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Monitoring organotins in marine biota

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This series presents detailed descriptions of methods and procedures relating to chemical and biological measurements in the marine environment. Most techniques described have been selected for documentation based on performance in ICES or other intercalibration or intercomparison exercises: they have been carefully evaluated and shown to yield good results when correctly applied. They have also been subject to review by relevant ICES working groups, but this is not to be construed as constituting official recommendation by the Council.

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Contents

Abstract

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These guidelines provide best practices for the measurement of organotin compounds in biota for monitoring programmes. Target compounds include tributyltin (TBT), dibutyltin (DBT), and monobutyltin (MBT) as well as triphenyltin (TPhT), diphenyltin (DPhT), and monophenyltin (MPhT). The bivalve *Mytilus edulis* can be a suitable target species for the monitoring of organotins. Sampling strategy as well as transportation and storage are important for the final quality of the data. Handling and pretreatment of the samples is also discussed. Several analytical methods can be used for the determination of organotin compounds. Critical steps, such as extraction and derivatization of the determinants, are discussed, followed by descriptions of the analytical detection techniques. Emphasis is placed on analytical quality control and quality assurance.

Keywords: Organotin, TBT, biota, sample pre-treatment, storage, extraction, clean-up, derivatization, calibration, gas chromatography, high-performance liquid chromatography

1 Introduction

Guidelines are required to support monitoring programmes for the marine environment under regional sea conventions, such as OSPAR and HELCOM (Roose and Brinkman, 2005), and European Commission directives, such as the Water Framework Directive (Quevauviller *et al*., 2008) and the Marine Strategy Framework Directive (Roose, 2008). This document is a supplement to general guidelines for monitoring contaminants in biota (e.g. OSPAR's JAMP Guidelines for Monitoring Contaminants in Biota (OSPAR, 1999)), focusing on specific techniques for organotin analysis. This is neither a complete description nor a substitute for detailed analytical instructions. The guideline recommends best practices for the measurement of organotin compounds in marine biota for monitoring programmes. Target compounds include tributyltin (TBT), dibutyltin (DBT), and monobutyltin (MBT), and triphenyltin (TPhT), diphenyltin (DPhT), and monophenyltin (MPhT).

2 Species

Target species for the monitoring of organotin compounds are shellfish, particularly bivalves such as *Mytilus edulis* or *Mytilus galloprovincialis*. *M. edulis* occurs in shallow waters along almost all coasts of the temperate North Atlantic and also in the Baltic Sea. It is therefore suitable for monitoring in near-shore waters. No distinction is made between *M. edulis* and *M. galloprovincialis* because the latter, which may occur along the coast from Spain and Portugal to the south coasts of the UK, cannot easily be discerned from *M. edulis*. The Pacific oyster (*Crassostrea gigas*) can be sampled in areas where *Mytilus* sp. are not available.

Gastropods can also be used as TBT indicators, for instance in relation to biological effect monitoring. However, gastropods do not feed as continuously as bivalves and have a higher capacity for TBT metabolism, which may result in a higher variability of TBT body burdens compared with bivalves. In addition, correlation between imposex and TBT body burdens in the environment can be low. This might be the result of imposex being induced irreversibly in early life stages when TBT levels were higher, or because of non-continuous feeding strategies. In some sensitive gastropod species, imposex can be induced by TBT at lower levels than analytical detection can generally achieve.

3 Sampling

General sampling guidelines for temporal trend and spatial monitoring are given in appropriate guidelines, such as HELCOM's *Manual for Marine Monitoring in the COMBINE Programme of HELCOM*, Part D (HELCOM, 2007a), and OSPAR's JAMP guidelines (OSPAR, 1999). References relevant to sampling and statistics include Gilbert (1987), Bignert *et al*. (1993, 1994), Nicholson and Fryer (1996), and Nicholson *et al.* (1997).

4 Transportation

Samples should be processed and kept cool as soon as possible after collection. Live mussels should be transported in closed containers at temperatures between 5°C and 15°C, preferably <10°C. Once samples have been depurated (see Section 5.2) and shucked (see Section 5.3), they may be frozen and then transported at temperatures

< −20°C. Samples for biological effects monitoring require more rigorous conditions (e.g. storage in liquid nitrogen).

5 Pretreatment and storage

5.1 Contamination

Sample contamination can occur during sampling, sample handling, pretreatment, and analysis (HELCOM, 1994), as a consequence of the environment, the containers or packing material used, the instruments used during sample preparation, or from the chemical reagents used during the analytical procedures. Controlled conditions are therefore required for all procedures, including the dissection of organisms on board ship.

5.2 Depuration

In order to remove any contaminated sediment particles from their intestinal system mussels should be depurated. They should be placed on a polyethylene tray elevated above the bottom of a glass aquarium. The aquarium should be filled with filtered subsurface seawater that has been collected from the same site as the samples and, if possible, not subjected to contamination from point sources. The aquarium should be aerated, and the mussels should be left for 20 to 24 h at water temperatures and salinities similar to those in the waters from which the samples were taken.

5.3 Opening of the shells

Mussels should be shucked live and opened with minimum tissue damage by detaching the adductor muscles from the interior of one valve. The mussels should be inverted and allowed to drain on a clean towel or funnel for at least 5 min in order to facilitate dry weight determination.

5.4 Dissection and storage

The soft tissues should be removed and deep-frozen (−20°C) as soon as possible in containers appropriate to the intended analysis. Although TBT is known to be stable in cockles and oysters stored at −20°C in the dark over a seven-month period, longer storage can cause a significant loss (Gomez-Ariza *et al*., 1999). Dissection of the soft tissue must be performed under clean conditions, on a clean bench, by trained personnel wearing clean gloves and using clean stainless-steel knives. Tools should be cleaned after the preparation of each sample. Washing in acetone or alcohol and high-purity water is recommended.

Homogenization should be performed immediately prior to any subdivision of the sample (see Section 6.5).

6 Analysis

6.1 Preparation of materials

Solvents, chemicals, and adsorption materials must be free of organotin compounds or other interfering compounds. Solvents should be checked for purity by concentrating the volume normally used in the procedure and then analysing it for the presence of organotin compounds and other interfering compounds. If necessary, the solvents can be purified by redistillation. Chemicals and adsorption materials should be purified by extraction and/or heating. Glass-fibre materials (e.g. thimbles for Soxhlet extraction) should be pre-extracted. Alternatively, full glass thimbles with a G1 glass filter at the bottom can be used. Generally, paper filters should not be used for filtration. Because all precleaned materials are prone to recontamination (e.g. by the adsorption of organotin compounds and other compounds from laboratory air), materials that are ready for use should not be stored for long periods. Materials containing PVC and/or silicone must be avoided. Glassware should be extensively washed with detergents, heated at >250°C, and rinsed with organic solvents (e.g. mixtures such as hexane/acetone) immediately before use. Alternatively, all glassware can be washed in 10% HCl (or even in concentrated HCl) and then rinsed with distilled water.

6.2 Lipid determination

Organotin data are not usually expressed on a lipid basis. Lipid content is not a good normalizer because of its poor correlations to organotin content. However, the determination of the lipid content of tissues can be useful in characterizing the samples. If required, the lipid content should be determined on a separate subsample of the tissue homogenate, because some of the extraction techniques used routinely for organotin determination may destroy lipid materials. The total fat weight should be determined using the method of Bligh and Dyer (1959), as modified by Hanson and Olley (1963), or an equivalent method such as that of Smedes (1999).

6.3 Dry weight determination

Dry weight determinations should be carried out by air-drying homogenized subsamples of the material to be analysed to constant weight at 105°C.

6.4 Calibration and preparation of standard solutions

6.4.1 External calibration

When using an external calibration, multilevel calibration with at least five calibration points is preferred to define adequately the calibration curve. Depending on the extraction/derivatization methods used, standards preparation can be done in either of two ways:

i) by using alkyltin salts and then proceeding to the derivatization step as for samples (for hydridization or ethylation followed by purge-and-trap analysis, there is no appropriate way other than using alkyltin salts);

ii) by using commercially readily available derivatized standards (e.g. QUASIMEME; available online at http://www.quasimeme.org/).

Standard solutions can be prepared in methanol, ethanol, or another solvent, depending on the instrumental method used. Addition of an internal standard (tripropyltin chloride (TPrTCl), or ^{13}C-labelled or deuterated TBT) to all standard and sample solu-

tions is recommended if using gas chromatography (GC) analysis with mass selective detection. When using TPrTCl, which is a non-derivatized standard, the recovery efficiency of the whole procedure can be determined. A new calibration solution should always be cross-checked to the previous standard solution. Standard solutions should be stored in a refrigerator in gas-tight containers to avoid evaporation of solvent. It is important to determine the expiry date of standard dilutions in order to avoid a concentration shift caused by deterioration of analytes or evaporation of solvents.

6.4.2 Isotope dilution–mass spectrometry

When using isotope dilution–mass spectrometry (ID-MS), external calibration is not required. Centineo *et al.* (2004) report a quantitative speciation method for analysis of MBT, DBT, and TBT using ID-MS.

6.5 Homogenization and drying

Homogenization should be carried out on fresh tissue. Care should be taken to ensure that the sample integrity is maintained during the actual homogenization and during drying. When the analysis is undertaken, all fluids, including lipids, which may initially separate on thawing, should be included with the materials that are homogenized. Freeze-drying is usually a good option because this process preserves concentrations of MBT, DBT, and TBT (Quevauviller, 1996). When grinding samples after drying, classic techniques using a ball mill can be used. References relevant to cryogenic homogenization include Iyengar (1976), Iyengar and Kasperek (1977), and Klussmann *et al.* (1985).

6.6 Extraction

The release of organotin compounds from the biological matrix is a critical step because of the strong matrix binding of the analytes and possible species degradation. Recovery standards should be added prior to extraction. However, correction procedures should be used with care because equilibration between the spiked and the target compounds is not always guaranteed.

Several extraction techniques are commonly used. Microwave-assisted extraction (MAE), as well as mechanical shaking, provides quantitative recoveries with negligible degradation of most butyltin compounds. However, degradation of DBT was reported with longer extraction times for MAE (Rodríguez-González *et al.*, 2004). Heating and/or sonication of the extract may enhance these extraction techniques. Alternatively, pressurised liquid extraction (accelerated solvent extraction) or solid-phase micro-extraction (SPME) can be used to extract organotin compounds (Brunori *et al.*, 2006).

Extraction is usually carried out in an aqueous methanolic acidic environment, with subsequent extraction to an organic phase, such as pentane or hexane. Acidic conditions enhance extraction efficiency; acetic acid is preferred to other acids in order to ensure the stability of butyltin compounds. Complexing agents, such as tropolone, are sometimes used to increase extraction yields; however, the effectiveness of the complexing agent should be carefully monitored (Narasaki *et al.*, 2000; Smedes *et al.*, 2000). Extraction can be performed on wet samples as well as on freeze-dried samples. However, wet tissue must be mixed with anhydrous sodium sulphate or other anhydrous materials prior to extraction.

6.7 Derivatization

The organotins in the sample extracts must be converted to alkylated or hydride derivatives for analysis.

6.7.1 Alkylation

There are three possible methods for generating alkylated derivatives of organotins.

1) **Grignard reagent**. A variety of Grignard reagents are used for alkylation reactions in derivatization. The smaller the alkylation group, the more volatile the products of derivatization, and the greater the losses that will occur during the transfer and work-up. This method is time consuming and requires very dry conditions and non-protic solvents. The use of Grignard reagents is hazardous because they react violently with protic solvents (e.g. water, acid, alcohol, and ketones) and therefore appropriate safety precautions must be taken. A liquid–liquid extraction step is necessary to isolate the derivatized organotins. However, unlike hydride derivatives of butyltins, which may degrade in hours or days, the tetra-alkyl derivatives formed with Grignard reagents are very stable (Morabito *et al.*, 2000). Derivatization with Grignard reagents include extra steps in the analytical procedure because a clean-up of excess Grignard reagent with acid is required.

2) **Sodium tetraethylborate ($NaBEt_4$)**. Derivatization with this complexing agent has been developed to reduce the analysis time. The $NaBEt_4$ procedure allows a simultaneous extraction–derivatization in a buffered medium (optimum pH 4–5). The derivatives produced by $NaBEt_4$ derivatization are more thermally stable. However, $NaBEt_4$ is extremely air-sensitive and, because it is considered pyrophoric, care must be taken to maintain its chemical integrity. Although solutions in water are stable for approximately 1 month at 4°C, it is advisable to prepare them freshly for use. Solutions of the reagent in an organic solvent (e.g. tetrahydrofuran, methanol, or ethanol) seem to be more stable (Smedes *et al.*, 2000). The determination of organotin compounds in complex matrices, such as biological matrices with high lipid content, has led to several problems, including low recovery and low derivatization efficiency. A clean-up step may be subsequently required. Sodium tetrapropylborate ($NaBPr_4$) can be used as an alternative derivatization agent similar to $NaBEt_4$.

3) **Sodium diethyldithiocarbamate (NaDDTC)**.This is preferable to Grignard reagents because it does not require anhydrous conditions, and it does not simultaneously derivatize and extract like $NaBEt_4$. This step can be combined with the Grignard reagent to provide better derivatization for a wider spectrum of organotins.

6.7.2 Hydride generation

The butyltin species are converted to a hydride form by sodium tetrahydroborate ($NaBH_4$). Hydride generation produces a large volume of hydrogen as a by-product, which facilitates the purging of butyltin hydrides from a large volume of sample.

6.8 Clean-up

The most commonly used clean-up methods involve the use of alumina or silica adsorption chromatography. For the latter, phenyltin compounds such as triphenyltin may not co-elute with butyltins. Gel-permeation chromatography (GPC) and similar

methods based on high-performance liquid chromatography (HPLC) methods are also employed. The major advantages of using HPLC-based clean-up methods are their ease of automation and reproducibility.

6.9 Preconcentration

Evaporation of solvents using a rotary evaporator should be performed under controlled temperature and pressure conditions. The sample volume should be kept above 2 ml; evaporation to total dryness should be avoided. To reduce the sample volume even more (e.g. to a final volume of 100 µl), solvents such as pentane or hexane can be removed by concentration with a gentle stream of nitrogen. Only nitrogen of a controlled high quality should be used. Iso-octane is recommended as a solvent for the final solution to be injected into the GC.

6.10 Instrumental determination

Most of the analytical techniques developed for the speciation of organotin compounds are based on GC. This remains the preferred separation technique owing to its high resolution and the availability of sensitive detectors. Alternatively, HPLC can be used, because it does not require derivatization of the organotin compounds.

6.10.1 Gas chromatography

The two injection modes commonly used in GC are splitless injection 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. A variety of detectors can be used, including: atomic absorption spectrometry (AAS); (pulsed) flame photometry ([P]FPD), using a 610 nm bandpass filter; electron-impact mode–mass spectrometry (EIM-MS) at 70eV; and inductively coupled plasma–mass spectrometry (ICP-MS; Takeuchi *et al.*, 2000). Helium must be used for GC-MS, GC-FPD, and GC-ICP-MS. The preferred column length is 25–30 m, with an internal diameter of 0.15–0.3 mm. Film thicknesses of 0.3–1 µm are generally used. The most commonly used stationary phase for organotin analysis is 5% phenyl methyl siloxane.

The ICP-MS and (P)FPD detectors have been applied widely because of their inherent selectivity and sensitivity. (P)FPD has been shown to have greater selectivity and lower detection limits (by a factor of 25 to 50 times) for organotin compounds other than those obtained with conventional FPD (Bravo *et al.*, 2004).

6.10.2 High-performance liquid chromatography

All stainless-steel parts of the HPLC system that come into contact with the sample should be replaced by polyether ketone (PEEK) components. Reverse-phase columns (e.g. octadecylsilane C18) are commonly used (Wahlen and Catterick, 2003), and the mobile phase can consist, for example, of a mixture of acetonitrile, water, and acetic acid with 0.05 % triethylamine, pH 3.1–3.4 (65 : 25 : 10 variable, depending on the columns used). Fluorescence or ultraviolet detection, as well as AAS, are commonly used. More recently, inductively coupled plasma–optical emission spectrometry (ICP-OES), inductively coupled plasma–mass spectrometry (ICP-MS), and MS detectors, such as atmospheric pressure chemical ionization–mass spectrometry (APCI-MS) and electrospray ionization–mass spectrometry (ESI-MS), have been used.

7 Quality Assurance

References of relevance to Quality Assurance (QA) procedures include QUASIMEME (1992), HELCOM (1994, 2007b), ICES (1996), OSPAR (1999), and Morabito *et al*. (1999).

7.1 System performance

The performance of the instrumentation should be monitored by regularly checking the resolution of two closely eluting organotin compounds. A decrease in resolution points to deteriorating instrumental conditions. A dirty MS source can be recognized by the presence of an elevated background signal together with a reduced signal-to-noise ratio. Chromatograms should be inspected visually by a trained operator.

7.2 Recovery

The recovery should be checked and reported. One method is to add an internal (recovery) standard (e.g. tripropyltin) to each sample immediately prior to extraction and a second (quantification) standard (e.g. tetrapropyltin) immediately prior to injection. Cassi *et al.* (2002) even used a third recovery standard to check the overall solvent-extraction efficiency. The recovery of MBT and MPhT may be lower than that of other organotin compounds, probably because of lower derivatization efficiency. For this, a monoalkyltin standard could be used as a recovery internal standard (Smedes *et al*., 2000).

When using the ID-MS technique, the loss of target analytes is compensated. However, the recovery should still be calculated and should be between 50% and 150%. Care also should be taken to ensure that complete isotope equilibration is achieved (Rodríguez-González *et al.*, 2004).

7.3 Standard solutions and calibration

See Section 6.4.

7.4 Blanks

A procedural blank should be analysed for each sample series and should be prepared simultaneously, using the same chemicals and solvents as for the samples. Its purpose is to indicate any contamination of the sample with interfering compounds, which will lead to errors in quantification. Even if an internal standard has been added to the blank at the beginning of the procedure, a quantification of peaks in the blank and subtraction from the values obtained for the determinants should be carefully considered.

7.5 Accuracy and precision

At least one laboratory reference material (LRM) should be included for each series of identically prepared samples. The LRM must be homogeneous and well characterized for the determinants in question, and stability tests must have demonstrated that it produces consistent results over time. The LRM should be the same type of matrix (e.g. liver, muscle tissue, fat or lean fish) as the samples, and the determinant concentrations should occur in a range comparable with those of the samples. If the range of determinant concentrations in the samples is large (> factor of 5), two reference materials should be included in each batch of analyses covering the lower and upper concentrations. 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 in order to check within-batch analytical variability. The primary pur-

pose of an LRM analysis is to check that the analytical method is under control and yields acceptable precision. In addition, a certified reference material (CRM, e.g. ERM-CE 477 (mussel, certified for TBT, DBT, MBT) or NIES No. 11 (fish tissue certified for TBT and non-certified reference value for TPhT)) of a similar matrix should be analysed periodically in order to check the method bias. A duplicate of at least one sample should be run with every batch of samples. Each laboratory should participate in interlaboratory comparison studies and proficiency testing schemes on a regular basis, preferably at an international level.

7.6 Data collection and transfer

Data collection, handling, and transfer must take place using quality-controlled procedures.

8 Data recording and reporting parameters

The calculation of results and the reporting of data can represent major sources of error, as has been demonstrated in intercomparison studies for organotin compounds (Wells, 2006). Control procedures should be established in order to ensure that data are correct and to avoid transcription errors. Data stored in databases should be checked and validated, and checks are also necessary when data are transferred between databases.

Data reporting should be in accordance with the requirements of the monitoring programme. Results should be reported according to the precision required for the programme. In practice, the number of significant figures is defined by the performance of the procedure.

The following parameters should be recorded.

1) Sampling and biological parameters (shellfish)
- location of sampling site (name, latitude, and longitude)
- date and time of sampling (GMT)
- sampling depth with respect to low tide (for subtidal sites only)
- irregularities and unusual conditions
- name and institution of sampling personnel
- number of pooled samples
- number of individuals in pool
- mean, minimum, and maximum length and standard deviation
- mean dry shell weight
- mean soft tissue weight (wet weight)
- condition index (CI), calculated as (mean soft tissue weight)/(mean length)^3

2) Analytical and quality assurance parameters
- LRM and CRM results for a set of organotin compounds, reported on a wet weight or dry weight basis
- descriptions of the extraction, cleaning, and instrumental determination methods
- mean tissue lipid weight and method of extraction
- the mean soft dry weight and method of determining water content if this differs from air-drying to constant weight at 105°C (if sufficient material is available)
- the detection limit or quantification limit for each organotin compound. Specific performance criteria, including detection (quantification) limits and precision, are usually set by the programme. A typical quantification limit for single contaminants is 1 $\mu g\,kg^{-1}$ wet weight, although this may be difficult to achieve for phenyltin compounds
- QA information according to the requirements specified in the programme

3) Lipids (if determined)

- total lipids (e.g. Bligh and Dyer, 1959, or Smedes, 1999) expressed as percentage or g kg^{-1} wet weight

4) Parameters

- organic contaminants of interest to monitoring programmes for which these guidelines apply: organotin compounds suite required for analysis
- butyltin compounds: TBT, DBT, and MBT, unit expressed as μg kg^{-1} as organotin-ion or as Sn-atom, reported on a wet weight or dry weight basis
- phenyltin compounds: TPhT, DPhT, and MPhT, unit expressed as μg kg^{-1} as organotin-ion or as Sn-atom, reported on the basis of wet weight or dry weight

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10 Abbreviations and technical terminology

AAS	atomic absorption spectrometry
APCI-MS	atmospheric pressure chemical ionization–mass spectrometry
CRM	certified reference material
EIM-MS	electron-impact mode–mass spectrometry
ESI-MS	electrospray ionization–mass spectrometry
FPD	flame photometric detector
GC	gas chromatograph
GC-FPD	gas chromatography–flame photometric detector
GC-ICP-MS	gas chromatography–inductively coupled plasma–mass spectrometry
GC-MS	gas chromatography–mass spectrometry
GPC	gel-permeation chromatography
HPLC	high performance liquid chromatography
ICP-MS	inductively coupled plasma–mass spectrometry
ICP-OES	inductively coupled plasma–optical emission spectrometry
ID-MS	isotope dilution–mass spectrometry
LRM	laboratory reference material
MAE	microwave-assisted extraction
MS	mass spectrometry
PEEK	polyether ketone
(P)FPD	(pulsed) flame photometric detection
QA	quality assurance
SPME	solid-phase micro-extraction

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