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Chemical measurements in the Baltic Sea: Guidelines on quality assurance

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Abstract

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-6 (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 the various annexes and, where applicable, their associated appendices, will, however, depend on the specific job description of each analyst.

It is the intention of the guidelines that other members of the *laboratory staff* can find use for specific parts of the guidelines, e.g., Annex B-5 (Sampling), which contains principles in relation to sampling procedures and documentation.

These guidelines have been prepared by the ICES/HELCOM Steering Group on Quality Assurance of Chemical Measurements in the Baltic Sea (SGQAC)[1] for use in association with the HELCOM Cooperative Monitoring in the Baltic Marine Environment (COMBINE) Programme, and the former Baltic Monitoring Programme. These QA guidelines have been

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[1] This document benefited from the contribution of several members of SGQAC, principally: Horst Gaul, Michael Gluschke, Uwe Harms, Sigfried Kreuger, Mikael Krysell, Elzbieta Lysiak-Pastuszak, Jill Merry, Ana-Liisa Pikkorainen, Christa Pohl, Elisabeth Sahlsten, Norbert Theobald, Peter Woitke, Jerzy Woron.

incorporated in the COMBINE Manual as Part B. Accordingly, in that manual, the section numbers of the main body of the guidelines have been prefixed by the letter B, which has not been carried over into this publication of the guidelines. The designation of the annexes with the letter B has, however, been used here.

Keywords: Quality assurance, quality audit, chemical measurement, sampling, sampling preservation, sample pre-treatment, calibration, blank, control chart, reference material, measurement uncertainty, seawater temperature, salinity, nutrients, nitrate, phosphate, silicate, dissolved oxygen, oxygen saturation, trace metals, organochlorine pesticides, polychlorinated biphenyls, PCBs, DDT, polycyclic aromatic hydrocarbons, PAHs, mussels, fish muscle, fish liver.

1 INTRODUCTION

1.1 Need for Quality Assurance of Chemical Analytical Procedures in Marine Monitoring

It has been seen that, although there has been considerable improvement in analytical chemistry over the past two decades, there was a large number of European laboratories which still had difficulties in providing reliable data in routine work (Topping, 1992). 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 past twenty years by the International Council for the Exploration of the Sea (ICES), and which have shown that there are large interlaboratory differences in the measurements of contaminants in marine samples.

As a consequence of improperly applied measures to assure the quality of analytical data, information about variations of contaminant 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, 1994a) 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, 1994b)

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.

1.2 Objective

The objective of the guidelines outlined here is to support laboratories working in marine monitoring to produce analytical data of the required quality. The guidelines may also help to establish or improve quality assurance management in the laboratories concerned. The technical part of the guidelines provides advice on more practical matters. The guidelines will not focus on sampling in detail, since this will be dealt with at a later stage.

1.3 Topics of Quality Assurance

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

a) A knowledge of the purpose of the investigation is essential to establish the required data quality.

b) Provision and optimization of appropriate laboratory facilities and analytical equipment.

c) Selection and training of staff for the analytical task in question.

d) Establishment of definitive directions for appropriate collection, preservation, storage, and transport procedures to maintain the integrity of samples prior to analysis.

e) Use of suitable pre-treatment procedures prior to the analysis of samples, to prevent uncontrolled contamination and loss of the determinand in the samples.

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

g) 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.

h) Participation in interlaboratory quality assessments (proficiency testing schemes) to provide an independent assessment of the laboratory's capability of producing reliable measurements.

i) 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.

2 THE QUALITY SYSTEM

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 that 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).

General requirements applicable to all types of chemical measurements (all types of objective testing) are specified in the European Standard "General Criteria for the Operation of Testing

Laboratories" (EN 45001), and in the "General Requirements for the Technical Competence of Calibration and Testing Laboratories" (ISO Guide 25).

Guidance on the interpretation of 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 a CEN Working Group (CEN/TC 230, 1995). Both 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.

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.

2.3 Organization, Management, and Staff

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:

1) Technical Manager, Quality Manager, and any deputies;
2) general lines of responsibility within the laboratory (including the relationship between management, technical operations, quality control, and support services);
3) the lines of responsibility within individual sections of the laboratory;
4) 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.

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 the 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.

2.3.3 Staff

The laboratory management should define the minimum levels of qualification and experience necessary for the 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.

2.4 Documentation

Necessary documentation in connection with analysis includes:

1) a clear description of the analytical methods;
2) a strict keeping of laboratory journals;
3) instrument journals;
4) laboratory protocols for sample identification;

5) 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.

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.

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 laboratory to ensure against bias of results.

General service equipment (e.g., sample containers, hot plates, stirrers, non-volumetric glassware) 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 volumetric equipment, analytical balances, thermometers, barometers, etc., is critical to analytical measurements and this equipment must be maintained, calibrated, and used in a manner consistent with the accuracy required of data. In certain situations, analysts 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.

2.7 Quality Audit

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.

The quality audit is the periodic check that a laboratory makes on its own Quality System to guarantee that it is effective, implemented, and adhered to. It is recommended to use an external assessor on a regular basis. Arrangements for implementing an audit may be based upon a check list developed by the EURACHEM/WELAC Working Group (EURACHEM/WELAC, 1992), which is attached as Annex B-3 to these Guidelines.

3 SPECIFYING ANALYTICAL REQUIREMENTS

3.1 General

The objective of analytical investigations is to obtain chemical information about materials or systems concerning their specific qualitative and quantitative composition and structure (Grasshoff, 1976; Danzer, 1992).

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

3.2 Determinand of Interest

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

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 pre-treatment and analysis, if sufficient knowledge about the basic properties of the sample is available. There may also be other, non-analytical factors to consider, including the nature of the area under investigation.

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 determinand. 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.

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.

4 VALIDATION OF ANALYTICAL METHODS

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.

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 are given.

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 determinand is regarded as specific.

Few analytical methods are completely specific for a particular determinand. This is because both the determinand 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 the determinand and interfering substances.

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

1) 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).

2) 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.

4.2.2 Sensitivity

Sensitivity is the difference in determinand 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) determinand concentration. Such a curve is obtained by plotting the instrumental response, y, versus the determinand 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 the samples to be analysed and the standards used to establish the calibration curve.

4.2.3 Detection limit

The detection limit of an analytical method is the smallest concentration (the smallest amount) that the analyst can expect to detect with a given degree of confidence.

The IUPAC (1978) has recommended that the limit of detection, defined in terms of either concentration (c_L) or amount (q_L), be related to the smallest measure of response (x_L) that can be detected with reasonable certainty in a given analytical method.

According to this definition, the detection limit is given by

$$c_L(\text{or } q_L) = k \cdot S_b/b,$$

where S_b = standard deviation of the blank (see Section 4.2.4) and b = sensitivity (the slope of the standard curve).

A value of $k = 3$ is strongly recommended by IUPAC (based on the confidence interval).

This concept is further clarified by Long and Winefordner (1983) and the ACS Committee on Environmental Improvements (ACS, 1983). The Analytical Methods Committee (1987) stressed that the estimation of the limit of detection must be based on measurements of a "field blank" (effectively a hypothetical sample containing zero determinand concentration). This implies that the matrices of the samples and the corresponding field blank are identical so that unique interference effects for individual samples can be excluded.

4.2.4 Range

The range of the method is defined by the smallest and the largest determinand 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 determinand 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/determinand concentration relationship is no longer linear.

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 determinand occur prior to analysis. Effective sample stabilization methods exist for many determinands 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 determinand

Many determinands 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 determinand concentration. Generally, calibration means the establishment of a function by mathematically modelling the relationship between the concentrations of a determinand 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 determinand 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 determinand, 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.

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 $\sqrt{(s_w^2 + s_b^2)}$. 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, when s_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 is given in the following paragraphs.

General scheme of Analysis of Variance (after Doerffel, 1989):

Source of variability	Sums of squares	Degrees of freedom	Mean squares (variances)	Variance components
Between batches	$QS_1 = \sum n_j(x_j - x)^2$	$f_1 = m - 1$	$s_{bm}^2 = QS_1/m - 1$	$s_{bm}^2 = s_w^2 + n_j s_b^2$
Within batches	$QS_2 = \sum\sum(x_{ij} - x_j)^2$	$f_2 = m(n_j - 1)$	$s_w^2 = QS_2/m(n_j - 1)$	
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 an 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.

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 a 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 CRMs, 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 determinand 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 determinand 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 determinand concentration x reported by the participant and the determinand 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).

5 ROUTINE QUALITY CONTROL (WITHIN-LABORATORY QUALITY CONTROL)

5.1 General

The objective of a quality assurance programme for chemical measurements is to reduce analytical errors to required limits and to assure that the results have a high probability of being of acceptable quality.

Having developed an analytical system suitable for producing analytical results of the required accuracy, it is of eminent importance to establish a continuous control over the system and to show that all causes of errors remain the same in routine analyses (i.e., that the results are meaningful). In other words, continuous quantitative experimental evidence must be provided in order to demonstrate that the stated performance characteristics of the method chosen remain constant.

According to international standards (ISO 9000, EN 29000, and EN 45000 series), 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.

5.2 X-charts

In marine chemistry, the X-chart is applicable for stable samples, e.g., certified reference materials for trace elements, nutrients and organics, stabilized biota for trace elements, or laboratory preparations of synthetic quality control materials, such as nutrient or trace metal solutions with a stable and preferably known theoretical content.

A simple X-chart is constructed in the following way:

1) Select an appropriate laboratory reference material (LRM) which, if possible, has been checked against a relevant certified reference material (CRM). This LRM is to be analysed later on a regular basis with environmental samples. See also Annex B-6.

2) Analyse the LRM at least ten times for the given determinand. The analyses should be done on different days spread over a period of time to ensure that the full range of random errors (for within- and between-batch analyses) is covered. This enables a calculation of the total standard deviation (s_t).

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

If the data for the LRM follow a Normal distribution, 95% of them should fall within $x \pm 2s_t$ (between the Upper Warning Limit and Lower Warning Limit) and 99.7% should fall within $x \pm 3s_t$ (between the Upper Control Limit (UCL) and Lower Control Limit (LCL)).

The X-chart as described here is relevant for checking that the chemical data produced are within statistical control for precision, expressed as the total standard deviation. The X-chart data can be used for periodic calculations of this statistical parameter.

5.3 Control Charts for Spiked Sample Recovery

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 analyses and in nutrient analyses 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:

1) Use the same spike concentration in all series of the same determinand, concentration range, and matrix.

2) Select and analyse a natural sample in each analytical series.

3) Spike by adding to the sample a known concentration of the analyte to be determined, and re-analyse. If possible, use a CRM concentrate.

4) Calculate the measured difference in concentration by subtraction and correction for dilution from spiking.

5) Plot the spike concentration in a chart of concentration versus time.

6) The average recovery (R) and the total standard deviation (s_t) can be calculated 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. The chart is relevant for checking that the chemical data produced are within statistical control for recovery of spikes and for precision, expressed as the total standard deviation. The chart data can be used for periodic calculations of these statistical parameters.

5.4 Cusum Charts

From the Cusum plots, the relative recovery and relative standard deviation may be calculated. The advantage of a Cusum chart is the possibility of combined control of two concentration levels, low and high, in one plot. This is useful when the concentration range of the samples varies from batch to batch, where the selection of reference samples with different concentrations is relevant. Typical samples that can be applied are CRMs, natural laboratory reference materials that have been checked against a CRM, or synthetic laboratory reference materials with known theoretical concentrations.

A Cusum chart enables the control of analyses done on a routine basis or sporadically. In this case, the control samples are prepared on the day of analysis. Control samples, containing constant or variable concentrations of a measured compound, are analysed in sequence with environmental samples. The number of control samples depends on the assumed level of quality assurance in the laboratory, e.g., 5% means one control sample per twenty environmental samples. The "cusum" (cumulative summation of differences—positive and negative—between the result of the analysis of the reference material/control sample (x_i) and the expected/true value (r_i)) value is calculated from the equation:

$$\text{cusum} = \sum_{i=1}^{n} \frac{x_i - r_i}{r_i} \times 100\%$$

where i = number of control samples.

The results of analyses are presented in the form of a table and a chart.

5.5 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).

5.6 Interpretation of Control Charts

The results of the analyses of the reference material analysed with each batch of environmental samples indicate whether the errors fall within acceptable limits. The results are satisfactory if they fall within the warning limits, i.e., between $\pm 2s_t$. If one result falls outside the warning limits, there is no reason for alarm, providing that the next result falls within the warning limits. The results of control analyses should not fall between the warning and control limits ($+2s_t/+3s_t$ and $-2s_t/-3s_t$) more frequently than once in twenty determinations. If the results fall outside the warning limits too frequently, particularly if the same warning limit has been crossed more than once on consecutive results, then the analyst needs to assess the source of this systematic error. If the results on more than ten successive occasions fall on the same side of the x line (above or below), then the analyst must check the analytical procedure to determine the source of this systematic error. If a result falls outside the UCL or LCL limits, the analyst should stop the analysis, and then check the analytical procedure to determine the source of error. The lines $+3s_t$ and $-3s_t$ are regarded as the permissible limits; the results should not cross these limits more often than once in 100 analyses. If any of the limits is crossed, the results of the analysis of this particular batch of environmental samples should be rejected. The analyst should not continue until the source(s) of the errors have been identified and the analysis is again under control. Excepted from this are analyses that cannot be deterred or delayed (e.g., due to lack of an appropriate preservation method for ammonia), where the analyst can choose to perform the analyses with a suboptimal quality. In this situation, data should not be reported unless a valid retrospective correction of data can be performed.

Control charts are ideal for daily routine analyses. When the batches of analyses are done at different times, e.g., on cruises, the analyst should recommence the analysis of environmental samples only when the procedure is still under control, i.e., replicate analyses of an LRM must be done before the routine work recommences. Only when the results fall within the acceptable limits on the previously constructed control chart can the routine work be restarted.

It has to be noted that the accuracy of the method can be checked with a CRM or a well-characterized LRM. But if the analyst only uses reference materials without documented accuracy, he/she is controlling only the precision of the measurements.

6 EXTERNAL QUALITY ASSESSMENT

For marine environmental monitoring programmes, it is essential that the data provided by the laboratories involved are comparable. Therefore, participation in an external quality assessment scheme 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 is 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 proficiency testing schemes are based on the distribution of 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.

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).

Analytical system. 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.

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 of a measurand.

HELCOM CMP. Coastal Monitoring Programme.

HELCOM COMBINE. Cooperative Monitoring in the Baltic Marine Environment.

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.

Quality Assurance. The term 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 previously been organized by the International Council for the Exploration of the Sea (ICES) and, since 1993, as Laboratory Performance Studies by QUASIMEME.

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.

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 pre-treatment 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.

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ANNEX B-1

PRINCIPAL 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 AN ESTABLISHED ANALYTICAL METHOD FOR CHEMISTRY

1 INTRODUCTION

According to ISO17025, Section 5.4.5.2 "The laboratory shall validate non-standard methods, laboratory-designed/developed methods, standard methods used outside their intended scope, and amplifications and modifications of standard methods to confirm that the methods are fit for the intended use. The validation shall be as extensive as is necessary to meet the needs of the given application or field of application. The laboratory shall record the results obtained, the procedure used for the validation, and a statement as to whether the method is fit for the intended use."

The very purpose of a method validation is to conclude whether the method is fit for the intended use. This annex describes the procedures used for the validation of methods according to the above, as well as for confirmation of the performance of standard methods.

Section 4 of these guidelines explains the various expressions used in this annex.

2 OUTLINE OF PROCEDURE

The method is based on the procedure described in EURACHEM/WELAC "Guide for method validation" (EURACHEM, 1992; CITAC/EURACHEM 2000), where a scheme for the validation of methods is described in some detail. The procedure is divided into two parts: a full validation of a non-standard or substantially modified standard method and a limited procedure used for confirmation of performance when using a standard method or an otherwise well-established method.

3 CAPABILITIES

The procedure described for a full method validation will generate information on:

- Detection limit (LOD);
- Accuracy;
- Precision (repeatability and reproducibility) over the concentration range;
- Linearity;
- Sensitivity;
- Range;
- Selectivity;
- Measurement uncertainty;
- Robustness/interference.

The procedure for a limited validation will generate information on:

- Limit of detection;
- Linearity;
- Range;
- Accuracy;
- Precision (repeatability and reproducibility) over the concentration range.

Subsequently, information on precision will be drawn from control charts once the method is in use.

4 WHEN TO USE WHICH METHOD

Full validation has to be applied when:

- A new, internally or externally developed, method is introduced in the laboratory;
- A standard method or other accepted method undergoes substantial change (e.g., change of important chemicals, automation, scaling down, etc.);
- A method is used outside its intended scope (e.g., for other matrices or other concentration intervals).

The limited validation procedure can be used for:

- Methods already in use in the laboratory (previously validated), where minor modifications have been carried out.
- Standard methods (ISO, EN or national standards).
- Standard methods that have been subject to minor modification.
- Other generally accepted methods, where information on the scope is given (e.g., methods taken directly from HELCOM or OSPAR guidelines or from Grasshoff *et al.* (1999), "Methods of Seawater Analysis").

5 FULL VALIDATION PROCEDURE

Initially, six replicate measurements of a blank and/or a sample with little or no content of the analyte are performed. The initial limit of detection is calculated at this stage (see Section 4.2.3), as it is needed in the further planning.

5.1 Plan

A short plan should be prepared. It should contain information on:

- which method is to be tested;
- which matrix;
- how many samples should be analysed (normally six at each level);
- how the samples are obtained or prepared;
- customer demands on the method (if they exist).

If possible, the plan should be based on natural samples with known concentrations, but as these are seldom available, the plan could be as shown in the diagram below:

Level	**N1**	**N2**	**N3**	**N4**	**N5**	**N6**
Number of replicates in a batch	2	2	2	2	2	2
Number of batches	6	1	6	6	6	1

The levels are as follows:

Level N1 A synthetic sample, or if possible, a natural sample at a concentration close to the initial limit of detection;

Level N2 A synthetic sample at a concentration between N1 and N3;

Level N3 A natural sample at a concentration approximately in the middle of the range (preferably a matrix reference material);

Level N4 The natural sample used for N3 with a known amount of analyte added (spiked sample);

Level N5 A synthetic sample at a concentration close to the upper end of the range;

Level N6 A synthetic sample at a concentration approximately 20% over N5.

Calibration is performed for at least one batch by duplicate measurements with a minimum of six concentrations.

If linearity is to be determined for the whole method, and if the natural samples are not reference materials and there is no knowledge of the true concentration of these samples, the experimental plan must be supplemented by synthetic samples of approximately the same concentrations as the natural samples for one of the batches.

The measurements should be made in random order as the repeatability will probably be underestimated if replicates of a sample are always analysed consecutively.

The data generated by this experimental plan are the following:

- limit of detection (Section 4.2.3);
- verification of precision close to the limit of detection (cf. Section 4.2.5);
- repeatability at four concentration levels for natural and synthetic samples (Section 4.2.5.1);
- between-batch standard deviation at four concentrations for natural and synthetic samples (Section 4.2.5.1);
- accuracy for two synthetic samples (N1 and N5) and one spiked sample (N4 minus N3) (Section 4.2.5);
- linearity of calibration (and possibly of the whole method);
- sensitivity (Section 4.2.2);
- range (Section 4.2.4).

If reference materials have been available as natural samples, the quality of the evaluation is naturally improved.

If the type and concentration of the samples used in the evaluation are well chosen, the data generated can be used as the basis for implementing internal quality control for the method, providing that the validation has resulted in the method being approved.

Frequent (semi-annual) participation in a proficiency testing scheme should take place to determine the accuracy of the method.

6 LIMITED VALIDATION PROCEDURE

Initially, six replicate measurements of a blank and/or a sample with no or little content of the analyte are performed. The initial limit of detection is calculated at this stage (see Section 4.2.3), as it is needed in the further planning.

If the approximate limit of detection is already known, use the existing value instead.

6.1 Plan

A short plan should be prepared. It should contain information on:

- which method is to be tested;
- which matrix;
- how many samples should be analysed (normally six at each level);
- how the samples are obtained or prepared;
- customer demands on the method, if they exist.

An example of an experimental plan is given below.

Level	**N1**	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**
Number of replicates	6	2	2	2	2	2	2

The levels are as follows:

Level N1 A natural sample, or if this is not possible, a synthetic sample at a concentration close to the limit of detection (e.g., standard S1);

Levels S1–S6 Six (at least) standard solutions used for preparation of the standard curve.

The information obtained from these measurements is

- limit of detection (Section 4.2.3);
- linearity of the standard curve;
- linear range (Section 4.2.4).

The procedure gives enough information for a laboratory to start analyses of unknown samples with an established or slightly modified method with reasonable safety. However, this validation only gives very limited information on accuracy and, for those methods where interference is a major problem, possible effects of interference should be monitored until sufficient knowledge is obtained.

If at all possible, a matrix reference material should be analysed in connection with the limited validation, in order to produce information on accuracy and possible matrix problems.

Information on the reproducibility (day-to-day variation) of the method will be drawn from control charts, once enough control samples (normally 20) have been analysed under reproducibility conditions. See Section 5 for further explanations.

Normally a method will not be fully authorized until the reproducibility has been checked and shown to be within specified limits (if existing).

7 REPORT

A Validation Report should be written, containing the results from the above calculations. Apart from this, the plan should be included, as well as a statement on whether the method fulfills customer/laboratory demand/intended scope or not. The report should clearly state that the values have been obtained using a limited validation, not a full method validation.

If the laboratory has a quality system that requires method authorization, a method authorization sheet/report should also be written.

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ANNEX B-3

QUALITY AUDIT

Areas of particular importance to a chemistry laboratory (drafted by the EURACHEM/WELAC WG (EURACHEM/WELAC, 1992)) are listed below.

1 STAFF

Staff are properly trained and up-to-date training records are being maintained.

Tests are only carried out by authorized analysts.

The performance of staff carrying out analyses is observed.

2 EQUIPMENT

The equipment in use is suited to its purpose.

Major instruments are correctly maintained and records of this maintenance are kept.

Equipment, e.g., balances, thermometers, glassware, time pieces, pipettes, etc., is calibrated, and the appropriate calibration certificates demonstrating traceability to national or international standards are available.

Calibrated equipment is appropriately labelled or otherwise identified.

Instrument calibration procedures are documented and records of calibrations are satisfactorily maintained.

Appropriate instructions for use of equipment are available.

Instrument performance checks show that performance is within specifications.

3 METHODS AND PROCEDURES

In-house methods are fully documented and appropriately validated.

Alterations to methods are appropriately authorized.

The most up-to-date version of the method is available to the analyst.

Analyses are following the methods specified.

4 STANDARDS AND CERTIFIED REFERENCE MATERIALS

The standards actually required for the tests are held.

The standards are certified or are the “best” available.

The preparation of working standards is documented.

Standards and reference materials are properly labelled and correctly stored.

New batches of standards are compared against old batches before use.

The correct grade of materials is being used in the tests.

Where reference materials are certified, copies of the certificate are available for inspection.

5 QUALITY CONTROL

There is an appropriate degree of calibration for each test.

Where control charts are used, performance has been maintained within acceptable criteria.

QC check samples are being tested by the defined procedures, at the required frequency, and there is an up-to-date record of the results and actions taken where results have exceeded action limits.

Results from the random re-analysis of samples show an acceptable measure of agreement with results from the original analyses.

Where appropriate, performance in proficiency testing schemes and/or interlaboratory comparisons is satisfactory and has not highlighted any problems or potential problems. Where performance has been unsatisfactory, corrective action has been taken.

6 SAMPLE MANAGEMENT

There is an effective documented system for receiving samples, identifying samples against requests for analysis, and showing progress of analysis and fate of sample.

Samples are properly labelled and stored.

7 RECORDS

Notebooks/worksheets include the date of test, analyst, analyte, sample details, test observations, all rough calculations, any relevant instrument traces, and relevant calibration data.

Notebooks/worksheets are completed in ink, mistakes are crossed out and not erased, and the records are signed by the analysts.

Where a mistake is corrected, the alteration is signed by the person making the correction.

The laboratory's procedures for checking data transfers and calculations are being complied with.

Vertical audits on random samples have not highlighted any problems (i.e., checks made on a sample, examining all procedures associated with its testing from receipt through to the issue of a report).

8 REFERENCE

EURACHEM/WELAC (Cooperation for Analytical Chemistry in Europe/Western European Legal Metrology Cooperation). 1992. Information Sheet No. 1 (Draft): Guidance on the Interpretation of the EN 45000 series of Standards and ISO Guide 25. 27 pp.

ANNEX B-4

TECHNICAL NOTE ON THE DETERMINATION OF MEASUREMENT UNCERTAINTY

1 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. The result of an analysis after careful consideration of all error sources may by chance be very close to the true value. However, the uncertainty can still be very large, simply because the analyst is very unsure of how close that result is to the true value. 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 programmes.

This technical note provides information on how the uncertainty of measurement of the analytical methods used in the COMBINE programme 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 programme. It should be taken into account that the requirements on accuracy depend on the aims and the purpose of the monitoring programme.

2 DEFINITIONS

In accordance with the current version of the International Vocabulary of Basic and General Standard Terms in Metrology (ISO, 1993), the ISO Guide (ISO, 1995) defines **Measurement Uncertainty** as a parameter, associated with the result of a measurement, that characterizes 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 result 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 $U = k \, x \, u_C$.

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%.

3 PROCEDURES TO ESTIMATE UNCERTAINTY

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

According to the original ISO approach (Horwitz, 1998), the uncertainty of analytical results is derived by listing all of the possible errors in the form of standard deviations. Using this error budget model, the combined standard uncertainty can then be calculated as the square root of the sum of squares of the individual error components. This "bottom-up" approach of ISO 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.

A "top-down" view on estimating the combined standard uncertainty through intercomparison tests was developed by the Analytical Methods Committee of the Royal Society of Chemistry of the UK (Analytical Methods Committee, 1995). Both systematic and random errors of individual laboratories become random in an intercomparison test or in a laboratory proficiency testing scheme, provided that identical and homogeneous samples are analysed. Following ISO 5725 (ISO, 1994), the within- and between-laboratory variance can be calculated and combined in the reproducibility of the intercomparison test. Only in case that the same analytical procedure is used, can the calculated reproducibility of the intercomparison test be considered as the standard uncertainty of measurement.

A further "top-down" approach is provided by the British VAM (Valid Analytical Measurement) programme (Barwick and Ellison, 2000). According to this proposal, the combined standard uncertainty is characterized as the internal pooled standard deviation calculated from method validation data or using information from internal quality assurance measures, e.g., analysis of certified reference materials.

A comprehensive description of all of these approaches can be found in a recently released EURACHEM/CITAC Guide (EURACHEM/CITAC, 2000).

3.1 The "Bottom-up" Procedure

The "bottom-up" approach to estimate the uncertainty of analytical results seems to be rather impractical (Horwitz, 1998), because it does not include the outcome from intercomparison exercises or from laboratory proficiency testing schemes in marine monitoring available today. Therefore, if information on the uncertainty of analytical data generated in the COMBINE Programme is needed, the "top-down" approach should be preferred. The procedure by which the uncertainty of measurements is calculated depends on the requirements of the monitoring programme.

3.2 The "Top-down" Model using Data from Intercomparison Exercises

As stated in the EURACHEM/CITAC Guide (EURACHEM/CITAC, 2000), the reproducibility standard deviation of intercomparison exercises which are carried out according to ISO 5725 (ISO, 1994) or Thompson and Wood (1993) can be used as the combined standard uncertainty for methods operating within their defined scope, provided that contributions of additional factors (i.e., sampling error, inhomogeneous distribution of the analyte) have been shown to be negligible. The sampling error and its inclusion in the uncertainty budget require special investigations. The same is true for the uncertainty of co-factor determinations carried out in the COMBINE Programme to support monitoring results. Attachment 1, Part 2 provides a practical example of the uncertainty estimation using data from an intercomparison exercise.

3.3 The "Top-down" Model for Individual Laboratories

In case an individual laboratory is requested to provide information on uncertainty of measurement in connection with reported data, the approach proposed by the British VAM programme (Barwick and Ellison, 2000) should be followed. The so-called internal reproducibility standard deviation can be easily derived from internal quality control charts or from special investigations carried out to determine the internal reproducibility.

This guide focuses on identifying uncertainty sources and quantifying uncertainty components, and in particular gives guidance on how uncertainty estimates can be obtained from method validation experiments. According to the EURACHEM/CITAC Guide (EURACHEM/CITAC, 2000), an uncertainty estimation based on validation studies and routine quality control requires the best available estimate of the overall bias and the precision together with a quantification of any uncertainties associated with effects incompletely accounted for in the method performance studies (e.g., matrix effects, robustness or ruggedness testing).

In that case, the combined standard uncertainty is the combination of the standard deviation or relative standard deviation containing all the possible sources of uncertainty. Attachment 2 is an example of this procedure.

4 REPORTING UNCERTAINTY

The expanded uncertainty (estimated combined standard uncertainty multiplied by the coverage factor k, usually $k = 2$ for a confidence limit of 95%) should be reported for individual monitoring parameters in the form of a standard deviation or confidence interval together with information on how it was determined.

5 REFERENCES

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ATTACHMENT 1

1 PROTOCOL FOR EVALUATION OF MEASUREMENT UNCERTAINTY FROM IN-HOUSE QUALITY CONTROL MEASUREMENTS

Estimating measurement uncertainty can be done by identifying all possible sources of uncertainty associated with a method, quantifying uncertainty components (estimating the magnitude of the uncertainty associated with each potential source), and calculating total uncertainty by combining the individual uncertainty components following appropriate mathematical rules ("bottom-up" approach, see, e.g., EURACHEM Guide (EURACHEM, 2003)).

Another approach uses data from routinely undertaken internal quality control measurements, e.g., results of the replicate analyses of certified reference materials (CRM), without identifying all potential sources of uncertainty associated with the method and quantifying uncertainty components ("top-down" approach).

This document provides guidance on how uncertainty estimates for a method can be obtained from replicate quality control measurements of a representative certified reference material. It is assumed that these measurements comprise the total analytical procedure and have been carried out with appropriate frequency and during a sufficiently long time period. In that way, it can be ensured that most relevant uncertainty components associated with the method will be covered (starting from the laboratory sample or analysis sample, excluding contributions associated with sampling and sample handling prior to analysis).

Following these assumptions, the total uncertainty of the method is composed of:

- a contribution from the precision of the method, and
- a contribution from the trueness of the method (recovery of the analyte from the CRM).

Both contributions can be easily quantified using data from routinely performed quality control measurements.

The mathematical equations 1 to 6, outlined below, can be applied for calculating measurement uncertainty on the condition that the relative uncertainty of measurement of the method expressed in percent is approximately constant within the working range.

This condition might apply in most cases as long as the lower limit of the working range is well above the limit of quantification (see Part 3, below).

This implies further that:

1) the precision expressed as relative standard deviation (RSD) is approximately constant within the working range considered. This denotes that the absolute standard deviation increases proportionally with increasing concentration of the analyte in the sample;
2) the relative uncertainty of the recovery of the analyte from the CRM $u(\overline{R}_m)$ is independent of the concentration of the analyte. This denotes that it is approximately constant, e.g., ±5% of the determined concentration.

Note: If this condition does not apply, modified mathematical equations adjusted to the specific circumstances need to be used. For details see Barwick (2000).

Then, the relative combined uncertainty, $u_c(y)$, of the method is obtained using the following equation:

$$u_c(y) = \sqrt{RSD^2 + u(\overline{R}_m)^2} \qquad \text{Eq. 1}$$

The relative standard deviation is given by:

$$RSD = \frac{s_{obs}}{\overline{C}_{obs}} \qquad \text{Eq. 2}$$

where $\overline{C}_{obs}$ is the mean of replicate analyses of the CRM and s_{obs} is the standard deviation of the results from the replicate analyses of the CRM.

The relative uncertainty of the recovery, $u(\overline{R}_m)$, is calculated using:

$$u(\overline{R}_m) = \overline{R}_m \times \sqrt{\left(\frac{s_{obs}^2}{n \times \overline{C}_{obs}^2}\right) + \left(\frac{u(C_{CRM})}{C_{CRM}}\right)^2} \qquad \text{Eq. 3}$$

where C_{CRM} is the certified concentration of the analyte in the CRM, n is the number of replicates ($n \geq 10$, see [2]) and $u(C_{CRM})$ is the standard uncertainty of the certified concentration for the CRM with a mean recovery, $\overline{R}_m$, given by:

$$\overline{R}_m = \frac{\overline{C}_{obs}}{C_{CRM}} \qquad \text{Eq. 4}$$

It is assumed that $\overline{R}_m$ does not differ significantly from 1 and, hence, no correction for recovery is made. To determine whether the recovery is significantly different from 1, a significance test is used. The test statistic t is calculated using the following equation

$$t = \left|1 - \overline{R}_m\right| / u(\overline{R}_m) \qquad \text{Eq. 5}$$

If the degrees of freedom associated with $u(\overline{R}_m)$ are known, t is compared with the two-tailed critical value, t_{crit}, for the appropriate number of degrees of freedom at 95% confidence.

If t is less than the critical value, then $\overline{R}_m$ is not significantly different from 1.

If the degrees of freedom associated with $u(\overline{R}_m)$ are unknown, for example, if there is a contribution from the uncertainty in the certified value of a reference material, t is compared with k, the coverage factor that will be used in the calculation of the expanded uncertainty (see Eq. 6) (Barwick and Ellison, 2000).

If $\left|1-\overline{R}_m\right| / u(\overline{R}_m) < k$, the recovery is not significantly different from 1.

If $\left|1-\overline{R}_m\right| / u(\overline{R}_m) > k$, the recovery is significantly different from 1 and the results are corrected for recovery. Guidance on how to proceed is given in Barwick and Ellison (2000).

To calculate the combined uncertainty, $u_c(y)$, both relative standard uncertainties RSD and $u(\overline{R}_m)$ are combined following equation 1.

The expanded uncertainty, $U(y)$, is obtained by multiplying the combined standard uncertainty, $u_c(y)$, by an appropriate coverage factor, k, (Eq. 6). For most cases, a coverage factor of 2 is recommended, which gives an interval containing approximately 95% of the distribution of values:

$$U(y) = k \times u_c(y) = 2 \times u_c(y) \qquad \text{Eq. 6}$$

The result y of an analytical measurement should be stated together with the corresponding expanded uncertainty, $U(y)$, in the following form:

(result): $x \pm U$ [units]

The stated uncertainty is an expanded uncertainty, calculated using a coverage factor of 2 This corresponds approximately to the 95% confidence interval (EURACHEM, 2003).

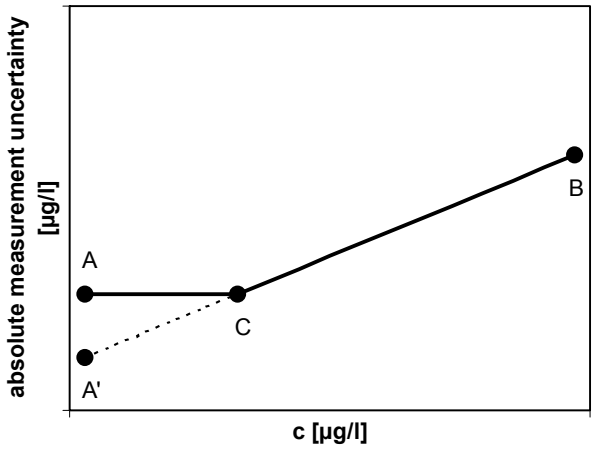
2 ESTIMATION OF MEASUREMENT UNCERTAINTY USING REPRODUCIBILITY DATA FROM INTERLABORATORY STUDIES

In principle, it is possible to use the relative reproducibility standard deviation, $CV_{R,}$ obtained in intercomparison studies as a basis for estimating the uncertainty of a method in a particular laboratory, if there is no significant difference between the relative repeatability standard deviation seen in the interlaboratory study and that observed in the laboratory. If so, this indicates that the precision achieved in the particular laboratory is similar to that obtained by the participants of the interlaboratory study (EURACHEM, 2003).

For estimating the laboratory`s expanded uncertainty, the relative reproducibility standard deviation $CV_{R,}$ obtained in the interlaboratory study is assumed to be an estimate of the combined standard uncertainty of the laboratory and multiplied with the coverage factor $k = 2$.

The uncertainty for a method, $U(y)$, obtained in that way can only be considered as a rough estimate for obtaining an idea about the order of uncertainty, but cannot replace estimating uncertainty from own measurements of, e.g., certified reference materials.

3 TRANSITION TO CONSTANT ABSOLUTE UNCERTAINTY OF MEASUREMENT AT LOW CONCENTRATIONS



A: lower limit of the working range

B: upper limit of the working range

C: threshold to be defined below that the assumption of constant uncertainty of measurement is accepted

--- shape of the function below C on the condition that relative uncertainty of measurement is constant over the whole working range

Figure B-4.1. Graphical representation of the absolute uncertainty of measurement as a function of analyte concentration.

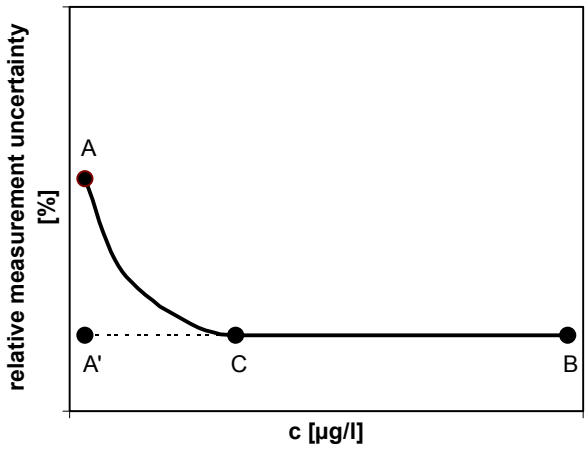


Figure B-4.2. Graphical representation of the relative uncertainty of measurement as a function of analyte concentration.

4 INTRODUCTION TO ADDITIONAL UNCERTAINTY COMPONENT IN CASE OF SIGNIFICANT DEVIATION FROM 1 OF THE RECOVERY OF THE ANALYTE FROM A CRM

If the recovery $\overline{R}_m$ of the analyte from the reference material differs significantly from 1 (*t*-test, $t \geq t_{krit}$), an additional uncertainty component is introduced.[2] Instead of Eq. 1, Eq. 7 and Eq. 8 apply.

$$u_c(y) = \sqrt{RSD^2 + u(\overline{R}_m)^2} \qquad \text{Eq. 1}$$

$$u_c(y) = \sqrt{RSD^2 + u(\overline{R}_m)^2 + \Delta^2} \qquad \text{Eq. 7}$$

$$\Delta = \overline{C}_{obs} - C_{CRM} \qquad \text{Eq. 8}$$

where Δ is the deviation of the measured concentration in the CRM from the reference value.

REFERENCES

Barwick, V.J., and Ellison, S.L.R. 2000. Development and Harmonisation of Measurement Uncertainty Principles – Part (d): Protocol for uncertainty evaluation from validation data; VAM Project (Version 5.1, Jan. 2000). http://www.vam.org.uk/publications (look under publications for the first author).

EURACHEM. 2003. EURACHEM/CITAC Guide: Quantifying Uncertainty in Analytical Measurement. www.uni-stuttgart.de/eurachem/pdf

EXAMPLE 1: ESTIMATION OF MEASUREMENT UNCERTAINTY USING THE RESULTS OF REPLICATE ANALYSES OF A CRM

During routine analyses of phosphate in seawater samples, a certified reference material was regularly analysed (30 times) as an AQC sample over a period of three months. The certified phosphate concentration in the reference material was 2.43 ± 0.41 µmol l^{-1} and assumed to be representative for the working range of the method.

According to manufacturer's specifications, the confidence interval of the phosphate concentration in the CRM was calculated using the reproducibility standard deviation obtained in the certification interlaboratory study multiplied by three. Hence, the standard uncertainty of the phosphate concentration, $u_c(PO_4)$, in the CRM is given by 0.41µmol l^{-1} / 3 = 0.14 µmol l^{-1}.

Note: Be aware that, depending on the producer of the CRM, different modes of calculation of the confidence interval of the certified concentration are in use. This must be taken into account when calculating the standard uncertainty of the certified concentration, $u_c(y)$, in the CRM.

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[2] If the recovery $\overline{R}_m$ of the analyte from the reference material differs significantly from 1, the analytical procedure is to be checked for the reason of the bias and, where applicable, the method has to be modified. But, in some cases, if the uncertainty of the certified concentration of the analyte in the CRM is extremely small, significant differences in the *t*-test can be observed even when the recovery, $\overline{R}_m$, is close to 1.

Certified concentration of the phosphate in the reference material C_{CRM} = 2.43 µmol l^{-1}

Standard uncertainty of the certified phosphate concentration $u(C_{CRM})$ = 0.14 µmol l^{-1}

From the results of the replicate analyses of the CRM, the following values can be determined directly:

Mean of replicate analyses of the CRM, $\overline{C}_{obs}$ = 2.34 µmol l^{-1}

Standard deviation of the results from the replicate analyses of the CRM, s_{obs} = 0.12 µmol l^{-1}

Then, the relative standard deviation of the mean of the phosphate concentration, RSD_{PO4}, is given by:

$$RSD = \frac{s_{obs}}{\overline{C}_{obs}} = \frac{0.12\ \ \mu mol/l}{2.34\ \ \mu mol/l} = 0.051$$

and the recovery, $\overline{R}_m$, is given by:

$$\overline{R}_m = \frac{\overline{C}_{obs}}{C_{CRM}} = \frac{2.34\ \ \mu mol/l}{2.43\ \ \mu mol/l} = 0.963$$

To calculate the relative standard uncertainty of the recovery, $u(\overline{R}_m)$, Equation 3 is used:

$$u(\overline{R}_m) = \overline{R}_m \times \sqrt{\left(\frac{s_{obs}^2}{n \times \overline{C}_{obs}^2}\right) + \left(\frac{u(C_{CRM})}{C_{CRM}}\right)^2} = 0.963 \times \sqrt{\left(\frac{0.12^2}{30 \times 2.34^2}\right) + \left(\frac{0.14}{2.43}\right)^2} = 0.056$$

To test whether the observed recovery is significantly different from 1, a statistical significance test (t-test) is performed following equation 5:

$$t = \frac{\left|1 - \overline{R}_m\right|}{u(\overline{R}_m)} = \frac{1 - 0.963}{0.056} = 0.661$$

If $t < k$ (coverage factor), it can be assumed that the recovery is not significantly different from 1 [2]. Since 0.661 is less than 2, the significance test indicates no significant difference between the observed recovery (0.963) and 1.

The relative combined standard uncertainty $u_c(PO_4)$ is than estimated as:

$$u_c(Phos) = \sqrt{RSD^2 + u(\overline{R}_m)^2} = \sqrt{0.051^2 + 0.056^2} = 0.076$$

Using the recommended coverage factor $k = 2$, the expanded uncertainty, $U(PO_4)$, is given by:

$$U(Phos) = k \times u_c(y) = 2 \times 0.076 = 0.152$$

Result: the relative expanded uncertainty, $U(PO_4)$, for the determination of phosphate in seawater samples within the considered working range is 0.152 and 15.2%, respectively.

This denotes for a theoretical result of 10.0 µmol l^{-1} phosphate:

Phosphate concentration: 10.0 ± 1.5 µmol l^{-1},

the stated uncertainty is an expanded uncertainty, calculated using a coverage factor of 2 (this corresponds approximately to the 95% confidence interval).

EXAMPLE 2: ESTIMATION OF MEASUREMENT UNCERTAINTY USING REPRODUCIBILITY DATA FROM INTERLABORATORY STUDIES

The results of the three QUASIMEME exercises on the determination of phosphate in sea water carried out in 2001 were as follows:

Relative reproducibility standard deviation: 4.67 / 4.47 / 6.30 %

Phosphate concentration - Assigned value: 9.71 / 13.08 / 1.88 µmol l^{-1}

Using this information, the averaged relative reproducibility standard deviation expressed as coefficient of variation, $CV(PO_4)$, for the intercomparison study on phosphate determination in seawater is, which can be equated with combined standard uncertainty, $u_c(PO_4)$, is given by:

$$CV(PO_4) = \sqrt{\Sigma x_i^2 / n} = \sqrt{\left(4.67^2 + 4.47^2 + 6.30^2\right)/3} = 5.2\%$$

Using the recommended coverage factor $k = 2$, the expanded uncertainty, $U(PO_4)$, is 10.4%.

This result is in satisfactory agreement with the estimated expanded uncertainty, $U(PO_4)$, of 15.2% obtained using the results of replicate analyses of a certified reference material.

ATTACHMENT 2

MEASUREMENT UNCERTAINTY IN ENVIRONMENTAL CHEMICAL ANALYTICAL PRACTICE: ESTIMATION INCLUDING ALL POSSIBLE SOURCES OF UNCERTAINTY

This estimation study of uncertainty is based on the British VAM (Valid Analytical Measurement) project, Development and Harmonisation of Measurement Uncertainty Principles (Barwick and Ellison, 2000). The guide focuses on how to identify and quantify uncertainty components, and in particular gives guidance on how uncertainty estimates can be obtained from method validation experiments.

One of the critical stages of any uncertainty study is the identification of all possible sources of uncertainty. The EURACHEM Guide (EURACHEM, 2000) discusses this process and a number of typical sources of uncertainty are given, including sampling, instrument bias, reagent purity, measurement conditions, sample, computational and random effects.

The next stage in the process is the planning of experiments which will provide the information required to obtain an estimate of the combined uncertainty for the method. Initially, two sets of experiments are carried out: a precision study and a trueness study. These experiments should be planned in such a way that as many of the identified sources of uncertainty as possible are covered.

However, further experiments may be required, for example, regarding sampling. Where sampling forms part of the procedure, effects such as random variations between different samples and any bias in the sampling procedure need to be considered. One test is the Robust ANOVA of analysed data of a replicate sampling experiment.

Precision study

In many cases, the method is used for the analysis of a single sample matrix type with a range of analyte concentrations. The precision should be investigated at concentrations covering the full range specified in the method scope. It is recommended that at least three concentrations are investigated (e.g., low, medium, and high), with at least four replicates at each concentration level. The replicates for each concentration level should be spread across different batches.

Calculate the standard deviation and the relative standard deviation of the results obtained for each concentration level. If there is no significant difference between the relative standard deviations for each concentration level, this indicates that the precision is proportional to the analyte concentration. In such a case, the relative standard deviations can be pooled to give a single estimate which can be applied to the concentration range covered by the precision study (see Eq. 1).

$$RSDpool = \sqrt{\frac{\left[\ (n_1 - 1) * RSD_1^{\ 2} + (n_2 - 1) * RSD_2^{\ 2} + ...)\ \right]}{(n_1 - 1) + (n_2 - 1) + ...}} \qquad \text{Eq. 1}$$

where RSD_1 is the relative standard deviation calculated for the sample at concentration level 1, n_1 is the number of replicates for that sample, etc.

However, it is common to find that the precision is not proportional to the concentration over the entire range specified in the method scope, especially if that range is wide. The situation is even more complicated when the method scope covers a range of sample matrices (for these cases, see the VAM project (Barwick and Ellison, 2000)).

Trueness study

In this protocol, trueness is estimated in terms of the overall recovery, i.e., the ratio of the observed value to the expected value. The closer the ratio is to 1, the smaller the bias in the method. Recovery can be evaluated in a number of ways, for example, the analysis of certified reference materials (CRMs) or spiked samples. The experiments required to evaluate recovery and its uncertainty will depend on the scope of the method and the availability, or otherwise, of suitable CRMs.

R_m is an estimate of the mean method recovery obtained from, for example, the analysis of a CRM or a spiked sample. The uncertainty in R_m is composed of the uncertainty in the reference value (e.g., the uncertainty in the certified value of a reference material) and the uncertainty in the observed value (e.g., the standard deviation of the mean of replicate analyses). The contribution of R_m to the overall uncertainty of the method depends on whether it is significantly different from 1, and if so, whether a correction is applied. Approaches to estimate recovery,

together with correction factors, are discussed in more detail elsewhere (Barwick and Ellison, 2000).

For estimating R_m and $u(R_m)$, a certified reference material should be chosen with a matrix and analyte concentration representative of those which will be routinely analysed using the method. Analyse at least ten portions of the reference material in a single batch. Each portion must be taken through the entire analytical procedure. Calculate the mean recovery $\overline{R}_m$ as follows:

$$\overline{R}m = \frac{\overline{C}_{obs}}{C_{CRM}} \qquad \text{Eq. 2}$$

where C_{obs} is the mean of the replicate analyses of the CRM and C_{CRM} is the certified value for the CRM.

Calculate the uncertainty in the recovery, $u(\overline{R}_m)$, using:

$$u(\overline{R}_m) = \overline{R}_m \times \sqrt{(\frac{s^2{}_{obs}}{n \times \overline{C}^2{}_{obs}}) + (\frac{u(C_{CRM})}{C_{CRM}})^2} \qquad \text{Eq. 3}$$

where s_{obs} is the standard deviation of the results from the replicate analyses of the CRM, n is the number of replicates and $u(C_{CRM})$ is the standard uncertainty in the certified value for the CRM. See VAM project (Barwick and Ellison, 2000) for information on calculating standard uncertainties from reference material certificates.

Calculation of combined standard uncertainties

How the individual uncertainty components are combined depends on whether or not they are proportional to the analyte concentration. If the uncertainty component is proportional to the analyte concentration, then it can be treated as a relative standard deviation. For a result y which is affected by the parameters p, q, r ....., which each have uncertainties $u(p)$, $u(q)$, $u(r)$ ....., the uncertainty in y', $u(y')$, is given by:

$$\frac{u(y')}{y'} = \sqrt{(\frac{u(p)}{p})^2 + (\frac{u(q)}{q})^2 + (\frac{u(r)}{r})^2 + ...} \qquad \text{Eq. 4}$$

If, however, the uncertainty is fixed regardless of the analyte concentration, then it should be treated as a standard deviation. The uncertainty in the result due to parameters that are not concentration dependent, $u(y'')$, is given by:

$$u(y'') = \sqrt{u(p)^2 + u(q)^2 + u(r)^2 + ...} \qquad \text{Eq. 5}$$

To calculate the combined uncertainty in the result, $u(y')$, at an analyte concentration y', the concentration-dependent and concentration-independent uncertainties are combined as follows:

$$u(y) = \sqrt{(u(y''))^2 + (y \times \frac{u(y')}{y'})^2} \qquad \text{Eq. 6}$$

where $u(y')$ is the combined concentration-dependent uncertainties calculated using Eq. 4 and $u(y'')$ is the combined concentration-independent uncertainties calculated using Eq. 5.

If only precision and trueness studies are carried out, Equation 6 may be simplified as follows:

$$u(y) = \sqrt{RSD_{pool}{}^2 + (y \times \frac{u(\overline{R}_m)}{\overline{R}_m})^2} \qquad \text{Eq. 7}$$

where RSD_{pool} is the relative standard deviation of the precision study and $u(\overline{R}_m)/\overline{R}_m$ is the relative mean recovery.

For a practical uncertainty estimation in monitoring programmes, it may be very elaborate to calculate the combined uncertainty for each new analytical result using Eq. 6. If the method is used for the analysis of a single matrix type, it is recommended that at least three concentrations are calculated using Eq. 6, for example, the same low, medium, and high concentrations as from the precision study. Note that the chosen concentrations should cover the full range specified in the method scope.

Calculate the relative combined uncertainty for each concentration level. If there is no significant difference between the relative standard deviations, then the mean combined uncertainty can be calculated for the analytical method. If there is a significant difference between the combined uncertainties, it may therefore be possible to give a range of combined uncertainties for the method, using the lowest and highest values.

Calculation of expanded uncertainty

The combined standard uncertainty $u(y)$ must be multiplied by an appropriate coverage factor, k, to give the expanded uncertainty. The expanded uncertainty is an interval which is expected to include a large fraction of the distribution of values reasonably attributable to the measurand. For a combined standard uncertainty $u(y)$, the expanded uncertainty $U(y)$ is given by:

$$U(y) = k \times u(y) = 2 \times u(y) \qquad \text{Eq. 8}$$

For most purposes, a coverage factor of $k = 2$ is recommended (however, see note below). For a Normal distribution, a coverage factor of 2 gives an interval containing approximately 95% of the distribution of values.

For reporting expanded uncertainty, the following form is recommended: (result) $y \pm U(y)$ (units), where the reported uncertainty is an expanded uncertainty calculated using a coverage factor of $k = 2$, which gives a level of confidence of approximately 95%.

REFERENCES

Barwick, V.J., and Ellison, S.L.R. 2000. Development and Harmonisation of Measurement Uncertainty Principles – Part (d): Protocol for uncertainty evaluation from validation data; VAM Project (Version 5.1, Jan. 2000). http://www.vam.org.uk/publications (look under publications for the first author).

EURACHEM. 2000. EURACHEM/CITAC Guide: Quantifying Uncertainty in Analytical Measurement. www.uni-stuttgart.de/eurachem/pdf

ANNEX B-5

GENERAL REMARKS ON SAMPLING FOR CHEMICAL ANALYSIS

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 determinands 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.

REFERENCES

ACS (American Chemical Society Committee on Environmental Improvement. Keith, L., Crummet, W., Deegan, J., Libby, R., Taylor, J., and 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.

ANNEX B-6

EXAMPLES OF REFERENCE MATERIALS FOR INTERNAL QUALITY CONTROL

In order to check the individual standards, the analyst needs reference materials (RMs) and certified reference materials (CRMs). Both the RM and CRM should have a similar matrix and concentration of the determinand as the sample(s) to be analysed.

The effect of matrix (salt effect) in the Baltic Sea with strong salinity gradients has to be determined experimentally and the analytical results corrected correspondingly, especially for silicate and ammonia.

The supply of CRMs is limited to a certain extent by the available quantities and sometimes by prices. In some areas, e.g., for some organic determinands, no certified materials with relevant matrix, concentration, and determinand are available.

Examples of 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
Eurofins (Denmark)	www.eurofins.dk (look under "Quality and management")
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 reports are available at: www.ices.dk/committee/mhc/mcwg.htm). Further information on CRMs can be obtained from the COMAR database, "The international database for certified reference materials" at www.comar.bam.de).

Table B-6.1. Examples of selected CRMs for marine monitoring programmes.

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)

Table B-6.1 (continued)**.**

Matrix	Certified values for	Material	Name	Manufacturer
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 sea water	BCR (Belgium)
Water	4 Metals	BCR 505	Trace elements in estuarine water	BCR (Belgium)
Water	Hg	BCR 579	Coastal sea water	BCR (Belgium)
Water	12 Metals	CASS-4	Nearshore sea water	NRC (Canada)
Water	10 Metals	NASS-5	Open ocean sea water	NRC (Canada)
Water	11 Metals	SLEW-3	Estuarine water	NRC (Canada)
Water	6 Metals	LGC6016	Estuarine water - metals	LGC (UK)
Water	NO_2, NO_3, NH_4, TN	QC SW3.1	Baltic/estuarine water (10 PSU)	EUROFINS (Denmark)
Water	PO_4, TP, SiO_4	QC SW3.2	Baltic/estuarine water (10 PSU)	EUROFINS (Denmark)
Water	NO_2, NO_3, NH_4, TN	QC SW4.1	Sea water (35 PSU)	EUROFINS (Denmark)
Water	PO_4, TP, SiO_4	QC SW4.2	Sea water (35 PSU)	EUROFINS (Denmark)

ANNEX B-7

UNITS AND CONVERSIONS

This annex 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 this annex, 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		$\mu mol/dm^3$	see Annex C-2
Nutrients		$\mu mol/dm^3$	as N, P, or Si; see Annex C-2
Total P and N	TP/TN	$\mu mol/dm^3$	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)		$\mu mol/dm^3$	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

Parameter	Symbol	Unit	Comment
▪ abundance		Counting units/dm^3	
▪ biomass		mm^3/dm^3	
Mesozooplankton			see Annex C-7
▪ abundance		Counting units/m^3	
▪ biomass		mm^3/m^3; mg/m^3	
Macrozoobenthos			see Annex C-8
▪ abundance		Counting units/m^3	
▪ 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	0.700 [$mg/dm^3 \times 0.700 = cm^3/dm^3$]
	cm^3/dm^3	mg/dm^3	1.429
	$µmol/dm^3$	cm^3/dm^3	0.0223916
	cm^3/dm^3	$µmol/dm^3$	44.658806
	mg/dm^3	$µmol/dm^3$	31.2519
	$µmol/dm^3$	mg/dm^3	0.0320 [0.0319988]
	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)

UNITS AND CONVERSIONS REGARDING OXYGEN SATURATION

Water saturation with oxygen is calculated from the following equation under the defined temperature and salinity status:

$$\{C(O_2)\} = \frac{O_2}{O_2'} \times 100$$

where:

O_2 is the oxygen concentration in the sample,

O_2' is the oxygen solubility in sea water at temperature and salinity, taken from the solubility tables (UNESCO, 1986).

An oceanographic calculator using this equation is also available on the ICES website (http://www.ices.dk/ocean/).

References

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ICES. 2000. Recommended equations for the calculation of solubility of dissolved oxygen in marine waters. *In* Report of the ICES Advisory Committee on the Marine Environment, 2000. ICES Cooperative Research Report, 241: 199–200.

Murray, C.N., and Riley, J.P. 1969. The solubility of gases in distilled water and seawater – II. Oxygen. Deep-Sea Research, 16: 311–320.

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

ANNEX B-8

TECHNICAL ANNEX ON THE DETERMINATION OF HYDROCHEMICAL PARAMETERS

APPENDIX 1

TECHNICAL NOTE ON THE DETERMINATION OF SALINITY AND TEMPERATURE

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

1 INTRODUCTION

Many investigations have been performed to present the salinity and chlorinity of sea water and the connection between salinity and chlorinity since 1884. Those investigations have been 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 ‰. 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 Technical Note on the Determination of Temperature and Salinity (CTD) in Part III of this Appendix.

4 ANALYTICAL PROCEDURE

4.1 Calibration and Stability of Calibration

Calibration of 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 (three 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 NOTE 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 carried out within the COMBINE programme, both thermometer types are suitable.

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:

a) 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.

b) 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 five 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 must 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

T_{corr} = the corrected, final, temperature,

T_{obs} = the observed temperature from the main thermometer,

I = correction according to the calibration,

V_0 = a constant, specific for each mercury thermometer (the volume of the mercury),

$1/\beta$ = 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 T_{obs}).

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 is 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 the 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

The following sampling equipment is needed:

- 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, 1991a; 1991b; UNESCO, 1988; 1994). Based on a combination of the previous protocols and field measurement experience from the COMBINE Programme, the protocol described below is proposed.

4.1 Sensor Quality Control

It is useful to control the function of the CTD conductivity sensors by analysing water samples, which 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 Appendix).

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 Appendix).

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

4.2.1 Stabilization

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

4.2.2 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.

4.2.3 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}.

4.2.4 Documentation

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

4.2.5 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 Appendix).

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 of 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 measurements. The COMBINE Programme requirements for the accuracy of temperature and salinity measurements are given in Annex C-2 of the COMBINE Manual.

5.2 Maintenance

It is recommended that exchangeable, pre-calibrated, spare temperature, conductivity, and pressure sensor modules are 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 toothbrush, 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 highly 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 (BMP is the offshore Baltic Monitoring Programme and CMP is the Coastal Monitoring Programme).

Parameter	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

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APPENDIX 2

TECHNICAL NOTE ON THE DETERMINATION OF DISSOLVED OXYGEN

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 DIN EN 25813 (DIN EN, 1993) and ISO 5813 (ISO, 1983) or using 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 (DIN EN, 1992) and ISO 5814 (ISO, 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 sub-sampling 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 sub-sampling and titration, only glass bottles with conical-shaped tops and with glass ground stoppers meet the requirements of the Winkler method. Sub-sample bottles must be calibrated and identified with their stoppers since they must not be interchanged. Sub-samples 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 the reagent and avoid 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 four 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 they should be taken 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 $cm^3\ dm^{-3}$ (ml l^{-1}) O_2 at NTP and/or in % of saturation (Weiss, 1970).

The calculation of saturation also requires the *in situ* temperature known to ±0.1 °C and salinity to 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:

- $cm^3\ dm^{-3} \times 1.429 = mg\ dm^{-3}$;
- $mg\ dm^{-3} \times 0.700 = cm^3\ dm^{-3}$;
- $cm^3\ dm^{-3} \times 0.0893 = \mu M\ O_2$;
- $\mu M\ O_2 \times 11.20 = cm^3\ dm^{-3}$.

8 PRECISION

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

9 REFERENCES

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APPENDIX 3

RECOMMENDED EQUATIONS FOR THE CALCULATION OF SOLUBILITY OF DISSOLVED OXYGEN IN MARINE WATERS

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 volume instead 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 equations that should be used for the computation of solubility values of dissolved oxygen, in various units, according to the UNESCO recommendation, are reproduced here. These equations (so-called B & K equations) are taken directly 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 (O_2), therefore, "micromole" means "micromole of O_2";
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
A =	–135.29996
B =	$+1.572288 \times 10^5$
C =	-6.637149×10^7
D =	$+1.243678 \times 10^{10}$
E =	-8.621061×10^{11}
F =	+0.020573
G =	–12.142
H =	+2363.1
	micromole per litre
I =	–135.90205
J =	$+1.575701 \times 10^5$
K =	-6.642308×10^7
L =	$+1.243800 \times 10^{10}$
M =	-8.621949×10^{11}
N =	+0.017674
P =	–10.754
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:

$$Cs_{(\mu mol\ kg^{-1})} = \exp [-135.29996 + (1.572288 \times 10^5) / (t + 273.15) - (6.637149 \times 10^7) / (t + 273.15)^2 + (1.243678 \times 10^{10}) / (t + 273.15)^3 - (8.621061 \times 10^{11}) / (t + 273.15)^4 - S \times (0.020573 - 12.142 / (t + 273.15) + 2363.1 / (t + 273.15)^2)].$$

and

$$Cs_{(\mu mol\ l^{-1})} = \exp [-35.90205 + (1.575701 \times 10^5)/(t + 273.15) - (6.642308 \times 10^7) / (t + 273.15)^2 + (1.243800 \times 10^{10}) / (t + 273.15)^3 - (8.621949 \times 10^{11}) / (t + 273.15)^4 - S \times (0.017674 - 10.754 / (t + 273.15) + 2140.7 / (t + 273.15)^2)].$$

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 REFERENCES

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APPENDIX 4

TECHNICAL NOTE ON THE DETERMINATION OF HYDROGEN SULPHIDE

1 INTRODUCTION

Hydrogen sulphide is a poisonous gas that readily dissolves in water. Hydrogen 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 the 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 have been taken, using the same sampling technique (cf. Technical Note on the Determination of Dissolved Oxygen, Attachment 2, above). 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 amounts 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:

1) 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.

2) 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).

3) 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.

4) 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 sub-sampling. Ideally, the result (Sample 1 – Sample 2) should be evenly distributed around zero. Any deviations from this suggest sub-sampling 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}.

8 REFERENCES

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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 pre-treatment) 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 the 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.

The addition of mercury or chloroform is an alternative preservation method for all nutrients except ammonia. However, 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 PRE-TREATMENT

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 B-2).

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 pre-treated 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 which 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:

a) 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).

b) 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.

6 REFERENCES

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ANNEX B-10

TECHNICAL NOTE ON THE ANALYSIS OF ANOXIC WATERS

1 INTRODUCTION

Anoxic sea water 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:

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

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

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

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 pre-treatment procedure.

Validation of the 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^{-1} 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^{-1} 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 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 the 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 ANNEX ON THE DETERMINATION OF HEAVY METALS AND PERSISTENT ORGANIC COMPOUNDS IN SEA WATER

APPENDIX 1

TECHNICAL NOTE ON THE DETERMINATION OF TRACE METALS (Cd, Pb, Cu, Co, Zn, Ni, Fe) INCLUDING MERCURY IN WATER

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 (Hg_R): A methodologically defined fraction consisting mostly of inorganic Hg(II).

Total mercury (Hg_T): Mercury content of an unfiltered sample, after digestion with an oxidizing compound (e.g., $KMnO_4$).

Total dissolved mercury: Mercury content of a filtered sample, after digestion with an oxidizing compound (e.g., $KMnO_4$).

Dissolved mercury (DM): This includes elemental mercury (Hg^0), 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 pre-treatment 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 (U.S. 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 m^3 (corresponding to class 100 U.S. Norm). For mercury analysis, additional air cleaning using activated charcoal filters is required.

2 PREPARATIONS

Chemicals

High purity water (e.g., "Milli-Q water", 18 MΩ 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 for purifying (clean) chemicals and reductant solutions for mercury analysis.

In order to avoid contamination problems, all plastic ware, bottles, and containers must be treated with acids (HCl or HNO_3) 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% HNO_3 (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 HNO_3 (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% $KMnO_4$, 0.1% $K_2S_2O_8$, and 2.5% HNO_3 and heated for 2 hours at 80 °C. The bottles are then rinsed with water and stored with 2% HNO_3 containing 0.01% $K_2Cr_2O_7$ or $KMnO_4$ until ready for use.

Filters

Polycarbonate filters (e.g., Nuclepore) (0.4 µm, 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 hours) to volatilize gaseous mercury. This procedure is described in detail by Quéremais and Cossa (1997).

If trace metals in suspended particulate matter (SPM) are to be determined, filters have to be placed in pre-cleaned 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 sub-sampling into the pre-cleaned quartz or Teflon bottles (Teflon has an extra low content of trace metals). Sub-sampling 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 pre-cleaned 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 of the sample during sampling and storage may be jeopardized by contamination and/or unexpected losses owing to volatilization.

4 FILTRATION PROCEDURE

In the environmental and geochemical scientific community concerned with water analysis, it is generally accepted that the term “dissolved” refers to that fraction of water and its constituents which have passed through a 0.45 µm 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 the 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 µm 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 HNO_3 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 ($Cr_2O_7^{2-}$) in addition to acidification or complexing agents (cysteine) to neutral or alkaline samples to prevent Hg losses during storage.

6 SAMPLE PRE-TREATMENT

6.1 Water samples

Depending on the expected concentration range (10^{-7}–10^{-9} g kg^{-1}) of trace metals (dissolved) in Baltic Sea water and because of the salt matrix interfering during the measurement process, pre-concentration 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).

6.2 Filters

Different methods for analysing the material on the filter are described by Hovind and Skei (1992) and Loring and Rantala (1991). Pressure decomposition with an acid mixture (HCl, HNO_3, 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-12, Appendix 1, 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 graphite furnace atomic absorption spectrometry (GFAAS), electrochemical methods, inductively coupled plasma-mass spectrometry (ICP-MS), inductively coupled plasma-atomic emission spectrometry (ICP-AES), or total-reflection X-ray fluorescence (TXRF).

Owing to 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 pre-concentration 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 (sulphur-containing waters), see Annex B-12, Appendix 2.

8 QUALITY CONTROL

The internal quality control is described in Section 5 of the General Guidelines.

8.1 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 pre-treatment procedures, including the addition of the same quantities of chemical substances as for the sample.

8.2 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).

The accuracy of the standard stock solutions can be ensured by the use of CRMs or participation in intercomparison exercises.

The working standard should be prepared from the standard stock solution for every batch of samples; it should preferably be prepared daily. Pre-cleaned 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).

8.3 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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APPENDIX 2

TECHNICAL NOTE ON THE DETERMINATION OF PERSISTENT ORGANIC POLLUTANTS IN SEA WATER

1 INTRODUCTION

These guidelines concentrate on the sampling and extraction of lipophilic persistent organic pollutants from sea water 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 (ICES, 1997);
- "Guidelines for the determination of chlorobiphenyls in sediments: Analytical methods", ICES ACME Report 1996 (ICES,1996);
- "Determination of Polycyclic Aromatic Hydrocarbons (PAH)s in Biota", ICES ACME Report 1998 (ICES, 1999); and
- Annex B-13 (these Guidelines).

As the same analytical methods can be used for the determination of lipophilic contaminants 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 contaminants are generally quite different in water and sediment samples. The concentration patterns of the contaminants are mainly influenced by their polarity, which can be expressed by their octanol/water coefficient (log K_{ow}; K_{ow} = Concentration in octanol phase / Concentration in aqueous phase). Thus, in water samples, the more hydrophilic compounds with log K_{ow} values of 3 to 4 predominate (e.g., 2- and 3-ring aromatics and HCH isomers), while in sediments and biota the contaminants with log K_{ow} 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 sea water with a log $K_{OW} > 3$. The analysis of POPs generally includes:

- sampling and extraction of the water;
- clean-up; and
- 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 onto the container material or contamination. Especially the very lipophilic compounds (4- to 6-ring aromatic hydrocarbons, DDT, PCBs) tend to adsorb onto every surface. Therefore, the seawater samples should not be stored longer than 2 hours 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. Glass fibre 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 super-cleaned 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 PAHs and chlorinated hydrocarbons in sea water 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 requires a number of additional quality control procedures (to reduce adsorption losses and contamination problems). There are two possible means of phase separation: filtration and centrifugation.

Filtration is done using 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 large amounts of suspended matter. A pump is necessary to force the water through the filter.

Centrifugation requires a high volume centrifuge which must be operable on-board 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 in the same way as sediment. The solute phase is analysed in the same way as 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 sea water, 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 methods 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.

Owing to the robustness of the method, there is a preference for 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, C_{18} 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 on-board 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 acetonitrile). 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 have been published by the IOC (1993), Ehrhardt (1987), and Bruhn and McLachlan (2002).

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; Kulovara, 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 concentrations 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 onto 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, CB185) 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 avoid 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 (e.g., 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 relatively 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 sea water (ICES, 1996, 1997, 1999).

A 3 ml glass column with glass fibre frit (commercially available for SPE) is filled with 500 mg of silica gel (dried for 2 hours at 200 °C) and subsequently washed with 30 ml of CH_2Cl_2 and 30 ml of hexane. The hexane sample extract (concentrated to 500 µl) is applied on top of the column and eluted with 5 ml of CH_2Cl_2/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 required, additional clean-up (HPLC, GPC) might become necessary.

7 CHROMATOGRAPHIC DETERMINATION

Details of 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 considerably lose sensitivity.

With GC-MS, detection limits of 5–30 pg l^{-1} can be achieved 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 for naphthalene and phenanthrene and, to a lesser extent, other PAHs); for these, 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 3 to Annex B-12: Technical note on the determination of polycyclic aromatic hydrocarbons in biota.

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 distributed regularly 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, 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 POPs in sea water 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;
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-6). 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, for both PAH and CB analyses, an additional internal standard is added for volume correction. Internal standards should be added in a fixed volume or weight 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 pre-deuterated 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., CB29, CB112, CB155, CB198 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 ANNEX ON THE DETERMINATION OF HEAVY METALS AND PERSISTENT ORGANIC COMPOUNDS IN BIOTA

APPENDIX 1

TECHNICAL NOTE ON THE ANALYSIS OF CHLORINATED BIPHENYLS, ORGANOCHLORINE PESTICIDES, AND METALLIC TRACE ELEMENTS IN MARINE FISH

1 SAMPLING AND SAMPLE HANDLING OF FISH MUSCLE AND LIVER TISSUE

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. 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.

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.

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 metallic trace element analyses. Stainless steel tweezers are recommended if biological tissue is dissected for the analysis of chlorinated biphenyls (CBs) and organochlorine pesticides (OCPs).

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 the 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 haemostats in diluted (1+6) acid.

   c) Rinse with high-purity water.

2) For the 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 ship, the dissection of fish should be carried out in the land-based laboratory under conditions of maximum protection against contamination.

For the 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 sub-samples 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 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 (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. 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.

2 STORAGE OF FISH SAMPLES

Material from single fish specimens should be packaged and stored individually.

- Samples for the analysis of metallic trace elements can be stored in polyethylene, polypropylene, polystyrene, or glass containers.
- Samples for the analysis of CBs and OCPs should be packaged in pre-cleaned aluminium foil or in pre-cleaned 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 for 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.

3 DETERMINATION OF CHLORINATED BIPHENYLS AND ORGANOCHLORINE PESTICIDES

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, determinand 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.

3.1 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 °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 redistillation 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 C_{18}-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).

3.2 Sample Pre-treatment

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 extractions 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 the different types and amounts of biological matrices to be investigated (see "Recovery" under Section 3.4, below).

3.3 Clean-up

The crude extract obtained from sample pre-treatment 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 Al_2O_3 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 heptachlorepoxides, which are easily broken down by H_2SO_4, are to be determined.

3.4 Determination by Gas Chromatography

Owing to 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 owing to 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 instrumental equipment), 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 approximately a 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 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 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 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 Geus *et al*., 1996; de Boer *et al*., 1995).

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 desiccator 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 multilevel 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-tetrachloro-naphthalene or homologues of dichloroalkylbenzylether can be used. For GC with mass selective detection (GC-MSD), ^{13}C-labelled CBs must be used as internal standards. With GC-MS, ^{13}C-labelled CBs should preferably be used as internal standards.

4 DETERMINATION OF METALLIC TRACE ELEMENTS

Metallic elements appear in different marine biological matrices in trace concentrations, ranging from the mg kg^{-1} through the µg kg^{-1} to the ng kg^{-1} 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.

4.1 Pre-treatment of Laboratory Ware and Reagents; Contamination Control

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 ft^3 or 3.6×10^3 per m^3 of 0.5 µm 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 the design, testing, and maintenance of clean rooms; it 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).

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 pre-treatment and handling, these materials meet the requirements of purity necessary for the required analytical investigations. Cleaning procedures for plastic and glass laboratory ware have been comprehensively dealt with by Moody and Lindström (1977), Tschöpel *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 (Tschöpel *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 solutions of analytes 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 the 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 systems are described in detail by Matthinson (1972), Kuehner *et al*. (1972), Dabeka *et al*. (1976), Tschöpel *et al*. (1980), Mitchell (1982b), Moody and Beary (1982), Moody *et al*. (1989), and Paulsen *et al*. (1989). Acids of extreme high purity are produced by multiple batch-wise distillation of reagent-grade acids in a silica apparatus, which is placed in a laminar-flow hood.

4.2 Sample Pre-treatment

If the determinands are heterogeneously distributed in the sample material, it may be preferable to homogenize prior to taking sub-samples 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).

4.3 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.

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; Würfels *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 the application of a carefully developed and controlled temperature program and modifying the matrix prior to the ashing procedure (addition of ashing aids) may be suitable to prevent losses of volatile elements. The use of special materials (quartz, titanium, stainless steel) for the construction of sample containers may be helpful to minimize 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 the 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), the loss of elements through volatilization 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 rapid energy transfer to the sample and a very rapid build-up of high internal vessel temperature and pressure, with the consequence that an enormous reduction in digestion time occurs. Furthermore, a reduction of acid volume (McCarthy and Ellis, 1991) and less contamination during the decomposition process were found (Dunemann, 1994; Sheppard *et al*., 1994).

The admittance 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.

4.4 Calibration

For calibration purposes, single element stock standard solutions at a concentration of 1000 mg l^{-1}, 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.

Single or mixed working element standard solutions for calibration purposes are prepared by dilution of the stock standard 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 $\mu g\ l^{-1}$ 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:

1) 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.

2) The required analytical precision must be achievable and known throughout the entire range.

3) The measured value (response) at the lower end of the range must be significantly different from the procedural analytical blank.

4) The chemical and physical properties of the calibration standards must closely resemble those of the sample under investigation.

5) The calibration standards must be processed through the entire analytical procedure in the same manner as the sample.

6) The standard addition technique should be used only under very special circumstances (Cardone, 1986a; 1986b).

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APPENDIX 2

TECHNICAL NOTE ON THE DETERMINATION OF TOTAL MERCURY IN MARINE BIOTA BY COLD VAPOUR ATOMIC ABSORPTION SPECTROMETRY

1 POSSIBILITIES OF USING COLD VAPOUR ATOMIC ABSORPTION SPECTROMETRY IN TOTAL MERCURY ANALYSIS

Total mercury refers to the amount of mercury measured in biological samples that have been subjected to a chemical digestion/decomposition process. 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^0) 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.

For the analysis of environmental samples, including biota, cold vapour atomic fluorescence spectrometry (CV-AFS) offers improved sensitivity compared with CV-AAS.

The cold vapour mercury determination with atomic absorption or atomic fluorescence detection can be performed manually using batch techniques or automatically using flow injection (FI) techniques. FI is a very efficient approach for introducing and processing liquid samples in atomic absorption spectrometric measurements. The FI technique, combined with a built-in atomic absorption spectrometer optimized 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 efficacy 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 the 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 sulphur-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 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-AAS. 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, HNO_3) and oxidants (H_2O_2, $KMnO_4$, $K_2Cr_2O_7$, $K_2S_2O_8$, Br^-/BrO^{3-}) 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; McLeod and Jian, 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-AAS techniques;
- stability of sample solutions during storage (at least short term);
- no formation of solid reaction products.

On-line sample pre-treatment 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 pre-treatment 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 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 onto 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(0); 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 will be less stable and will 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 efficacy of this mixture is 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 the 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

A general problem with cold vapour mercury determination is the condensation of moisture on transfer tube walls, and it can also cause fogging of the optics and adsorption cell windows. Moisture is generally removed by the use of desiccants, which require constant renewal, but a more effective method involves passing the vapour through a semi-permeable polymeric membrane tube.

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 effect was a suppression, broadening or even splitting of the mercury signal. The authors postulated that the 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. Section 5 in the General Guidelines).

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 Sections 3.5, 4.2.5, and 4.2.5.2b in the General Guidelines).

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APPENDIX 3

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 molecular weight 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 dibenzo-thiophenes) 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 also requires 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.

2.2 First Choice Shellfish Species

Mytilus spp. (mussel)

The blue mussel *(Mytilus edulis)* occurs in shallow waters along almost all coasts of the Baltic Sea. It is therefore suitable for monitoring in nearshore waters. No distinction is made between *M. edulis*, *M. gallopovincialis*, and *M. trossulus* because the latter species fill a similar ecological niche. A sampling size range of 20–70 mm shell length is specified to ensure availability throughout the whole maritime area. 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.

3 SAMPLING

Two alternative sampling strategies can be used: sampling to minimize natural variability and length-stratified sampling. Only details of length-stratified sampling are described in this document, as this strategy has been used in monitoring programmes for temporal trends of contaminants in biota in the Northeast Atlantic.

3.1 Shellfish

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.

3.2 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).

4 TRANSPORTATION

Fish samples should be kept cool or frozen (at a temperature of −20 °C or lower) as soon as possible after collection. Live mussels 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). 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.

5 PRE-TREATMENT AND STORAGE

5.1 Contamination

Sample contamination may occur during sampling, sample handling, pre-treatment 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 ship. 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.

5.2 Shellfish

5.2.1 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.

5.2.2 Dissection and storage

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 homogenized as soon as possible, and frozen in glass jars at −20 °C 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.

5.3 Fish

5.3.1 Dissection and storage

The dissection of fish muscle and internal organs should be conducted as soon as possible after collection. To sample fish muscle, care should be taken to avoid including any epidermis or subcutaneous fatty tissue in the sample. Samples should be taken underneath the red muscle layer. In order to ensure uniformity, the right side dorso-lateral muscle should be sampled. 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.

6 ANALYSIS

6.1 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, 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.

6.2 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 sub-sample of the tissue homogenate, as some of the extraction techniques routinely used 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.

6.3 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.

6.4 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; Düring and Gäth, 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.

6.5 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.

6.6 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 concentrates 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 l. Compounds of interest for environmental monitoring for which the guidelines apply.

Compound	MW	Compound	MW
Naphthalene	128	C_2-Phenanthrenes/Anthracenes	206
C_1-Naphthalenes	142	C_3-Phenanthrenes/Anthracenes	220
C_2-Naphthalenes	156	Fluoranthene	202
C_3-Naphthalenes	170	Pyrene	202
C_4-Naphthalenes	184	C_1-Fluoranthenes/Pyrenes	216
Acenaphthylene	152	C_2-Fluoranthenes/Pyrenes	230
Acenaphthene	154	Benz[*a*]anthracene	228
Biphenyl	154	Chrysene	228
Fluorene	166	2,3-Benzanthracene	228
C_1-Fluorenes	180	Benzo[*a*]fluoranthene	252
C_2-Fluorenes	194	Benzo[*b*]fluoranthene	252
C_3-Fluorenes	208	Benzo[*j*]fluoranthene	252
Dibenzothiophene	184	Benzo[*k*]fluoranthene	252
C_1-Dibenzothiophenes	198	Benzo[*e*]pyrene	252
C_2-Dibenzothiophenes	212	Benzo[*a*]pyrene	252
C_3-Dibenzothiophenes	226	Perylene	252
Phenanthrene	178	Indeno[1,2,3-*cd*]pyrene	276
Anthracene	178	Benzo[*ghi*]perylene	276
C_1-Phenanthrenes/Anthracenes	192	Dibenz[*ah*]anthracene	278

6.7 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 0.2–10 μg kg^{-1} wet weight for individual PAH compounds should be achievable by both GC-MS and HPLC-UVF techniques.

6.8 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.

6.9 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. Owing to 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.

7 CALIBRATION AND QUANTIFICATION

7.1 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-6). 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.

7.2 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.

7.3 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.

8 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 Materials. 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-6; 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.

9 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-13

TECHNICAL ANNEX 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 SEDIMENTS

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 fibre 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 super-cleaned 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 PRE-TREATMENT

Before taking a sub-sample 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 Na_2SO_4 or $MgSO_4$ 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 hours. If there is any doubt, a second extraction step should be performed and the 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 (Dean *et al.*, 1995; Reimer and Suarez, 1995).

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 owing to 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 cm^3. 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 semi-polar 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 cm^3. 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 cm^3 acetonitrile for each cm^3 of extract and subsequent evaporation to 1–2 cm^3. 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 cm^3 of hexane must be added for each cm^3 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 always

to 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-12 (Appendix 3, 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 programs, 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 (polyether-etherketone) 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 of the column 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

Helium should preferably 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 (EI) 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, the reproducibility of retention times may not be as 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 an 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 distributed regularly 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 Section 4.2.3 of the General Guidelines (Part B-4.2.3 of the 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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APPENDIX 2

TECHNICAL NOTE ON THE DETERMINATION OF CHLOROBIPHENYLS IN SEDIMENTS

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* substituted 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 endosulfan. 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 criterion 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", Appendix 3 in Annex B-13.) Plastic materials (except polyethylene or polytetrafluorethene) must not be used for sampling due to adsorption of determinands to the container material.

The samples should be transported in closed containers, and 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. Glass fibre 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 PRE-TREATMENT

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 sub-sample 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 non-polar 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 a non-polar 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 a non-polar 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 a non-polar 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 non-polar 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 Na_2SO_3 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 be easily 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 removes 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 a non-polar extract over a column containing silica loaded with concentrated sulphuric acid.

5.3 Further Clean-up

As CBs are non-polar, clean-up using normal phase chromatography is the most appropriate technique for their separation from other compounds. Using a non-polar 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 non-polar 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. Owing to the use of aqueous solvents in reversed-phase HPLC, the samples have to be transferred several times between polar and non-polar 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

Owing to 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.

6.1 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.

6.2 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 non-polar, 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 because they do not provide sufficient reproducibility.

6.3 Carrier Gas

Hydrogen should preferably 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}.

6.4 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 autosampler 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.

6.5 Temperature Programming

The temperature program 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 program, 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.

6.6 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 deca-chlorinated 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 that for ECD.

6.7 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.

6.8 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., CB29, CB112, CB155, CB198, 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, the 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 achieved. The method for calculating the

limit of determination should follow the advice in Section 4.2.3 of the General Guidelines (Part B-4.2.3 of the 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.

9 REFERENCES

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APPENDIX 3

TECHNICAL NOTE ON THE DETERMINATION OF HEAVY METALS IN MARINE SEDIMENTS

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). These 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, clean glass or polyethylene (polypropylene/polystyrene) containers, which have been carefully labelled and pre-weighed. 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 sea water.

The tools and containers must be stored dust-free when not in use. A comprehensive description of cleaning procedures for plastic and glass laboratory ware can be found in Annex B-11, Appendix 1 “Technical notes on the determination of trace metals (Cd, Pb, Cu, Co, Zn, Ni, Fe) including mercury in sea water” 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 –20 ºC or below.

2 SAMPLE PRE-TREATMENT; 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 to 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 placing 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 B-16 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 the 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 in parallel with the samples.

3 CALIBRATION

For calibration purposes, single stock standard solutions at a concentration of 1000 mg dm^{-3}, purchased from a qualified manufacturer, can be used. Fresh stock standard 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 to 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 laboratory ware 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 spectrometry), 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 pH MEASUREMENT IN SEA WATER

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/Hg_2Cl_2). 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 (ISO, 1994).

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

4.1 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 bulb is by 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 HNO_3 for 30 minutes. Change the inner filling solution and let the electrode condition in storage solution for at least 1 hour before use.

4.2 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 (Radiometer, Copenhagen). 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, CO_2-free reagents and water must be used. CO_2 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 into 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 buffer 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

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ANNEX B-15

TECHNICAL NOTE ON MEASUREMENT OF TOTAL ALKALINITY IN SEA WATER

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 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 pH 4.5. The method is described in detail by Anderson *et al*. (1999), APHA (1995), and IS0 9963 (ISO, 1994).

3 SAMPLING

Seawater 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 should be prepared using CO_2-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 Annex B-14 “Technical Note on pH Measurement in Sea Water”.

Calibration

For calibration of the pH meter, see Annex B-14 "Technical Note 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.

Preparation of Hansson buffer:

Chemical	Solution A, 1 litre	Solution B, 1 litre
NaCl	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)
$MgCl_2(H_2O)_6$	54.4 mM (11.060 g)	54.4 mM (11.060 g)
$CaCl_2(H_2O)_2$	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 of solution A. Dilute to 50.00 ml with solution B.

The stability of the solutions is:

- 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 the colorimetric and the potentiometric methods. Check the accuracy of titrant addition in automatic titration systems.

Check the pH meter pH, for example, 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[3].

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 °C 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.

[3]Information regarding the nearest dealer of VKI Reference Materials can be obtained from ProLab, Denmark (telephone no.: (45) 45 76 79 76, fax no.: (45) 45 76 26 02).

6 REPORTING

Results are normally reported in mmol [HCO_3^{2-}] dm^{-3} (equals meq dm^{-3}) or in mg $CaCO_3$ dm^{-3}. Formulas for the calculation (APHA, 1995) are:

Total Alkalinity (meq dm^{-3}) = $1000 \times v_{HCl} \times t_{HCl}/v_b$

Total Alkalinity (mg $CaCO_3$ dm^{-3}) = $50{,}000 \times v_{HCl} \times t_{HCl}/v_{b,}$

where

v_{HCl} is the volume of HCl in ml,

t_{HCl} 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

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ANNEX B-16

TECHNICAL NOTE ON QUALITY ASSURANCE OF THE DETERMINATION AND DOCUMENTATION OF CO-FACTORS

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 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.

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 D.5 of the COMBINE Manual.

3 CO-FACTORS IN WATER ANALYSIS

Particulate material

The quantity of particulate material is determined by filtration through a filter according to the ISO 11923 standard (ISO, 1997).

Organic carbon

The method recommended is described in Annex B-17 of the COMBINE Manual.

Salinity

Salinity (and temperature) may be defined as a co-factor in investigations where the mixing of different water masses is studied or takes place. The same standard oceanographic equipment as described in the "Technical Note on the Determination of Salinity and Temperature of Sea Water" (Annex B-8, Appendix 1) is used, and the performance requirements will also be the same.

4 QUALITY ASSURANCE 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).

5 REFERENCES

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ANNEX B-17

TECHNICAL NOTE ON THE DETERMINATION OF ORGANIC CARBON IN SEA WATER

1 INTRODUCTION

1.1 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.

1.2 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 is there 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-}.

2 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 (see Grasshoff *et al*., 1999 and ISO, 1999).

It is important to obtain a representative sample, which under 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.

3 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.

4 SAMPLE PRE-TREATMENT

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.

5 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 frequently been 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 nitrogen 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 the 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.

6 CALIBRATION AND THE BLANK

The analysis of POC is most often carried out together with the analysis of PON (particulate organic nitrogen). For POC and PON determinations, the instrument is calibrated with high-purity acetanilide (analytical grade reagent). 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.

7 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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