replicates if present)

$4.5*10^{-13}$=conversion factor (dpm ==> Ci)

v= sample volume (cm^3)

Δt= incubation time (hours)

SA= specific activity for [^{3}H]-thymidine (Ci mol^{-1})

Bacterial growth in cells (P_c) is calculated as

$$P_c = \Delta n_{ty} \times TCF \times 24 \times 1000 \quad (2)$$

where *TCF* is the thymidine cell conversion factor. A conversion factor empirically determined for the Baltic Sea area of 1.4 x 10^{18} cells [mol thymidine]$^{-1}$ (n=73, ±SE=0,1 x 10^{18}) is recommended. This factor seems independent of growth rate and is close to the theoretical factor for coastal environments [4, 8-12].

The factors 24 and 1000 transform cells cm^{-3} h^{-1} to cells dm^{-3} day^{-1}.

5.1.2 Bacterial biomass production

Cell production is transformed to bacterial biomass production (P_b, mol carbon dm^{-3} day^{-1}) with the function

$$P_b = P_c \times m_b \quad (3)$$

The factor m_b is the carbon content of cells on average in the sample in µmol C cell^{-1}. See the standard operating procedure for bacterioplankton biomass for a definition.

5.1.3 BACTERIAL OXYGEN CONSUMPTION

Bacteral oxygen consumption, ΔO_2^{bact}, may be calculated from P_b , bacterial growth efficiency, *BGE*, and the respiration quotient, *RQ*, according to:

$$\Delta O_2^{bact} = P_b \times \frac{1-BGE}{BGE} \times RQ \quad (4)$$

Estimates of BGE are currently uncertain and vary with at least nutrient status. Recalculation of bacterial growth to bacterial oxygen consumption is therefore a crude estimate of the latter. The best estimate of BGE is probably obtained by the function reported by Del Giorgio and Cole, 1998 [13]

$$BGE = \frac{0.037 + 0.65 \times P_b}{1.8 + P_b} \quad (5)$$

where Pb is the bacterial growth rate in µg C dm^{-3} h^{-1}. The bacterial growth efficiency average 0.27 in the reported data set, which is close to constants used in the literature. The uncertainty of the function has not been reported.

Values of 0.9 has been used for the respiration quotient (RQ), based on a weighted average for respiration of carbohydrate (weight 0.5), protein (weight 0.33) and fatty acids (weight 0.17) [14]. This RQ is also in good accordance with results from experiments on a marine bacterium [15].

5.1.4 Standard deviation

The standard deviation (SD_{tot}) for replicates at one sample depth is calculates as the square sum of both sample and background treatments according to:

$$SD_{tot} = \sqrt{\left(SD_s^2 + SD_b^2\right)} \quad (6)$$

where SD_s och SD_b are the standard deviation for samples and backgrounds, respectively.

5.1.5 THE VARIATIONS COEFFICIENT

The variation coefficient (CV_{tot}) is calculated as:

$$CV_{tot} = \frac{SD_{tot}}{\overline{m}} \quad (7)$$

where m is the average netto dpm based on the difference between samples and background.

5.2 CALCULATIONS

Values for assimilated thymidin (dpm), background and other factors are entered in a database or calculation software according to table 2. Calculation functions according to item 5.1 are applied.

The calculation should return parameters and units according to Table 3 in item 7.

5.3 MEASUREMENT UNCERTAINTY

The measurement uncertainty has been determined according to the standard of measurement uncertainty in chemical analysis of the European Union [16].

The standard uncertainty corresponds to standard deviation and is estimated from several identified variance components of the method. The assimilation of thymidine shows a low expanded uncertainty of ±21%, approximately corresponding to a 95% confidence interval (Table 1). Conversion factors contribute with the greatest uncertainty.

- **Table 1:** Measurement uncertainty for bacterial growth rate. U is the expanded uncertainty with a factor 2.
-

Parameter	Unit	Value	U (%)
Bacterial growth	µmol C dm^{-3} day^{-1}	0.29	±21

The coefficient of variation (±CV) for netto dpm should stay below 20% in productive waters. During the winter season values may be somewhat higher. Values above ±60 % should be scrutinized.

Background values should stay below 100 dpm and average 30 dpm.

The detection limit corresponds to 100 dpm netto uptake of thymidine (+2 x SD). This corresponds to 1 x 10^7 cells dm^{-3} day^{-1} or 0.02 µmol dm^{-3} day^{-1}, approximately the same in carbon or O_2. Typical growth rates in mesotrophic environments are 20 times higher.

6. QUALITY ASSURANCE AND EVALUATION

6.1 CONTROL CHARTS

Duplicate samples should be run regularly corresponding to about 10% of the samples. Plot the standard deviation of duplicates against date in a control chart.

Background values are plotted in control chart.

6.2 EVALUATION

For evaluation of all charts use alarm (2 x SD) and action limits (3 x SD). Values above the action limit should be evaluated for potential error sources. If errors are found, they are corrected with date, motivation and signature added. If no error can be identified, values are labelled as extreme values or questionable values.

It's recommended plotting a full year of data at the end of the year of measurement, to get a good view of the seasonality and depth variation. Sample dpm and growth rate parameters may be plotted against date and depth. Values should be plausible and not differ more than 3 x SD from the average values during a given season. Values should also show an expected variation with depth, where surface values typically are greater than those in deeper water.

Correct found errors and note date, motivate change and sign the change made. Label deviating values as extreme or questionable values if no errors can be identified. Avoid deleting values without proper reason.

7. REPORTING

Enter data in a data base or calculation program as described in Table 2, together with logistic data to identify the sample according to recommendation by the International Council for Exploration of the Sea (ICES).

- **Table 2:** Primary database variables and units.

Parameter	Unit	Digits	Function	Category	Acronyme	Value ex..
Sample radioactivity	dpm	3	-	Depth	BGSAMDPM	1000
Background radioactivity	dpm	3	-	Depth	BGBKGDPM	40
Specific activity	Ci mol^{-1}	3	-	Depth	BGSPACTY	82000
TCF†	cells mol^{-1}	3	-	Depth	BGTCF	1.4x10^{18}
Sample volume	cm^{-3}	3	-	Depth	BGSAMVOL	1
Start of incubation	tt.mm	4	-	Depth	BGINCST	10.18
End of incubation	tt.mm	4	-	Depth	BGINCEN	11.2
Respiration quotient	-	2	-	Depth	BGRQ	0.9

Bact. growth efficiency	%	2	5	Depth	BGGREFF	0.3
Date of calibration (scint.)	01-09-2026	6	-	Depth, standard	BGICD	01-10-2004

† Thymidin conversions factor transforming uptake of thymidine in mol to cells produced.

- **Table 3:** Calculated parameters of bacterial growth rate.

Parameter	Unit	Digits	Function	Category	Acronym	Value ex.
Thymidine uptake rate	mol cm^{-3} h^{-1}	3	5.1.1.	Depth	BGTHYUP	5.11´10-15
Thym. uptake rate ±SD	mol cm^{-3} h^{-1}	2	5.1.1, 5.1.5	Depth	BGTHYSD	4.13x10^{-16}
Bacterial cell production	cells dm^{-3} day^{-1}	3	5.1.1	Depth	BGCELLP	1.84x10^{8}
Bact. Prod. ±SD	cells dm^{-3} day^{-1}	2	5.1.4, 5.1.5	Depth	BGCCELSD	1.12x10^{7}
Bact. Carbon production	µmol C dm^{-3} day^{-1}	3	5.1.2	Depth	BGCARPR	0.29
Bact. Carbon prod. ±SD	µmol C dm^{-3} day^{-1}	2	5.1.4, 5.1.5	Depth	BGCARCV	0.02
Bact. oxygen	µmol dm^{-}	3	5.1.3	Depth	BGCOXYCO	0.6

consump.	^{3} day^{-1}					
Bact. oxygen ±SD	µmol dm^{-3} day^{-1}	2	5.1.4, 5.1.5	Depth	BGCOXYSD	0.05

Use quality codes according to ICES directives.

Use ICES format when reporting logistic information with each value.

Valuable variables of explanation include bacterial biomass (whole community, µmol C dm^{-3}), bacterial volume (median, µm^{3} cell^{-1}), bactivorous flagellates (flagellates dm^{-3}), temperature (°C), total phosphorus (µmol dm^{-3}), total nitrogen (µmol dm^{-3}) and oxygen (µmol dm^{-3}). Substrate variables may also be used if available, where total DOC is a crude indicator of substrate availability.

8. EQUIPMENT

Plastic- and glass ware

- Polypropylene tubes (50 ml) with lid (e.g. Falcon®)
- 1.5 ml micro-centrifuge tubes of polypropylene (e.g. Eppendorf®)
- Scintillation vials (6 ml). (e.g. Beckman Mini Poly-Q-vial)
- Pipette tips 0.5-10 µl, 10-100 µl, and 100-1000 µl
- Pasteur pipettes of glass with a narrow tip. Narrow the tip by melting the pipette over a gas burner, gently pulling each end of the pipette apart. Break the pipette at the narrowest position.

Refrigerated centrifuge

A refrigerated centrifuge for micro-centrifuge tubes (1.5 ml) that can achieve the desired g-force and 4 °C is required. One example is a Beckman GS-15R with rotor F2402. Centrifuges that do not manage refrigeration may be run inside a refrigerator. The rotor should be chilled before applying the samples.

Water vacuum device

A vacuum pump with capacity of at least -400 mmHg is required. A Pasteur pipette with narrowed tip is connected to a water vacuum device attached to a regular tap to remove the supernatant. This also discards the radioactive liquid directly into the sink. Alternatively a water trap may be installed between the vacuum source and the Pasteur pipette.

Automatic pipettes

Calibrated pipettes covering volume ranges of 0.5-10 µl, 10-100 µl and 100-1000 µl is required. A motor driven pipette is recommended for dispensing liquid to many samples.

Refrigerated incubator

A refrigerated incubator with room for at least 14 micro-centrifuge tubes is recommended to use (e.g. Eppendorf ThermoStat plus, prod nr. 5352 000.010 + 5364 000.016). A temperature of +2°C has been found to give an optimal precipitation of cell material by TCA. The incubator should be pre-chilled for 30 min.

Cooling rack

A solid cooling rack (e.g. plastic) with holes for 1.5 ml micro-centrifuge tubes may be used to chill samples, as an alternative to a refrigerated incubator. The rack should be chilled at -20°C. The rack keeps sufficient cooling capacity for 15 min. at room temperature. Store the rack in the freezer when not in use.

Scintillation counter

A scintillation counter with internal quench correction is recommended. One example is Beckman Coulter™ LS 6500 Multi-Purpose Scintillation Counter (cat.nr. 510656). Beckman's™ software for digital collection of data to a computer file is used.

Tube blender

A laboratory blender for tubes is recommended. One example is Vibrofix VF1, Jankel & Kunkel, IKA-Labortechnik.

9. CHEMICALS AND SOLUTIONS

Isotope

A fresh solution of tritiated thymidine less than 8 weeks old from activity date should be used. Make sure the isotope has the desired specific activity (about 80 000 Ci mol^{-1}) Note the date of arrival and volume used on the vial. Store the isotope in the refrigerator.

Tritiate [methyl-^{3}H] thymidine (e. g. Amersham order no. TRK 686) with specific activity of 80 000 Ci mol^{-1} and concentration of 12.5 µmol dm^{-3} (1mCi ml^{-1}) is used. Withdraw the volume required for one station to a clean micro-centrifuge tube to minimize the risk of contamination. Use sterile tips with the pipettes.

The [methyl-^{3}H] thymidine is a ß-emitter where electrons have a range of about 10 mm in water. Minimise handling of the concentrated stock solution. Use protective gloves and a laboratory coat. Discard the diluted isotope according to local regulations.

Trichloroacetic acid (TCA)

Trichloroacetic acid contains large amounts of crystal water. 100% (w/v) of TCA is made by mixing 500 g of TCA powder (e.g. Merck, ProAnalysi) with 227 cm^3 of Milli-Q water. Diluted solution of 50 % and 5 % are made from the stock solution by dilution with Milli-Q water.

TCA is corrosive on eyes, skin and mucous membrane. Vapour and dust may cause irritation and damage on the lungs. Use mouth protection and protective gloves and a laboratory coat. Work in a ventilated hood with TCA powder.

Scintillation liquid

Toluene- and Xylen free scintillation liquid is recommended (e.g. Pharmacia OptiPhase HiSafe 3, Wallac OY, prod. no. 1200-437).The scintillation liquid should be possible to mix with water.

Milli-Q water

Milli-Q water is made from deionized water that is further purified through an ion exchange resin and 0.2 µm filter. Devices producing Milli-Q water is manufactured by e.g. the Millipore ® Company.

Ice

Crushed ice may be use as a cooling medium for tubes with TCA.

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ANNEX C-12: GUIDELINES CONCERNING BACTERIOPLANKTON ABUNDANCE DETERMINATION

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2.

1. INTRODUCTION

The total biomass of bacterioplankton constitutes an indicator of nutrient status in aquatic environments and thereby an indicator of eutrophication. The variable is estimates by direct microscopy and image analysis following methodology published in refereed international scientific journals.

Bacterioplankton biomass analysed by manual direct microscopy is relatively simple and economic. It may therefore be conducted by most laboratories. Image analysis provides a better estimate of biovolume, save counting effort and is operator independent. As a state variable changes in bacterial biomass is subjected to more complicated interpretation than bacterial growth rate, as both growth, mortality and e.g. competition may constitute reasons to observed variations. Ideally therefore both variables are included in a monitoring programme, but may be evaluated also individually.

1.1 BACKGROUND

Bacterioplankton constitutes about half of the living mass of secondary producers in many aquatic environments. Bacteria also account for half of the secondary production in many aquatic environments, and are comparable to the productivity of phytoplankton in some environments [1].

Bacterioplankton are osmotrophs feeding on dissolved organic carbon and dissolved mineral nutrients. They often live like solitary cells free floating, but may also grow attached to particle surfaces. Bacterioplankton typically divide by binary fission, are rod shaped, spherical or c-shaped with an average dimension of 0.6 µm. Small heterotrophic flagellates are their main predators.

Both the biomass and productivity of bacterioplankton is shown to increase with increasing nutrient status in aquatic environments [2, 3]. Time series of bacterioplankton biomass also show proper power to detect trends (J. Wikner unpubl. data). Monitoring of bacterioplankton is therefore motivated to follow the nutrient status of marine environments.

1.2 PRINCIPLE

Bacterioplankton in aquatic environments are rather small (≈0.5 µm) and at low abundance (109 dm-3) as compared to bacteria in culture. To create sufficient contrast in the microscope samples are stained with a fluorescent dye like acridine orange (AO)[4].This is a planar aromatic molecule that binds unspecifically to nucleic acid, but also other cellular components. Staining of the whole cell allow estimation of cell size. AO fluoresce in visible light (red/orange) at illumination with wave lengths 450 – 490 nm.

The sample is filtered on to a blackened filter and stained in the filter funnel. All bacteria will thereby be placed in one focal plane, concentrated and become easier to focus. The filter is mounted on a glass slide and a drop of paraffin oil added, before the cover slip is applied to achieve minimal light diffraction.

A statistically determined number of microscopic fields are counted by aid of an ocular grid or image digital camera. The size of each field is determined by a micrometer scale. The filtered area is determined by the inner diameter of the filter funnel. The average number of bacteria per microscopic field is calculated, and the number of bacteria on the filtered area is determined by multiplying with the ratio between filtered area and microscopic field area.

By image analysis cell volume and morphology type may also be estimated [5]. The bacterial volume may also be estimated manually by comparison with an ocular scale. The volume is calculated by geometric functions for cylinders and spheres. The bacterial volume may then be used to calculate the cell biomass from known carbon-to-volume relationships.

1.3 EXTENT

Samples from oceanic, brackish and fresh waters may be analysed. Filtration of a sample volume providing about 30 cells per microscopic field mean that cell concentrations from 1 x 10^7 cells dm^{-3} may be detected.

1.4 DISTURBANCES

The sample should be preserved prior to filtration and filtration the same day as sampling should be aimed at.

Use well rinsed sample containers. Sample bottles and solutions added to the samples must be free from contaminating particles that may be stained. Make sure that the filtering proceeds at an expected rate. To rapid filtration may indicate a broken filter, erroneously applied filter or too high vacuum.

The background should typically be near black with a good contrast to the bacterial cells. Try to find a remedy to high background before counting the sample.

Two different qualities of filters have occurred at the market. The darker is required for proper microscopy.

Check the settings of the microscope at each counting session. Changes in type of objective, ocular or other lenses may change the magnification factor. The settings should match the directives under item 8.7 or your customized settings.

If many organic aggregates occur in the sample, a significant occurrence of particle bound cells may be found. The total concentration of bacteria may then be under estimated. In this case pre-treat the sample with detergent and sonication according to item 4.3

1.5 CONTAMINATION RISK

See to that the sampling towers are well rinsed with particle free water after each usage to reduce remaining bacteria. Background preparations from Milli-Q purified deionized water, as described under item 4.3 C, should be made at each filtration occasion. By experience contamination from filter funnels, air or other sources is negligible if this standard operating procedure is followed.

1.6 SAFETY

Acridine orange and formaldehyde are harmful substances. Handling of these chemicals shall be done in a ventilated hood and with gloves. Preferably formaldehyde should be used on a ventilated bench as the gas phase is heavier than air. Mouth protection should be used when working with acridine orange powder as it is may cause cancer.

2. PREPARATION

2.1 CLEANING AND PURIFICATION

Sample bottles

Sample bottles are washed with detergent and warm water, followed by rinsing with Milli-Q water. The bottles are air dried and stored with cap or stopper to avoid contamination.

Filter equipment

The filter equipment should be washed after each filtering occasion. The filter manifold is rinsed with warm water followed by Milli-Q water. The same cleaning procedure is done with the filter funnel. Pay specific attention to the filter supports. Strive for lack of remaining colour from the stain.

Glass slide and cover slip

Glass slides and cover slips directly from the packages may be used without further cleaning. See to that no larger particles remains that may interfere with planar application of the cover slip.

Microscope

The ocular and objective should be cleaned annually or as required by competent staff. Use lens paper and chemical pure gasoline. For more comprehensive cleaning consider to use microscope technician from the supplier.

2.2 SAMPLE IDENTIFICATION

All sample bottles should be labelled with cruise identity, sampling station, variable, sample depth and year. Use water resistant tape and marker pen. The glass slide is labelled with water resistant marker pen with cruise identity, sampling station, sample depth, filtered volume and filtration date.

2.3 REAGENTS

Formaldehyde crystals may form with time in its concentrated form (37%). Therefore, concentrated formaldehyde is filtered through 25mm Ø Acrodisc®filter, 0,2 µm pore size at earliest 1 week before sampling.

Mix 30 mg acridine orange in 10 cm^3 of Milli-Q water. Use mouth protection and gloves.

2.4 PRIOR TO SAMPLING

Pure sample bottles (e.g. 50 cm^3 glass bottles) are added 2.0 cm^3, 0.2 µm filtrated, 37% formaldehyde. Close with a rubber stopper and plastic wrapping (Parafilm™) or other closure. Provide an aluminium seal for bottles lacking a screw cap, to be applied after sampling.

2.5 PROTOCOL

A protocol for logistic data (Station, coordinates, date, time, etc.) should be used during the sampling

3. SAMPLING

3.1 SAMPLING

3.1.1 SAMPLING STRATEGY

It is recommended to take at least 2 samples at different representative depths of the monitored layers of the water column. Surface layer and deepest layer are made priority to. Layers are defined by hydrographic profiles. Required power of the data and natural variability set the required number of samples.

A sampling frequency of 8 samples per year is required to get confident annual estimates (J. Wikner, unpubl. results). Samples should be distributed in the seasonal curve to provide a good coverage of different levels (more samples during the productive season).

An economic alternative is to allocate at least 2 samples to a representative month with limited inter-annual variation. This strategy, however, result in a lower power to detect trends and less ability to cover changes in seasonality. August is recommended based on current experience. Low frequency stations should preferably be evaluated together with high frequency stations located in the same sea area.

It is advocated that at least one high frequency station of 18 samples per year is monitored in each country. This allows an analysis of intra-annual variation and follow changes in seasonal dynamics.

3.1.2 SAMPLING METHOD

Sampling may be performed with a rosette sampler or Niskin bottles attached to a wire.

Rinse a Vogel-pipette with sample water and discard. Add new sample liquid and pour 50 cm^3 to a labelled sample bottle with formaldehyde added. Mix the sample by turning the bottle up-side-down 5 times.

3.2 PRESERVATION/PROCESSING

Preservation is provided by the filtered formaldehyde (1.4 % final conc.) in the prepared sample bottle.

3.3 STORAGE

Store the preserved samples at 4 °C. A preserved sample may be stored in the refrigerator (4 °C) for 7 days before making the microscope slide. Microscope slides with filter mounted may be stored for at least 70 days in the freezer (- 20 °C) without loss of cells [6]. Store the glass slides horizontally to avoid that the immersion oil pours away.

Dry filters may be stored for at least 70 days in the freezer (- 20 °C).

4. METHOD DESCRIPTION

4.1 REAGENTS

Prepare acridine orange, immersions oil, and Milli-Q water according to item 9.

4.2 CALIBRATION SOLUTION

Calibration standard with fluorescent beads are primarily used for image analysis systems, but may be applied at manual counting to control settings and magnification. Prepare fluorescent micro-spheres according to item 9.

4.3 SAMPLE PROCESSING

Collect fresh Milli-Q water in two sterile plastic tubes (e.g. Falcon 50 cm^3). One tube (A) is used for rinsing the pipette between the transfer of samples to the filter funnels. The other tube (B) is used to provide Milli-Q water when rinsing filters after staining.

If the samples have a large amount of aggregates and particle bound bacteria they need to be sonicated before analysis. This may be the case for coastal stations during some seasons [7]. A preserved sample is added Triton X-100 (0.001% final conc.) and natriumpyrophosphate (10 mM final conc.). Mix the sample by a vortex for 5 s. Sonicate the sample in ice bath for 30 s at 75 Watt power.

1. Clean the filter support by a paper towel, close the valve and place a few drops of Milli-Q water on the support
2. Mount a GF/C filter and wet it with Milli-Q water. Apply a black 0.2 µm polycarbonate filter (e.g. Poretics©). Apply vacuum so that the filter lies flat on the GF/C filter. Close the vacuum source, let the pressure disappear and close the valve
3. Make a background sample where a filter without sample addition is stained and destained with Milli-Q water in the same way as the other samples
4. Shake the sample flask violently for 10 s to homogenise the sample. Open the lid and transfer the appropriate volume of sample (typically 3-7 cm^3), aiming at least 30 cells per microscopic field (same tip as under item **C** may be used). Use a sample volume table for different stations and seasons developed by experience. See an example for the Gulf of Bothnia in item 11.1. Rinse the pipette tip with Milli-Q water (Falcon Tube A)
5. Filter all samples at a vacuum of -13 kPa (-100 mm Hg, 0.87 bar, 14 psi) to the filter surface appear dry. Close the vacuum let the pressure disappear and close the valve
6. Fill a syringe with acridine orange. Mount a 0.2 µm Acrodisc® filter. Add 15 drops (about 0.75 cm^3) of acridine orange to each sample. The filter should be completely covered by the stain. Incubate for 5 min.
   In the mean time label the glass slides according to item 2.2. Apply immersion oil according to item **I**. See to that the background-filter is on the same glass slide as a sample to aid focusing

7. Filter the stain through the filters. Close the vacuum, let the pressure disappear and close the valve. Wash the filter by adding 1 cm^3 of Milli-Q water from Falcon tube B with a clean tip. Filter the liquid through the filter until it appear dry and leave the vacuum on. Remove the filter funnels

8. Pick up the filter with forceps. Air-dry the filter 45 s by slowly moving it in the air until dry. The filter may be labelled and stored in this condition

9. Apply a drop of immersion oil where filters are supposed to be mounted. Two 25 mm filters may be mounted on each glass slide. Spread the immersion oil drop on an area larger than the filter, by using the filter itself. Place the filter on the oil film. Add a drop of oil on top of the filter. Mount the cover slip and let the oil spread under the whole cover slip by capillary forces. Occasionally a slight pressure on the cover slip by the forceps may aid the spreading of the oil. If two filters are mounted large cover slips covering both may be used. Store the glass slides in slide holders at -20 °C until microscopic analysis

10. Wash the filter funnels after the filtration according to item 2.1. See item 3.3 for storage of samples

11. Preserved liquid samples and glass slides are stored until the annual quality assurance is done

4.4 CALIBRATION

Check that the settings of the microscope (lenses and filter sets) match with that expected (cf. item 8.6 and 11.4). Use a standard preparation of fluorescent beads with known size to calibrate the microscope and image analysis system if applied. Run standard beads in the beginning of each session and compare the results with a control diagram.

The control diagram should include bead abundance and bead size as a function of analysis date.

4.5 ANALYSIS

Analysis of microscope slides with bacterioplankton on filters may be done manually or by image analysis.

4.5.1 IMAGE ANALYSIS

Microscopic fields may be photographed by a digital video camera, images stored on digital media and the particles analysed by image analysis software. This is a preferred analysis as it is operator independent, possible to calibrate, reduce manual counting effort and is economic.

Specific procedure and settings should be done according to specification for camera and software used. An example of an image analysis system routinely applied for monitoring analysis for 5 years is available in item 11.4

4.5.2 MANUAL ANALYSIS

1. Manual analysis may be used if image analysis can not be accomplished. This will introduce operator dependence and less accurate biovolume estimates. Check that the correct equipment

is installed and settings used (item 8.6). Check that the run time of the mercury lamp is not exceeded (max. 200 hours). Record the run-time in a log journal

2. Count the number of cells in 20 microscopic fields, distributed in a representative way over the filter surface. Avoid looking in the microscope while moving position. If areas with markedly heterogeneous distribution occur, a new slide should be prepared
3. Count cells that are rod-shaped, spherical and c-shaped particles, cells appearing solitary or in colonies. Irregular particles are likely not bacteria and should be excluded. Extremely tiny particles may be viruses and should be neglected
4. Use the large square in the ocular grid. Choose a sample volume resulting in an average of 30 cells per field (use item 11.2). Count 20 cells per sample or more than 300 cells in total. Standard error of the average of microscopic fields should not exceed 10 %. Count more fields in that case

5. CALCULATIONS

5.1 CALCULATION FUNCTIONS

5.1.1 TRANSFORMATION OF CELL NUMBERS

The average number of cells per microscopic field (BA_f) and particles on the corresponding background filter (BA_{bkg}) is used to calculate the concentration of bacteria (N_b) in the sample according to:

$$N_b = F_f \times \frac{(BA_f - BA_{bkg})}{V \times d} \quad (1)$$

where V is the filtered volume, F_f the magnification factor (5.1.2), and d the dilution factor due to formaldehyde (50/52=0.9615).

5.1.2 MAGNIFICATION FACTOR

The magnification factor F_f is the filtered area (i.e. inner diameter of the filter funnel) (A_t) divided by the area of the camera image or the ocular grid (a) according to:

$$F_f = \frac{A_t}{a} \quad (2)$$

This factor depends on the ocular, lenses and objective on the microscope and camera according to item 8.6. Use a micrometer scale to measure the area of the captured image from the CCD camera or ocular grid square (item 8.5).

5.1.3 VOLUME CALCULATION

The volume estimate is made on each sample with image analyses. When doing manual microscopy an estimate should be made at 4 occasions during the year.

The bacterial (V_b) is calculated from estimated cell length and cell radius assuming a cylinder with two half spheres according to:

$$V_b = \frac{4\pi r^3}{3} + \pi r^2 (l - 2r) \quad (3)$$

where l is the cell length and r the cells radius. For cocci the length is <1.5 times the width. The radius is calculated by an estimate of the cell area in the image analysis software according to Blackburn et al. 1998[5].

5.1.4 CARBON DENSITY

The carbon density per cell is determined from biovolume by a volume dependant function [8, 9] according to:

$$m_b = 0.12 \times v_b^{0.7} \quad (4)$$

where m_b is the carbon content of the bacterium in pg cell^{-1} and v_b the bacterial volume in µm^3 cell^{-1}. The constant 0.12 is a conversion factor with the unit pg carbon µm^{-3}. The carbon biomass may be converted to mol dm^{-3} by dividing with the molar weight for carbon (12 g mol^{-1}). Bacterial volume may be interpolated between samplings if missing. Literature values (e.g. 0.07 µm^3, [5]) may provide a rough estimate of the biovolume as within year variation has been shown to be low (±SD 10%)[5].

- 5.1.5 Bacterial biomass

The bacterial biomass (B_b) is determined as

$$B_b = N_b \times m_b \quad (5)$$

5.2 CALCULATIONS

Primary data from the image analysis or manual count is readily calculated to appropriate parameters in an calculation software like Microsoft Excel (item 11.1). Calculated data can be aggregated using a pivot table procedure. Compiled data are entered into a database according to item 7.

5.3 PRECISION AND ACCURACY

The detection limit of the method is estimated to 5.0 × 10^6 cells dm^{-3}. The calculation is based on 1 cell per 5 fields on average multiplied by the magnification factor.

The standard error for microscopic fields should be less than 17 % (action limit of 3 × SD) when performing image analysis and 10 % at manual count (due to fewer but larger fields in the former technique). Otherwise count more fields. At high heterogeneity consider making a new slide or sonicate the sample according to item 4.3. The standard deviation between 2 homogenous samples has been estimated to ±2 %.

The accuracy of the method is based on the difference in morphology between, other plankton cells bacteria and abiotic particles. For manual count specific care should be taken to distinguish coccoid cyanobacteria (1-2 cells) during juli-august, when they may approach 10% of the heterotrophic bacterial community. Inter-calibration with other laboratories is desirable as objective standards are missing.

In image analysis coccoid cyanobacteria are discarded based on size criteria. The accuracy is not operator sensitive, but depend on the ability of the neural network to distinguish bacterial cells from similarly sized and shaped particles.

The measurement uncertainty has been determined according to the standard of measurement uncertainty in chemical analysis of the European Union [10].

The standard uncertainty corresponds to standard deviation and is estimated from several identified variance components of the method. The bacterial biomass shows a low uncertainty of ± 23 %, approximately corresponding to a 95 % confidence interval (Table 1). The estimate of average number of cells per microscopic field contributes with the greatest uncertainty (i.e. variation between microscopic fields). The uncertainty is therefore similar in the unit cells dm^{-3}.

Table 1: Measurement uncertainty for bacterial biomass. The expanded uncertainty (*U*) is presented based on the expansion factor 2.

Parameter	Unit	Value	U (%)
Bacterial biomass	µg carbon dm^{-3}	28	23

6. QUALITY ASSURANCE AND EVALUATION

When performing manual counting the standard error for the average number of cells per microscopic field should be below 10% in manual analysis. Increase the number of counted field otherwise.

6.1 CONTROL CHARTS

Run a standard of fluorescent beads at each session when performing image analysis. Bead abundance and volume is plotted against date.

The Milli Q background should show values of 4×10^5 cells $[\text{filtered area}]^{-1}$ ($\pm 6.4 \times 10^5$) based on image analysis estimates. The background value should be divided by the sample volume prior to subtraction from the corresponding sample. When the image analysis system finds no particles an error message may be returned. These background values should be set to zero manually.

Standard deviation (i.e. variance between microscopic fields) for samples and Milli-Q backgrounds are plotted versus date.

Duplicate samples should be analysed regularly amounting to about 10% of the total number of samples. Plot the standard deviation of duplicates against date.

6.2 EVALUATION

After a full year of data set has been collected, values are scrutinized by plotting bacterial concentration for each station versus date and depth, respectively.

Evaluate estimated bacterial concentration versus empirically derived seasonal and depth abundance. Coupling to substrate and local Values should show an expected seasonal variation with highest values during late summer. Bacterial abundance should typically be higher in the trophic layer than in the deep water.

For all control charts it’s advised that samples differing more than 2 × SD from average values are checked, and actions should be taken for samples deviating more the 3 × SD. If causes of errors can be identified they are attended to. If no source of error can be identified the sample maybe left as an extreme value or is labelled by quality code “questionable value”. Be careful not to delete values without proper reason.

6.3 HARDWARE CALIBRATION

The camera image size has been determined with a microscopic scale with the 63x objective (item 11.4). The image area for the camera was determined with aid of the micrometer scale. Height and width was determined to 107.4 µm and 132.8 µm, respectively (14258 μm^2), using a scale to measure dimensions on the computer screen image.

The ocular counting square size is determined in a similar way when performing manual counting. A typical size for a large counting square in the ocular is 70 × 70 µm, and 21 × 21 µm for a small square.

Filtered area was determined from the diameter of the filter funnel (21 000 µm). The filter area (πr^2, 3.464×10^8 μm^2) and image area was used to determine the magnification factor according to equation under item 5.1.2 to 24292.

Number of pixels per µm for the CCD camera was determined with the ”Analysis: Line” option in the image capture software *Wasabi*. The microscopic scale was projected with a regular light source and captured in *Wasabi* as an image. A line measure in *Wasabi*determined the length in pixels between two scale lines. The distance between the scale lines was divided by the length in pixels giving 0.0986 µm pixel^{-1} for the system. This value should be entered in the “Calibration” cell in LabMicrobe (item 11.4.4). LabMicrobe round this value to 0.1 but use the precise value.

7. REPORTING FORMAT

Make required calculation in a calculation software like Microsoft Excel and aggregate data from different images using pivot tables. Variable specific parameters are given in table 2. A similar table may be set up for Milli-Q and bead results. Also add logistic and quality code information according to standards of the International Council for the Exploration of the Sea (ICES) and HELCOM.

For internal laboratory data handling it is recommended to record preparation date and time of the samples (link Milli-Q background with samples), and analysis date and time which link bead standards with samples.

Table 2: Reporting format of bacterial biomass shown with units and typical values.

Parameter	Unit	Digits	Function	Categories	Acronym	Value ex.
Bacterial abundance	cells dm^{-3}	3	5.1.1, 5.1.2	Depth, Standard	BACTABU	1.30E+09
±SD Bact. conc.	cells dm^{-3}	2	Statistics	Depth, Standard	BASDCON	1.10E+08
Number of fields	number	1	-	Depth, Standard	BANPIC	5
Counted cells	cells	3	-	Depth, Standard	BACOUCEL	500
Bacterial biovolume	µm^3 cell^{-1}	3	5.1.3	Depth, Standard	BACVOL	0.07
±SD Biovolume	µm^3 cell^{-1}	2	Statistics	Depth, Standard	BASDVOL	0.006
Preparation date-time		10	-	Depth, Standard	BAPDAT	05.09.2026 13:50
Analysis date-time		10	-	Depth, Standard	BAADAT	05.10.2001 14:30

Table 3: Calculated parameters.

Parameter	Unit	Digits	Function	Categories	Acronym	Value ex.
Carbon density	fg cell^{-1}	3	5.1.4	Depth	BACTDENS	19

Bacterial biomass	µg carbon dm^{-3}	3	5.1.5	Depth	BACTBIOM	24

”Statistics” mean that established statistical functions are used.

Valuable variables of explanation encompass bacterial growth rate (whole community, cells dm^{-3} day^{-1}), temperature (°C), total phosphorus (µmol dm^{-3}), total nitrogen (µmol dm^{-3}) bactivorous flagellates (cells dm^{-3}) and oxygen concentration (mol O_2 dm^{-3}).

Data should be reported to national and international databases. If primary data is not archived by these databases the operator should store primary data locally.

Images and primary data may be archived on digital media for time determined by quality assurance directives.

8. EQUIPMENT

8.1 FILTER

Blackened polycarbonate filters with pore size 0.22 µm, diameter 25 mm are used to capture bacteria. Glassfiber filter (e.g. Whatman GF/C), 25 mm diam. are used as filter supports.

Two qualities of blackened filter have occurred on the market. The blacker type is required for epifluorescence microscopy.

8.2 MULTI-FILTERING UNIT

A multifilter-unit with 12 filter sets for 25 mm diameter filters is recommended. Filter funnels may be made of stainless steel or glass.

8.3 SUPPLIES

Plastic syringe: 5 cm^3, Plastipak, Becon and Dickonson. Pincett, Millipore, Glass slides, Menzel-Gläser, 76 x 26 mm. Cover slip, Menzel-Gläser, 24 x 60 mm, #0, Sample bottles: Glass, 50 cm^3. Gloves: Pwder free vinyl gloves.

8.4 STERILE FILTER

0.2 µm glassfiber filter, sterile, non-pyrogenic (e.g. Acrodisc®, GelmanScience).

8.5 MICROSCOPIC SCALE

A microscopic scale constitutes the basis for abundance estimates and size determination of bacteria in the microscope and image analysis system. One example is S8-Stage Mic., (Graticules Pyser-SIG LTD., Great Britain). A calibration certificate should be provided with the microscope scale.

8.6 MICROSCOPE

An example of a microscope system is given below. Other systems with similar capacity may be applied.

Zeiss Axiovert 100:

Ocular: 10 x/20, adapted for glasses, prod. nr. 44 40 32
Oil-objective: Planapo 63 x/1.4, oel, 160, prod. nr. 44 04 81
Colour filter set: Acridine orange: FS 09: 450-490, FT 510, LP 520
  FS15: BP546, FT580, LP590
  DAPI: FS 02; G 365, FT 395, LP 420
Ocular holder: ∞/1x

This setting gives a magnification factor of 24292 for the Camera ORCA-ER (Hamamatsu®). Zeiss counting grid (Pl10x/18 nr. 44 41 32) gives a factor for the small square 318054. The large square has the magnification factor 29695.

For manual counting and other camera systems a 100x objective (e.g. Zeiss Neofluar 100 x/1.30, oel, 160/-, Ph 3, prod. nr. 46 18 21-9903) may also be used. Determine the magnification factor for each system.

8.7 SONICATOR

A sonicator disrupts aggregates based on high frequency sound. A typical tip size is 5 mm diameter. The sonicator should be able to generate the given power.

9. CHEMICALS AND SOLUTIONS

Acridine orange

30 mg acridin orange (Merck®, best. nr. 1.14281.0010) is dissolved in 10 cm^3 Milli-Q water. The solution is filtered through a 0.2 µm sterile filter (Acrodisc®) directly into the filter funnel. Working solution is stored at 4 °C in the dark for at most 8 weeks.

Always use gloves when handling acridine orange, as it’s a carcinogen. When working with its powder form use a mouth protection. Working solution of acridine orange should be disposed of according to local directives.

Formaldehyde

37 % formaldehyde is filtered through a 0.2 µm sterile filter (Acrodisc®). Use gloves and eye protection. A ventilated hood preferably with evacuation downwards should be used, when formaldehyde vapour is heavy.

Immersions oil

Cargille non-drying Immersion oil for microscopy., Type A, formula code 1248. Cat. No. 16482 (R.P. Cargille laboratories, Inc. Cedar Grove, N.J. 007009, USA). The oil may be stored at 4 – 40 °C. It does not contain solvents or polychlorinated biphenyl (PCB) compounds.

Triton® X-100

The final concentration in samples to be sonicated is 0.001 % (v/v).

Natriumpyrophosphate

Tetra-$Na_2P_2O_7$*10 H_2O, is diluted in Milli-Q water. The final concentration in a sample to be sonicated is 10 mM.

Suspension of fluorescent beads

Fluorescent latex beads (Duke Scientific, Polymere Microspheres™ Green Fluorescing, 1% solids, 1,4*10^{11} beads cm^{-3}, diameter 0.519 µm, CV < 5 %, Cat. No. G500) are used to calibrate the image analysis system. The average volume of the bead is 0.073 $µm^3$(0.063-0.085 $µm^3$). The small bacterial cells cover only 5 pixels, and larger deviation may occur in reality.

Bead standards are prepared by filtering beads at an expected concentration of 1x10^9 beads dm^{-3}. Make two standards that are given unique codes. Store in standards the refrigerator at 4 °C. Standards prepared from the same solution should have ±CV of 21 % (n=4) for cell numbers and 15 % (n=4) for cell volume. The same standard has been applied for many years without clear changes in abundance or size of beads.

10. REFERENCES

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11. APPENDICES

11.1 CALCULATION OF PARAMETERS

Primary data from the image analysis or manual microscopy may be transformed to desired parameters by calculation in LabDatabase (calculation module to LabMicrobe, Bioras®) or Microsoft Excel. Calculation of cell concentration, carbon density per cell and carbon biomass is in the latter case done in same sheet as primary data. In a second sheet data for each image is aggregated to give average values per samples by a pivot table. Processed data are stored together with logistic data in a local data base. Report to national and international databases are done according to directives from ICES.

11.2 SAMPLE VOLUME

The tables below give volumes of sample in millilitre that by experience gives desired cell density per microscopic field on the filters as a function of month. Upper row show depth in meters

Bothnian Bay															**Öre estuary**						
Month/m	**0**	**1**	**4**	**8**	**14**	**15**	**20**	**40**	**60**	**80**	**90**	**100**	**120**		**Month/m**	**0**	**1**	**4**	**8**	**14**	**20**
Jan	5	5	5	5	5	5	5	5	7	7	7	7	7		Jan	5	5	5	5	5	5
Feb	5	5	5	5	5	5	5	5	7	7	7	7	7		Feb	5	5	5	5	5	5
Mar	5	5	5	5	5	5	5	5	7	7	7	7	7		Mar	5	5	5	5	5	5
Apr	5	5	5	5	5	5	5	5	5	5	5	5	5		Apr	5	5	5	5	5	5
May	5	5	5	5	5	5	5	5	5	5	5	5	5		May	4	4	4	4	4	4

Jun	5	5	5	5	5	5	5	5	5	5	5	5	5		Jun	4	4	4	4	4	4
Jul	4	4	4	4	4	4	4	4	4	4	4	4	4		Jul	3	3	3	3	3	3
Aug	4	4	4	4	4	4	4	4	4	4	4	4	4		Aug	4	4	4	4	4	4
Sep	4	4	4	4	4	4	4	5	5	5	5	5	5		Sep	4	4	4	4	4	4
Oct	4	4	4	4	4	4	4	6	6	6	6	6	6		Oct	4	4	4	4	4	4
Nov	5	5	5	5	5	5	5	6	6	6	6	6	6		Nov	4	4	4	4	4	4
Dec	5	5	5	5	5	5	5	6	6	6	6	6	6		Dec	5	5	5	5	5	5

Botnian Sea													
Month/m	**0**	**1**	**4**	**8**	**14**	**15**	**20**	**40**	**60**	**80**	**90**	**100**	**200**
Jan	5	5	5	5	5	5	5	5	7	7	7	7	7
Feb	5	5	5	5	5	5	5	5	7	7	7	7	7
May	5	5	5	5	5	5	5	5	7	7	7	7	7
Apr	5	5	5	5	5	5	5	5	5	5	5	5	5
May	5	5	5	5	5	5	5	5	5	5	5	5	5
Jun	5	5	5	5	5	5	5	5	5	5	5	5	5

Jul	4	4	4	4	4	4	4	4	4	4	4	4	4
Aug	4	4	4	4	4	4	4	4	4	4	4	4	4
Sep	4	4	4	4	4	4	4	5	5	5	5	5	5
Oct	4	4	4	4	4	4	4	6	6	6	6	6	6
Nov	5	5	5	5	5	5	5	6	6	6	6	6	6
Dec	5	5	5	5	5	5	5	6	6	6	6	6	6

11.3 Import format

The table shows the structure for bacterioplankton concentration data to be imported to the local database.

Station	CruiseCode	SampDateTime	Depth	BactCon	BactConq	BaSDCon	BaNPic	BaCounCel	BaVol	BaVolq	BaSDVol	PrepDateTime
Sampling station	Cruise code (YYYYKnn)	Date and time for sampling (yyyy-mm-dd_hh:mm)	Sampling depth (meter)	Bacteria conc. in (celler dm^{-3})	Qualit code	Standard deviation for bacterial conc. (celler dm^{-3})	Number of analysed images per filter	Total number of cells counted	Bacterial volume in µm^{3}	Quality code for volume	Standard deviation for volume (µm^{3})	Time and date of filtration (yyyy-mm-dd_hh:mm)

Corresponding data are archived for Milli-Q backgrounds and beads. A column for Analysis date-time and type of particle should then be added. Preparation date-time connect samples with the corresponding sample. Analysis date-time connect samples and Milli-Q backgrounds with a standard estimate.

11.4 EXAMPLE OF IMAGE ANALYSIS

In the following section an example of a procedure and settings for image analysis of microscope slides with aquatic bacteria is presented. The protocol has been applied for routine monitoring of bacterioplankton at Umeå Marine Sciences Centre for 5 years.

The system employs a digital charged coupe device (CCD) camera and specifically developed software for image analysis of bacterioplankton. The software is developed in a LabView® environment and applies a neural network technology to recognize bacterial cells among other particles in the sample (LabMicrobe™, Manufacturer: DiMedia®). This presentation may aid tuning of also other image analysis systems.

11.4.1 CAPTURE OF IMAGES

A Check that the correct equipment is installed and settings applied on the microscope, as it may affect the magnification. Record the burning-time for the mercury lamp via the counter - max. 200 hours). The computer screen should be set at "highest 32 bits" and a resolution of 1280x1024, 60Hz.

B Start the main current on the camera control unit. Start the software *Wasabi* . The window "ORCA Control" should appear. Check that the settings match item 8.6 and 8.7.

C Run a standard slide with fluorescent microspheres of known abundance and size according to item 9.4. Compare analysis results and check that they stay within 3 standard deviations of the average. Typical values for a standard are 1.6×10^9 cells dm^{-3} (SD ± 0.13×10^9) and 0.093 μm^3 (SD ±0.0096).

D In the "ORCA control" menu typical start values are 200 ms for fluorescent beads and 350 ms for sea water samples. "Gain" and "Offset" should be 0.

Image analysis is done on all samples and a background slide. Add one drop of immersion oil on the cover slip. Use first filter set 09 (Zeiss) according to item 8.5. Focus the bacteria with the oculars. Close the light passage to the oculars and open it to the camera. Filter set 15 may be used if better contrast and lower background is achieved.

E Choose "Live Image" in the "Image" menu. A window with the live video image of the sample should appear. Fine tune the focus based o the live image.

G Open the "LUT" function measuring the level of exposure of the image. The exposure meter should be yellow and cover more than 50% of the full range. Red bar mean over exposure. Adjust the exposure if required to meet the criterion above.

A check of particle intensity can be made by the procedure "Analysis: Line". The intensity of the strongest particle should not exceed 2500 units. The background should be even and about 300 units. Other settings of the LUT function need not to be changed.

H When you are satisfied with the image click "Image:Aquire". Label the file with station<space>cruise number (NN)<space>depth with fullstop and one decimal according to the format "A13 04 6.0m". Use space between all categories, but not between 6.0 and m. In the LabMicrobe module "Analyze" station, cruise and depth will be distributed into columns. Image number is added by *Wasabi*automatically. Morphology type is added by "Analyze". Do not use a hyphen. Extra information of 61512 bytes is always stored with the image (always 1344 × 1024 pixel, 16 bits signed).

I estimates of sea water sample require 5 images to be taken, distributed over a representative area of the filter. Exit *Wasabi* when images have been stored.

11.4.2 ANALYSIS OF IMAGES

A Open the program “LabMicrobe” and window “BatchMicrobe”. Choose the appropriate log file. No file extension should be added.

Press the right arrow in the menu bar. A new navigation window should appear. Select the desired folder by entering it and press ”Select current directory”. Make the corresponding choice for the result file. Binary images (less memory requirement) do not need to be saved, and loose information of dividing cells.

Check that the settings of the log file for BatchMicrobe are correct when required (item 8.7). This is done in LabMicrobe by opening the log in question by “Operate:Datalogging:Retrieve”. Choose log number and press “OK”.

B The image analysis is starting automatically when the target folder is chosen. All files in the defined source folder will be processed (0.1 s per image). The image analysis system chooses and characterizes particles according to the settings in the log file. The selection done by the neural network is based on training sets of natural brackish water bacteria used for the development of the software 1995. See Blackburn et al. 1998 for further specification.

11.4.3 PROCESSING OF PRIMARY DATA

A Images analyzed by BatchMicrobe requires further processing. Start the program “Biovolume”. Start extraction of biovolume data by clicking the arrow button in the upper left corner. Select folder where the result files from the BatchMicrobe analysis are stored. One file per image should be present. Select name for the result file and its folder. The biovolume extraction will start immediately. If required the result files can be open in a calculation program like Excel and file names adjusted.

B Bacterial abundance and biovolume of the whole community is aggregated (median value) from primary data with the module ”Analyze”. Use values for field data according to item 11.4. Choose settings for largest and smallest cell volume by the cells provided. Start the analysis with the arrow button in the upper left corner. Give name to the source file and result file.

All files created under item 11.4.3B can should then be compiled in an calculation software like Excel to give average values per volume for all morphology types in the community. Calculations according to item 5 should be used. Results are reported according to item 5.2. Also the Milli-Q background is analyzed in the same way and subtracted from the sample values. Note that Milli-Q values should be divided by the same volume as the corresponding sample to be correct.

11.4.4 IMAGE ANALYSIS INSTRUMENTATION AND SETTINGS

CCD camera: C4742-80-12AG (synonymous with ORCA ER, Hamamatsu®) with control unit may be used. A C-screw thread connects the camera with the microscope. The camera can be cooled to -30°C.

Computer: Personal computer, Pentium processor 2.66 GHz , 1 Gbite RAM memory, TEAC.

Control unit for the CCD camera

Control of the CCD camera is done by the software Wasabi in the window “ORCA control”. The following settings had worked well for routine use of acridine orange stained water samples:

Buttons

Focus mode: Off, **Light mode**: Off, **Auto exposure**: Off

Camera

The exposure time is chosen so that the coloured bar in the LUT-window is light green to yellow, and cover at least 70% of the range. The exposure is then optimal relative the dynamic range of the camera. The exposure time is not expected to vary extensively between samples. Auto exposure may be used to make a first adjustment of the exposure time. Typical values achieved are:

Exposure time: 350 ms for sea water samples, 200 ms for fluorescent beads
Gain: 0
Offset: 0

Auto exposure (AE)

Sensitivity: 0.0% Set minimal frequency for light strength used for calculation of exposure time.

Hysteresis: 5.0% Lock light strength according to *Sensitivity* in relationship to maximum possible value.

Format

Set the size of the shown image. Large image size should be used.

Superpixel: 1x1
Subarray: Large

Trigger

Camera timing: Intern

Temperature

Control the working temperature of the camera.

”Temperature” show the actual temperature of the camera.

Cooling: ON **Auto**: *Unclear*

Target temperature: 20°C

Software

Image capture: WASABI 1.4.0.4 control software for the camera unit.

Image analysis: LabView 3.1, Image analysis library. Concept Vi (LabView macro) with copy protection, LabMicrobe 1.0 och BatchMicrobe 1.0 by Nicholas Blackburn, DiMedia®.

Settings for WASABI

Images are saved with “Aquire” in WASABI. Correct settings are done with “Aquire” with preview.

Aquisition area: Top: 0, Left: 0, Width: 1344, Height: 1024.
Resolution: Full
Bits per pixel: 16BPP
Average: 1
Clip negative pixel to zero: unmarked, **Aquire to new window**: Unmarked, **Keep live image**: Marked.

A captured image is stored as a .TIFF-fil (6.0) with 16 bits per pixel with no compressin. **Strip size**: 10. Typical size is 2 755 532 bytes for an image file (see under ”Properties” for the file).

11.4.5 SETTINGS FOR LABMICROBE

Standard setting for LabMicrobe is given below. They are entered in LabMicrobe and saved as a log-file. Call the correct log-file when performing an analysis.

Only **Pixel size (calibration), Neural network** parameters, **Classifier tolerance**, **Threshold values** and **File options** in LabMicrobe’s log files are used by BatchMicrobe. “Threshold parameter’s” is not used when the flip for ”Parameter threshold” is off.

At delivery of LabMicrobe the neural network is trained to select bacterial morphology types (Short rod (=cocci), Long rod and C-shaped). The standard cell sets are those used during the development of the software. See Blackburn et al. 1998 for details.

The LabMicrobe window

The following settings should be used for the camera and microscope systems presented above:

Status window: Gives current activity

Save binary image: Save an image with less information and memory requirement. Not routinely used when counting bacteria.

Threshold data: Min. 2 Max. 10000

Palette: Binary

Data params: Area (calibrated)

Calibration: 0,0986 (rounded to 0,10 by the software. Entered value is used)

Processing: Show the image file under processing. End at final image file when the image set is done.

Particle: 1

Classifier tolerance: High.

All particles, **Plot species, Select species** and **Identified particles** change depending on image analysed.

Parameter threshold: Set to desired interval during manual analysis. Determine what particles that are shown with a large symbol in the diagram. Does not affect the analysis with done with BatchMicobe. The handle should typically be down (off).

”Other options->” (extend the window to the right).

Read raw file 1 **Read Raw file**
16 bits signed 5 **File data type**
Offset to data=3020. The offset value may be required to be adapted to a specific image type. TIFF format is complex and save also other information with the image. The off set value is determined as the total file size in bytes subtracted with the pixel area times 2.

Offset = 2 755 532 -(1344x1024x2)=3020

Don´t use min max 0 **Use Min max**
0,00 **Optional min. value**
0,00 **Optional max. value**
Little Endian (Intel) 1 **Byte Order**
Width=1344, **Height**=1024.
Mark local maxima: Down
Network: 0; 0,0; 5,13
Size independency: Down
Labels: 0,?

Reconstruct and collect window

Number of frequencies: 4
Other: 4
Plot particles: 1
Continue: Green

Parameters window

CVI Parameters: Area (calibrated) and Number of holes labelled black.

User Parameters: Particle# and Hemisphrod labelled

Morphology window

Minimum particle size: 1

See also the manual of the image analysis software provided be DiMedia®.

Batch Microbe window

Save binary Image: Off (=down).

LabMicrobe log number: 0

Mark local maxima: Down

Data file extension: (blank)

Analyze window

Min and max set the limits for the biovolume range that is included in the analysis.

Min. size: 0.01
Max. size: 0.40
# bins (mode): 10.

Last updated 10.4.2008 (appendices added)

PART D. PROGRAMME FOR MONITORING OF CONTAMINANTS AND THEIR EFFECTS

D.1. Objectives and goals for contaminants monitoring

D.2. Selected compartments
  D.2.1 Biological compartments
  D.2.2 Sea water
  D.2.3. Sampling sites

D.3. Selected contaminants
  D.3.1. Open sea
  D.3.2. Coastal zone (EC MON 1/96, Annex 7)

D.4. Supporting studies

D.5. Selection and number of specimens

D.6. Field sampling and storage (for mussels also sample preparation for analysis)

D.7. Sample preparation for chemical analysis

D.8. Statistical comments

D.9. Spatial distribution studies

D.10. Establishing of national Specimen banking programmes

D.11. Biological effects monitoring

D.12. General strategy for the study of new contaminants

D.13. Sampling programme as committed by the Contracting Parties

References

D.1. OBJECTIVES AND GOALS FOR CONTAMINANTS MONITORING

More specifically the aims of COMBINE mean:

For contaminants:

To compare the level of contaminants in selected species of biota (including different parts of their tissues) from different geographical regions of the Baltic Sea in order to detect possible contamination patterns, including areas of special concern (or ´hot spots´).

To measure levels of contaminants in selected species of biota at specific locations over time in order to detect whether levels are changing in response to the changes in inputs of contaminants to the Baltic Sea.

To measure levels of contaminants in selected species of biota at different locations within the Baltic Sea, particularly in areas of special concern, in order to assess whether the levels pose a threat to these species and/or to higher trophic levels, including marine mammals and seabirds.

Into the aims of this monitoring programme the assessment of quality of seafood with regard to the human consumption is not included. That is the responsibility of appropriate national authorities.

Measurements of contaminants in Sea Water are an important tool to detect trends in space and time in a nonbiotic matrix. The data provide a basis to understand the bioaccumulation pattern of contaminants and to establish mass balances or contaminants.

The contaminant monitoring programme can never achieve the full extent of the geographical resolution so the all parts of the Baltic environment is covered. This mainly because the cost of the analytical programme but also because of zoogeographical reasons. Still there is a need to cover the main subregions with comparable results but because of above mentioned reasons the sampling network has to be sparsely distributed. The core variables within the contaminant programme are thus variables that are studied over the entire area and provide the best available comparable information on time trends as well as spatial distribution.

D.2. SELECTED COMPARTMENTS

D.2.1. BIOLOGICAL COMPARTMENTS

The following criteria are *taken into account for* the selection of monitoring species:

* the species should *be of reasonable size to* allow analyses of individuals
* the species *collected for core studies* should have broad distribution in the Baltic Sea
* the species should represent the sampling site *or defined areas*
* biological effects studies should be possible
* good knowledge of the ecology, physiology etc. should be available
* the species should have good accessibility.

Selected species: Open Sea

Herring (*Clupea harengus)*
Cod (*Gadus morhua)*
Guillemot (*Uria algae)*

Selected species: Coastal zone, to be decided

Blue mussel (*Mytilus edulis)*
Bladder wrack (*Fucus vesiculosus)*
Macoma balthica
Saduria entomon
Flounder (*Platichtys flesus)*
Perch (*Perca fluviatilis)*
Viviparous blenny (*Zoarces viviparus)*
Common tern (*Sterna hirundo)*
Grey Seal (*Halichoerus grypus)*

Ringed seal (*Pusa hispida)*
Common seal (*Phoca vitulina)*
White tailed sea eagle (*Haliaetus albicilla)*

Table D.1 shows an overview of sampling requirements for contaminants.

Selected tissues

All details concerning tissue selection with regard to the various analytes are presented in Table D.2. For the analysis of lipid-soluble compounds the concentration shall be reported both on lipid weight basis as well as fresh tissue basis. For metals concentrations shall be reported both on fresh weight basis and dry weight basis.

- Pooled samples of the growth of the year of algae are analysed for contaminants.
- In invertebrates pooled homogenised samples of soft tissues are analysed.
- In fish chemical analysis on individuals are carried out on muscle and liver tissues.
- Bird eggs are analysed on an individual basis as homogenised egg content.

Table D.1. Overview of sampling requirements for contaminants

Species (n)	Location	Depth (m)	Time	Age, year (N or S)	Size cm	Sex
Herring (12-15)	open sea	n.s.	Aug - Sept	N/2+,3+	n.s.	female
Cod (12-15)	open sea	n.s.	Aug - Sept	S/1+,2+	24-35	female
Macoma baltica 80 g	open sea	n.s.	Sept	n.s.	>0.5	n.s.
Saduria ent. 80 g	open sea	n.s.	Oct - Nov	n.s.	4-6	n.s. (***)
Uria aalge (10) eggs	islands		1-15 May	n.s.	n.s.	n.s.
Flounder (10-15)	coast (**)	< 20 m	Aug -	2+	n.s.	female

			Sept			
Viviparous blenny (10-15)	coast (**)	< 20 m	Nov - Dec	n.s.	17-30	males (*)
Perch (10-15)	coast	n.s.	Aug - Sept	n.s.	15-20	females
Sterna hir. (10) eggs	coast		June - July		n.s.	n.s.
Mytilus 80 g	coast	N/2-5 S/<15	Oct - Nov	n.s.	3-4	n.s.

* Males collected for chemical analysis before spawning (Aug), females collected in October for studies on reproductive outcome and fry development
** Samples shall be collected away from river mouth
*** Egg-carrying females avoided

S Southern Baltic
N Northern Baltic
n.s. not specified

D.2.2. SEA WATER

Contaminants occur in the dissolved phase as well as associated with suspended particulate matter. Since both fractions are taken up by biota in a specific manner, it is recommended to analyse dissolved and particulate contaminants separately. Surface sea water should be analysed since it is directly affected by input of contaminants via rivers and atmospheric deposition.

D.2.3. SAMPLING SITES

Sampling sites for fish, biota, bird eggs and water for contaminant analysis are shown in Figures D.1, D.2 and D.3 and Tables D.2, D.3 and D.4. In Figures D.1 and D.2 selected species at the various sampling sites are shown and in Table D.2 responsible countries are indicated. For the coastal areas a selection of sites covering hot spots as well as reference areas needs to be elaborated. The reference area system should be internationalized as far as possible, and be available for comparison for all Contracting Parties. The establishment of Baltic Sea Protected Areas (BSPA) implies a need for monitoring in these areas and they should be considered in the Coastal Monitoring Programme. Thus for the coastal areas the programmes are still under development. However, in some countries there are already programmes running.

TABLE D.2.Information on variables and matrices committed to be measured in the contaminants programme by the Contracting Parties (Danish monitoring programme included in the coastal programme)

OPEN SEA

Species	Matrix	Variable	DK	EE	FI	DE	LV	LT	PL	RU	SE
Core programme											
Herring	liver	Cu, Cd, Pb, Zn		+	+	+		+	+		+
	muscle	Hg;		+	+	+		+	+		+
		DDTs;		+	+	+		+	+		+
		CBs (IUPAC Nos.28, 52, 101,118,138, 153 and 180);		+	+	+		+	+		+
		HCB; alpha + gamma HCH		+	+	+		+	+		+
Main programme											
Cod	liver	Cu, Cd, Pb, Zn;		+	+	+		+	+		+
		DDTs;		+	+	+		+	+		+
		CBs (IUPAC Nos.28, 52, 101,118,138, 153 and 180);		+	+	+		+	+		+
		HCB; alpha + gamma				+		+			

		HCH									
	muscle	Hg		+	+	+		+	+		+
Macoma baltica	homogenized soft tissue	Hg, Pb, Cd, Cu;		+			+(metals)	+			
		DDTs;		+				+			
		CBs(IUPAC Nos. 28, 52, 101, 118, 138, 153 and 180);		+				+			
		alpha + gamma HCH		+				+			
Saduria entomon	homogenized whole organism	Hg, Pb, Cd, Cu;		+							
Guillemot	egg content	Hg, Pb, Cd, Cu;									+
		DDTs;									+
		CBs(IUPAC Nos. 28, 52, 101, 118, 138, 153 and 180);									+
		HCB; alpha + gamma HCH									+
Sea water	dissolved phase	Cu, Cd, Pb, Zn				+		+			
	particulate matter	Cu, Cd, Pb, Zn				+					

	total water	Hg, DDTs;				+		(+)			
		CBs(IUPAC Nos.28, 52, 101, 118, 138, 153 and 180); HCB; alpha-,beta-, gamma-HCH, PAHs				+		(+)			
Supporting programme											
Herring	different age classes	Hg, Pb, Cd, Cu;			+						
		DDTs;			+						
		CBs (IUPAC Nos. 28, 52, 101, 118, 138, 153 and 180);			+						
		HCB; alpha + gamma HCH;			+						
Blue mussel	homogenized soft tissue	DDT, DDE, DDD, CBs, HCH		+							
Sea water		tot. oil hydrocarbons (fluorom.)		+	+	+	+	+			
Herring;		brominated flame retardants									+
guillemot eggs											

[COASTAL ZONE] [will be updated according to the information from the CMP]

Species	**Matrix**	**Variable**	**DK**	**EE**	**FI**	**DE**	**LV**	**LT**	**PL**	**RU**	**SE**
Core progrramme											
Mytilus	homogenized soft tissue	Hg, Zn, Cu, Cd, Pb;	+		+	+		+	+		+
	homogenized soft tissue	DDTs;	+	(+)	+	+		+	+		+
		CBs(IUPAC Nos.28, 52, 101, 118, 138, 153 and 180)	+	(+)	+	+		+	+		+
		HCB; alpha +gamma HCH;	+	(+)	+	+		+	+		+
Viviparous blenny	liver	Cu, Cd, Pb, Zn	+			+	+		+		+
	muscle	Hg;	+			+	+		+		+
		DDTs;	+			+			+		+
		CBs(IUPAC Nos.28, 52, 101, 118, 138, 153 and 180);	+			+			+		+
		HCB; alpha +	+			+			+		+

		gamma HCH;									
Perch	liver	Cu, Cd, Pb, Zn				+	+		+		+
	muscle	Hg;			+	+	+		+		+
		DDTs;			+	+			+		+
		CBs(IUPAC Nos.28, 52, 101,118, 138, 153 and 180);			+	+			+		+
		HCB; alpha + gamma HCH;			+	+			+		+
Main programme											
Flounder	muscle	Hg	+					+			
	liver	Zn, Pb, Cd, Cu	+					+			
Supporting programme											
Seals	blubber	Hg, Pb, Cd, Cu;			+						+
		DDTs;			+						+
		CBs(IUPAC Nos. 28, 52, 101, 118, 138, 153 and			+						+

		180);									
		HCB; alpha + gamma HCH;			+						+
		biomarker studies on			+						+
		population			+						+
Mytilus	homogenized soft tissue	TBT	+								
	homogenized soft tissue	PAH	+	+							
Fucus	growth of the year	Cu, Cd, Hg, Pb, Zn		+							
Macoma	homogenized soft tissue	total oil hydrocarbons		+							
Mytilus		(fluorom.)		+							

[COASTAL ZONE] [will be updated according to the information from the CMP]

Biological effect monitoring for supporting programme

Species	**Variable**	**DK**	**EE**	**FI**	**DE**	**LV**	**LT**	**PL**	**RU**	**SE**
Perch	physiology, population parameters, reproduction, biomarkers			+	+			(+)		+

Viviparous blenny	physiology, biomarkers							+		+
	reproduction, population parameters							+		+
Fish community	population parameters			+				+		+
Seals	population dynamics, reproduction			+				+		+
White-tailed eagle	population dynamics, reproduction			+						+
Whelk Buccinum	imposex	+								

TABLE D.3. Areas for collecting biota for contaminant analysis

Species	Stat. Rect.
Herring	60/H2
	57/H2
	51/H0
	50/G8
	49/H6
	48/H6

	48/H4
	48/H3
	45/H3
	46/G7
	39/G8
	38/G6
	40/G5
	38/G4
	43/G1
Cod	57/H2
	51/H0
	49/H6
	48/H6
	48/H4
	48/H3
	45/H3

	42/G8
	39/G8
	38/G6
	38/G4
	43/G1
Viviparous blenny	57/H1
	43/H3
	44/G7
	38/G8
	38/G2
	40/G2
Perch	57/H1
	43/H3
	44/G7
Flounder	38/G2
	40/G2

	39/G0
Mytilus edulis	48/H4
	45/H4
	43/G1
Macoma baltica	43/H3
	48/H4
	45/H4
Saduria entomon	48/H4
Guillemot eggs	43/G7

TABLE D.4. Stations for collecting sea water for contaminant analysis

(At the stations with BMP designation, samples are taken for depth profiles, at the stations without BMP designation, only two depths above the halocline are sampled.)

Heavy metals			
BMP designation	**National designation**	**Latitude**	**Longitude**
	225005	54E42,90'	10E08,00'

N3	225006	54E36,00'	10E27,00'
	225059	54E27,55'	10E14,70'
	225057	54E06,10'	11E10,50'
M2	225058 / O12	54E18,90'	11E33,00'
M1	46	54E28,00'	12E13,00'
K8	30	54E43,40'	12E47,00'
K7	69	55E00,00'	13E18,00'
K5	113	54E55,50'	13E30,00'
K4	109	55E00,00'	14E05,00'
K2	213	55E15,00'	15E59,00'
	222	55E13,00'	17E04,00'
	256	55E19,60'	18E15,10'
K1	259	55E33,00'	18E24,00'
	253	55E50,40'	18E52,00'
	250	56 05,00'	19E10,00'
	263	56E20,80'	19E22,70'

	260	56E38,00'	19E35,00'
	272	57E04,30'	19E49,80'
J1	271	57E19,20'	20E03,00'
	WB3	53E57,00'	11E24,50'
	UW4	54E10,00'	12E06,00'
	KHM	53E49,50'	14E06,00'
	OB4	54E00,40'	14E14,00'

Organic contaminants			
BMP designation	**National designation**	**Latitude**	**Longitude**
	701	54E50,00'	09E30,00'
	704	54E50,00'	09E54,00'
N3	706	54E36,10'	10E27,00'
	708	54E33,00'	10E12,00'
	710	54E25,10'	10E13,30'
	715	54E03,00'	10E50,90'

	FB	54E36,00'	11E09,00'
N1	717	54E30,50'	11E25,00'
M2	718 / O12	54E18,90'	11E33,00'
M1	719 / O46	54E29,10'	12E17,00'
K8	30	54E43,40'	12E47,00'
K7	69	55E00,00'	13E18,00'
K5	113	54E55,50'	13E30,00'
K4	109	55E00,00'	14E05,00'
K2	213	55E15,00'	15E59,00'
	222	55E13,00'	17E04,00
	256	55E19,60'	18E15,10'
K1	259	55E33,00'	18E24,00'
	253	55E50,40'	18E52,00'
	250	56E05,00'	19E10,00'
	263	56E20,80'	19E22,70'
	260	56E38,00'	19E35,00'

	272	57E04,30'	19E49,80'
J1	271	57E19,20'	20E03,00'

D.3. SELECTED CONTAMINANTS

D.3.1. OPEN SEA

Core variables, herring:

- mercury, copper, cadmium, lead, *zinc*
- DDT and metabolites,
- CBs (Nos. 28, 52, 101, 118, 138, 153, and 180),
- hexachlorobenzene (HCB), and
- alpha- and gamma-hexachlorocyclohaxane (HCH)

The core programme for contaminants is given in Table D.2.

Main variables, cod, guillemot eggs:

- mercury, copper, cadmium, lead, zinc
- DDT and metabolites,
- CBs (Nos. 28, 52, 101, 118, 138, 153, and 180),
- hexachlorobenzene (HCB), and
- alpha- and gamma-hexachlorocyclohaxane (HCH)

Main variables, sea water:

Concentration in suspended particulate matter

- copper, cadmium, lead, zinc

Figures not avaiable yet.

FIGURE D.1. Sampling sites for fish

FIGURE D.2. Sampling sites for biota

FIGURE D.3. Sampling sites for water

Concentration in the dissolved phase

- copper, cadmium, lead, zinc

Total concentration

- mercury
- DDT and metabolites,
- CBs (Nos. 28, 52, 101, 118, 138, 153, and 180),
- hexachlorobenzene (HCB), and
- PAH
- alpha-, beta-, and gamma-hexachlorocyclohaxane (HCH)

D.3.2. COASTAL ZONE (EC MON 1/96, ANNEX 7)

This programme is still under development and the recommendations here are only tentative.

Contaminants in Mytilus, Macoma, perch viviparous blenny, eggs of common tern, guillemot and seal tissue are:

- mercury, copper, cadmium, lead, zinc,
- DDT and its metabolites,
- CBs (Nos. 28, 52, 101, 118, 138, 153, and 180),
- hexachlorobenzene (HCB),
- alpha- and gamma-hexachlorocyclohexane (HCH)

Contaminants in flounder, Danish coastal programme

- mercury, copper, cadmium, lead, zinc,
- DDT and its metabolites,
- CBs (Nos. 28, 52, 101, 118, 138, 153, and 180),
- hexachlorobenzene (HCB),
- alpha- and gamma-hexachlorocyclohexane (HCH)

The compilation of the contaminants to be analysed in the BMP and in the CMP is contained in Table D.2.

D.4. SUPPORTING STUDIES:

- determination of contaminants in herring of different age classes to follow the accumulation
- brominated flame retardants in selected species
- studies aiming to give information on effects of contaminants on Baltic top predators
- toxaphene in selected species of fish and invertebrates, guillemot eggs, seal tissues. Toxaphene is transported via the atmosphere and has a well-documented toxicity
- dioxins and furans are very toxic and among the most serious risk substances in the Baltic. Monitoring is, however, very expensive and there are few laboratories carrying out these determinations on a routine basis. The national time series already started need to be continued, as this is the only information available on these substances
- planar CBs are recommended by ICES not to be included in the monitoring programmes, but should be the subject of research programmes
- baseline study of TBT in biota, sediment and water
- determination of petroleum hydrocarbons (UVF) in sea water as well as biota.

D.5. SELECTION AND NUMBER OF SPECIMENS

Detailed information on sample size, age and size of collected specimens, sex and sampling time is given in Table D.1. At the analysis of fish samples females shall be used. Exception to this is viviparous blenny where males are used for chemical analysis and females for determination of biomarker parameters.

Experience from the BMP shows that individual data in long-term monitoring increase the possibilities of detecting both temporal and spatial variations. This explains the criteria that have been taken into account for the selection of species. In addition analysis of individual specimens will allow studies of the relationships between different contaminants. This will also provide the possibility of calculating ratios between contaminants and allow correlations to be made with biological variables such as length, body condition, etc.

Herring

Core variables for contaminant monitoring in biota of the Baltic Sea is herring. A narrow definition of sampling population is required. Young herring would more likely represent the sampling area compared to old ones showing a migration behaviour. Since individual analysis shall be performed, herring large enough to allow analysis of organic contaminants instead of individual fish should be selected. In practise, 1+-3+ year (north-south) old herring should be selected, depending on the geographical location of the sampling site. Sampling of spring spawning herring should be carried out in autumn to avoid changes of the physiology during spawning. The maturity of the gonads should be recorded and the same degree of maturity should ideally be sampled every year. The number of specimens could be reduced from the present recommendation (20) to at least 12 specimen. However, when new sampling localities are established where the within-year variation should be investigated

20 specimens are recommended. Depending on the rather low within-year variation, this would in general not cause any significant loss in the reliability of the average values. In order to reduce the influence of small-scale spatial and temporal variations on the inter-annual variation, it might be beneficial to collect samples several times during the sampling .

Flounder

At studies of flounder, age is a selection parameter. For flounder analysed specimens shall be 2+ years. 10-15 specimens shall be collected at each sampling site.

Cod

Cod can only be found on an regular basis southern and central Baltic Proper. Thus sampling and analysis for monitoring purposes shall only be carried out in these areas. For cod length-stratified sampling may be maintained where it has been successfully applied in the past, and then 25+/- 10% specimens shall be analysed. For new time series, however , it may be more appropriate to sample and analyse individually at least 12 fish of a limited size range (24-35 cm) from each sampling site in order to minimize natural variability within the sample.

Perch, viviparous blenny

To obtain samples from homogenous populations, a short length interval shall be used at the collection of these two species. For perch the length interval is 15-20 cm and for blenny 17-30 cm. 10-15 specimens shall be collected at each sampling site.

Invertebrates

Because of the variation in salinity within the Baltic no general advice can be given as to size of the invertebrates. However, in Kattegat 20 specimens of Mytilus at a size of 3-6 centimetres shall be used. This imply a possibility to compare obtained data with data recorded within OSPARCOM (OSPARCOM, 1996). For the rest of the study area it is important to keep to the same size at the specific sampling site between years and most probably the number of specimens in the pools has to be increased in certain areas to obtain a sample big enough to allow chemical analysis. For all invertebrates a pooled sample of 80 g is needed to allow the chemical analysis.

Bird eggs

Guillemot eggs shall be collected in the early part of the reproduction period to avoid analysis of replacement eggs. This will minimize the within year variation in contaminant concentrations of the collected samples. Ten eggs shall be collected per sampling site.

Below are listed the biological measurements that shall be taken on the various matrices.

- Age: fish
- Total weight: fish, mytilus, bird eggs
- Total length: fish, Mytilus. Macoma, bird eggs
- Total width: bird eggs
- Liver weight: fish

- Gonad maturity: fish
- Sex: fish

D.6. FIELD SAMPLING AND STORAGE (FOR MUSSELS ALSO SAMPLE PREPARATION FOR ANALYSIS)

Fish samples can be dissected for chemical analysis immediately after they have been caught. However, of practical reasons this can often be difficult and the laboratory conditions at the locality of collection might imply a risk for contamination during the preparation or available personal are untrained or lack sufficient practical experience. It is then more convenient to deep freeze the fish specimens before transport to a laboratory with adequate conditions. If the samples are deep frozen before transport, the outstretched specimens shall be put individually in polyethylene plastic bags and the bags labelled individually. The samples shall be kept frozen during the transport. If the specimens are prepared for chemical analysis in field recommendations given in Part D.7 have to be followed.

For bivalves, it is best to carry out the initial postsampling procedures on board the vessel to avoid a two-step procedure of freezing and re-freezing (which causes variable water losses). Thus, it is recommended that a person skilled in these procedures collect the bivalves and carry out the initial procedures as soon as possible thereafter.

When the organisms have been collected, they should be rinsed externally in clean water from the area of collection to wash away sediments and other foreign matter. They should then be allowed to remain in clean sea water from the area of collection for 12-24 hours to allow them to remove sediments and other foreign matter as pseudofaeces. The specimens should be kept alive at a temperature similar to that observed at the sampling site (preferably in a refrigerator). The storage tank should preferably be of glass.

When this time is over, the total length of each organism should be measured and the information recorded.

After draining off the shell liquor, the whole soft body of the organism including the adductor muscle should be carefully removed from the shell and combined with the others to be included in the sample. Care should be taken to avoid excessive tissue damage and thus cause water loss during this procedure.

Eggs of birds shall be transported to the laboratory for preparation immediately for further preparation. Egg measurements (weight, length and width) shall be done before opening of the egg with drilling a hole at the equatore. The egg content shall be blown out with a glass pipette (the egg shall not contain an embryo) and brought to the freezer, where it shall be kept until final preparation for chemical analysis. The empty eggshell shall be stored and dried in room temperature for future measurements of eggshell parameters. Homogenized egg content shall be analysed.

Storage of specimens or individual tissue samples as well as pooled samples

Material of single specimens shall be stored in polyethylene plastic bags.

Material from single tissue samples or pooled tissue samples shall be stored in the following way:

- Samples for trace element analyses can be stored in precleaned polyethylene, polypropylene, polystyrene or glass containers.
- Samples for analysis of organic contaminants should be stored in precleaned glass containers.

Tissues can deteriorate in a rather short time span at room temperature. Consequently, samples should be frozen as soon as possible after packaging. They can be frozen rapidly by immersion in liquid nitrogen or blast freezing, but both these techniques need care. Whatever system is used, freezing a large bulk of closely packed material must be avoided. The samples in the centre will take longer to cool and will therefore deteriorate more than those in the outer layer.

Once frozen, samples can be stored in a deep freezer at temperatures of -20 °C or below. The laboratories should validate their storage procedures. Each sample should be carefully labelled. The label should contain at least the sample's identification number, the type of tissue, and the date and location of sampling.

Mussel samples shall always be prepared for chemical analysis without freezing. After that the soft tissue samples can be frozen.

D.7. SAMPLE PREPARATION FOR CHEMICAL ANALYSIS

General remarks

Tissue samples have to dissected while they are in good condition. Uncontrollable losses of determinands or cross-contamination from other deteriorating tissues and organs may occur, because biological tissue deteriorates. To avoid this, individual fish specimens must be dissected at sea if adequate conditions prevail on board, or be frozen immediately after the collection and transported frozen to the laboratory where they are dissected later. The dissection room should be kept clean and the air should be free from particles. If clean benches are not available on board, the dissection of fish should be carried out in the land-based laboratory under clean room conditions. If the option chosen is dissection on board the ship, two criteria must be met:

1. the work must be carried out by personnel capable of identifying and removing the desired organs according to the requirements of the investigations, and
2. there must be no risk of contamination from working surfaces or other equipment.

Equipment at preparation

Crushed pieces of glass or quartz knives, and scalpels made of stainless steel or titanium are suitable dissection instruments.

Colourless polyethylene tweezers are recommended as tools for holding tissues during the dissection of biological tissue for trace metal analysis. Stainless steel tweezers are recommended if biological tissue is dissected for analysis of organic contaminants.

After each sample has been prepared, including the samples of different organs form the same individual, the tools should be changed and cleaned.

The following procedure is recommended:

a) for analysis of inorganic contaminants:
   1) Wash in acetone or alcohol and high purity water.
   2) Wash in HNO_3 (p.a.) diluted (1+1) with high purity water. Tweezers and haemostats in diluted (1+6) acid.
   3) Rinse with high purity water.
b) for analysis of organic contaminants:
   1) Wash in acetone or alcohol and high purity water.

The glass plate used during dissection should be cleaned in the same manner. The tools must be kept dust-free between working hours.

Treatment of the specimens

Before any tissue preparation start, the individual specimens shall be weighted and the total length shall be determined (outstretched specimens)

Muscle tissue preparation

For analysis of fish muscle, the epidermis and subcutaneous tissue should be carefully removed from the fish. Samples should be taken under the red muscle layer. In order to ensure uniformity of samples, the right side dorso-lateral muscle should be taken as the sample. If possible, the entire right dorsal lateral filet should be used as a uniform sample, from which subsamples can be taken after homogenizing for replicate dry weight and contaminant determinations. If the fish is small both entire filets shall be used. If, however, the amount of material so obtained would be too large a sample, a specific portion of the dorsal musculature should be chosen for the sample. It is recommended that the portion of the muscle lying directly under the first dorsal fin be utilized in this case. As both fat and water content vary significantly in the muscle tissue from the anterior to the caudal muscle of the fish, it is important to obtain the same portion of the muscle tissue for each sample. This is necessary in order to ensure comparability (Oehlenschläger, 1994).

Muscle tissue samples collected from frozen fish specimens shall always be prepared from samples that are half frozen. Preparation of tawned tissue will imply a risk of body liquid losses and shall be avoided as much as possible. Determination of sex and preparation of liver tissue shall also, as much as possible, be done when the fish specimen is only half tawned.

Liver tissue preparation

To sample liver tissue, the liver must be identified in the presence of other organs such as the digestive system or gonads. The appearance of the gonads will vary according to the sex and the season and the status shall be recorded (sex, maturity). After opening the body cavity with a scalpel, the connective tissue around the liver should be cut away and as much as possible of the liver is cut out in a single piece together with the gall bladder. The bile duct is then carefully clamped and the gall bladder dissected away from the liver.

Either the fish have been frozen before preparation (and the preparation is done on half frozen tissue) or sampling preparation of liver is done on unfrozen fish samples, the entire liver shall be weighted and after that brought to the freezer before further preparation for analysis. This is particularly important at preparation of the fatty cod liver. Tawned cod liver tissue will loose fat at the preparation because of squeezing. Any loss of body liquid or fat at the preparation before analysis will make the determination of fat and dry weight incorrect.

Bivalves and bird egg

Preparation procedure presented under the Chapter "Field sampling and storage (for mussels also sample preparation for analysis)".

Reference

Oehlenschläger, J. 1994. Quality assurance during sampling onboard. *In* ICES/HELCOM Workshop on Quality Assurance of Chemical Analytical Procedures for the Baltic Monitoring Programme. Ed. By G. Topping and U. Harms. Balt. Sea Environ. Proc. No. 58: 82-84.

D.8. STATISTICAL COMMENTS

Based on an assessment carried out by the Statistics and Data Treatment group of EC BETA the detection of a 5% annual change over a time period of 15-20 years is required in temporal trend monitoring programmes. This stress the need of long term studies when changes are not expected to be dramatic. The existing BMP monitoring has a rather long history, and the time period already covered by this series provides an excellent platform for future trend studies.

The Statistics and Data Treatment group of EC BETA recommend that the annual samples at individual sites can be reduced from the present 20 - 25 specimens to 10-15. The OSPARCOM ad hoc Working Group on Monitoring (MON) recommend at least 12 specimens per site and year. This is why 12 specimens at each sampling site and year have been recommended for the open sea programme. The advice by ICES (1995) is that based on the fact that calculations have been conducted on only a very limited data set and that there is a need for better estimates of the variance components associated with time series data, no definitive advice on optimal sample size for temporal trend monitoring programmes can be provided at the present time.

A power analysis was conducted by German scientists on basis of the German time series for Cd and Cu in surface sea water of the Baltic proper. It could be shown that 10-20 samples taken at a fixed season are sufficient to detect a 10% trend within 10 years with a power of >0.9.

D.9. SPATIAL DISTRIBUTION STUDIES

New studies are under way to develop statistical aspects of geographical distributions of contaminants. Information on statistical aspects of spatial distribution will be included at a later stage when available. The present programme of the core variable herring will provide us a possibility to make a spatial distribution study of the contaminants investigated in the Baltic area. In a similar way the main variables cod and blue mussel will give us a possibility to compare the contamination burden as well as a comparison of the ecological risk for the species of concern in the North Sea area and the Baltic.

D.10. ESTABLISHING OF NATIONAL SPECIMEN BANKING PROGRAMMES

There is often need for material from periods in the past to assist retrospective studies. A recognition of "new" contaminants and the ability to carry out retrospective studies to rapidly obtain information on trends has to be stressed. Also, there is often a need of re-analyse earlier collected samples in long time series to confirm the reliability of previously performed analyses. The establishment of specimen banking programmes provides the possibility of meeting these two needs.

D.11. BIOLOGICAL EFFECTS MONITORING

More specifically the aims of COMBINE mean:

For the effects of contaminants:

"To carry out biological effects measurements at selected locations in the Baltic Sea, particularly at sites of special concern, in order to assess whether the levels of contaminants in sea water and/or suspended particulate matter and/or sediments and/or in the organisms themselves are causing detrimental effects on biota (e.g., changes in community structure)."

The objectives of the biological effect monitoring programme is to study the relationships between concentrations and effects.

In the Baltic Sea area, studies related to the effects of contaminants in biota have often been performed as monitoring of changes at population or community level. However, to reach an understanding of the actual causes of these changes, knowledge on health parameters is essential. Although in an operative use in various sea areas, the effect studies have been sporadic in the Baltic Area.

Biological effects monitoring should integrate measurements from the level of effects of contaminant concentrations at the tissue level up to effects at the population level. It shall also cover different levels in the food web as well as different time scales in manifestations of the effects of exposure (acute and chronic responses). All studies should include simultaneous measurements of the levels of relevant contaminants in the study organism and relevant environmental matrix. The species chosen so far for the chemical analysis programme have, to a large extent, been selected on the basis of experiences from pilot studies.

Recommended studies

With regard to biological effects monitoring and being aware of the existing projects among the Contracting Parties to the Helsinki Convention, ICES was invited to advise on methods for determining effects primarily on reproduction, immunology and metabolism of marine organisms. In addition, the recommendations of OSPARCOM on the parameters used should be taken into considerations to harmonize the programmes and to make use of the expertise relevant for Baltic species and the Baltic environment. It is important that monitoring data, whether they are contaminant or effect data, are produced with high enough quality. QA is an important requirement to ensure a consistently high data quality.

Methods

Since the applicability of several of the contamination-related biomarkers in current use (e.g. EROD induction, histopathology) has not been adequately investigated in most Baltic Sea organisms that are potentially useful as monitoring species, studies providing information on this are of high value.

AchE (acetylcholinesterase) inhibitors such as organophosphate and carbamate pesticides may be substances of concern in some highly contaminated areas of marine environment (as pointed out by ICES). At present, however, the importance of these pesticides as marine pollutants is not known. Studies focusing of the applicability of AchE activity inhibition in different group of organisms should be encouraged.

In addition, the development of chronic sediment and water bioassays are considered useful for studies in heavily contaminated areas.

Organisms

Bivalves (*Macoma balthica*, *Mytilus edulis*) are regarded as the most useful indicators of the degree of regional contamination and in this view recommendable for the studies of biological effects.

Coastal fish (e.g. *Perca fluviatilis*, *Zoarces viviparus*) are good candidates for biological effects monitoring. Physiological monitoring has been carried out since 1989 on national level.

Top predators (e.g. seabirds, seals) are very sensitive indicators of contaminant influence. Continuation of national studies on pathology and population size of the three Baltic seal species is considered important.

D.12. GENERAL STRATEGY FOR THE STUDY OF NEW CONTAMINANTS

A general strategy for new contaminants should include the conduct of a baseline study in which biota or other media should be sampled from both reference sites and expected hot spots, providing a first indication of the maximum range of spatial variation within the Baltic Sea area.

D.13. SAMPLING PROGRAMME AS COMMITTED BY THE CONTRACTING PARTIES

The sampling programme as committed by the Contracting Parties is summarized in Table D.2.

Denmark

The Danish monitoring programme is under revision. The sampling strategy of the new suggested preliminary programme (not yet approved by the appropriate authorities) is based on five different groups of harmful substances taking into account the known sources. These five groups of substances are:

1) Persistent, toxic, bioaccumulating substances (half life of 2-10 years)

- hexachlorobenzene (HCB), DDT, hexachlorocyclohexane (HCH) (i.a. chlordane, dieldrin), PCBs,
- heavy metals Hg, Cd, Pb, Cu and Zn,
- PAH and organotin

2) Substances via waste water

- p-nonylphenols (+ethoxylates), phthalates (DEHP), Linear Alkyl Sulphonates (LAS) (detergents), PAH, certain metals (Be, Li, Ag, Sb, Tl) and organotin compounds

3) Substances from different diffuse sources, mainly agriculture

- pesticides (atrazine, simazine), organotin compounds

4) Anti-fouling agents

- Tributyltin, Cu-compounds, Irgarol

5) New problem substances (other sources)

- brominated flame retardants, tris(4-chlorophenyl)methanol and tris(4-chlorophenyl)methane, planar CBs, toxaphene

Group 1 substances will be analysed in biota from 5-7 sampling sites once per year. Only coastal areas will be monitored. Biological effects monitoring (imposex in whelk, Buccinum) from about 5-7 stations once per year. For substances in group 2-5, preliminary surveys of existing concentrations will be made before any new monitoring programme is started covering these substances.

Estonia

The Estonian programme includes:

- monitoring of fish (herring and if available cod): (Cu, Cd, Pb, Hg, Zn) and organochlorines (DDTs, PCB,"-,$- and (-HCH) once per year at 3 locations
- monitoring of invertebrate and algae (*Macoma*, *Saduria*, *Fucus*): Cu, Cd, Pb, Hg, Zn once per year at 3 locations
- monitoring of total oil hydrocarbons in sea water (fluorometric analysis) twice a year

Finland

The Finnish programme includes:

- monitoring of total oil hydrocarbons in sea water (fluorometric analysis) annually from the representative stations (6 stations)
- monitoring of biota (herring and if available, cod): heavy metals (Cu, Cd, Pb, Hg and Zn) and organochlorines (DDTs, CBs, HCB, "-,$- and (-HCH) at 4 locations

- in addition contaminants for different age classes of herring will be analysed annually from one sampling site
- the selected organisms at the coastal area are Macoma, Mytilus, Saduria, perch, pike and herring; and they are collected from altogether seven areas at every or every third year. Analyses: Cd, Cu, Hg, Pb, Zn, DDT compounds, CBs, HCHs and HCB

Germany

The German programme includes:

A. for open sea monitoring:

- monitoring of biota (herring and cod): heavy metals (Cu, Cd, Pb, Hg and Zn) and organic contaminants, DDTs, CBs, "-,$- and (-HCH, HCB from one sampling site once per year (area west of Bornholm),
- monitoring of sea water:

Basic programme:

- heavy metals (Cu, Cd, Pb, Zn and Hg) and organic contaminants (9 CB congeners, DDTs,"-,$- and (-HCH, HCB, PAHs (15 compounds)) once a year at 10 stations along the line J1, K1 and K2, and at M2, M1, K8, K5, K7 and K4
- Additionally: heavy metals (basic programme) at N3 and M2, twice a year;
- organic contaminants (basic programme + 30 petroleum hydrocarbons) at stations N3, N1, M2, M1 and FB, once a year.

B. for coastal monitoring:

monitoring of biota:

- perch: heavy metals Cd, Hg, Pb, Cu, Zn and organic contaminants HCB, HCHs and PCBs at three sampling sites twice a year
- Mytilus: heavy metals Cd, Hg, Pb, Cu, Zn, Cr, Ni, As and organic contaminants HCB, HCHs, DDTs and PCBs at four stations once a year and at two stations twice a year. Additionally at one station twice a year heavy metals, pesticides, PCBs and PAHs using different methods
- Viviparous blenny: heavy metals, pesticides, PCBs and PAHs using different methods at one station once a year
- Herring Gull eggs: heavy metals, pesticides and PCBs using different methods at one station once every second year

monitoring of seawater:

- heavy metals: Cu, Cd, Pb, Zn, Ni and Hg at four stations 8 to 12 times a year AND Cu, Cd, Pb, Zn and Hg at two stations once a year and at one station more than 15 times a year

- organic contaminants: 9 CB congeners, DDTs, "-,$- and (-HCH, HCB, PAHs (15 compounds) and 30 petroleum hydrocarbons at 5 stations once a year

C. coastal zone biological effect monitoring

perch: population parameters from three sampling sites twice a year

Latvia

The Latvian programme includes:

The Gulf of Riga

sea water:

- total oil hydrocarbons (fluorometric, determination); 7 stations sampled 4 times per year (February, May, August, November)

fish:

- Viviparous blenny, liver, metals - Zn, Cu, Cd, Pb (muscle Hg); 2 stations, once in August
- Perch, liver, metals - Zn, Cu, Cd, Pb (muscle Hg); 2 stations, once in August

molluscs:

- Macoma baltica, metals - Zn, Cu, Cd, Pb (muscle Hg); 2 stations, once in August

sediments:

- metals - Zn, Cu, Cd, Pb, (Hg); 9 stations, once in August

Lithuania

The Lithuanian programme includes:

In water:

- Hg - 3 stations;
- Cu, Cd, Pb, Zn - 3 stations;
- Organochlorines - 3 stations;

In biota:

- Hg - 1 station once per year;
- Cu, Cd, Zn, Pb - 1 station, once per year;
- Organochlorines - 5 stations, 1 time per year.

Poland

The Polish programme includes:

In biota:

- heavy metals (Hg, Cd, Pb, Cu, Zn) in herring muscle and cod muscle and liver, perch (muscle and liver), viviparous blenny (muscle and liver) and Mytilus edulis; from 6 sites
- DDTs, CBs; -HCH, HCB in herring muscle, cod muscle and liver, perch (muscle and liver), viviparous blenny (muscle and liver) and Mytilus edulis; once per year from 6 sampling sites

In sediment:

- metals (Cu, Zn, Cd, Hg, Pb) and organic toxicants once in five years from 9 sites

Russia

No information available.

Sweden

The Swedish programme for contaminant monitoring includes:

- Selected organisms in coastal areas: perch, viviparous blenny, blue mussels, all from two locations.
- Selected organisms in open sea: herring (5 sites), cod (2 sites) and guillemot egg (one site).
- Contaminants studied: Cd, Cu, Hg, Pb, Zn, DDT compounds, CBs, HCHs, HCB in perch, viviparous blenny, blue mussel, herring, cod and guillemot egg. Dioxins and planar CBs in herring (3 sites) and brominated compounds in guillemot (1 site).

Study area, Bothnian Bay, Bothnian Sea, the Baltic Proper, the Kattegatt. Samples collected in autumn but two time series started in the beginning of 1970s are collected in spring as well as the guillemot egg. All sampling sites are located in areas locally unaffected from local pollution.

The contaminant monitoring programme is integrated with the ecological and physiological fish monitoring programmes.

The Swedish programme for studies on biological effects of eutrophication and toxic substances monitoring includes:

- ecological coastal fish monitoring twice a year (July-August and October) in the Gulf of Bothnia and the Baltic Proper (minimum number of stations: 6 for gill nets and maximally 18 for fyke nets). Variables: stock analysis (species composition, catch per unit effort, age composition) and individual analysis (growth, gonad weight, fecundity, condition factor, external indication of diseases
- physiological coastal fish monitoring once a year (summer) in the Gulf of Bothnia and the Baltic Proper. Samples of stationary fish collected in one coastal area. Variables: gonadosomatic index, liver somatic index, hematocrit value, leucocyte count, plasma ions, cytochrome P-450, EROD activity, blood lactate and tissue glycogen.

Monitoring of population status of top predators (white tailed sea eagle, ringed, common and grey seals) are incorporated in the Swedish Marine Monitoring Programme. Reproduction as well as population size is followed by annual countings.

References

ICES, 1995. Report of the ICES Advisory Committee on the Marine Environment, 1995. ICES Cooperative Research Report No. 212.

OSPARCOM, 1996. Summary record of the ad hoc Working Group on Monitoring (MON), MON 96/9/1, Annex 5.

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