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COMMON IMPLEMENTATION STRATEGY FOR THE WATER FRAMEWORK DIRECTIVE (2000/60/EC)

Guidance Document No. 33

ON ANALYTICAL METHODS FOR BIOTA MONITORING UNDER THE WATER FRAMEWORK DIRECTIVE

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This technical document was drafted by the Joint Research Centre of the European Commission and edited to take account of comments from members of the Water Framework Directive Common Implementation Strategy (CIS) Working Group Chemicals. The document does not necessarily represent the official, formal position of any of the partners. Hence, the views expressed in the document do not necessarily represent the views of the European Commission.

Foreword

This Technical Guidance Document on Analytical Methods for Biota Monitoring aims to facilitate the implementation of environmental quality standards (EQS) in biota under the Water Framework Directive by addressing the extraction and analytical processes required for the priority substances to be determined in biota samples. It is Guidance Document No. 33 in the series of guidance documents prepared to support the Common Implementation Strategy (CIS) for the Water Framework Directive.

It complements Guidance Document No. 32, the Technical Guidance Document on Biota Monitoring (the Implementation of EQS_{biota}), which covers sampling strategies, and the two documents together thereby address the requirement for guidance on biota monitoring mentioned in Article 3(8a) of Directive 2008/105/EC as amended by Directive 2013/39/EU. They go beyond the information presented in Guidance Document No. 25 on Chemical Monitoring in Sediment and Biota under the Water Framework Directive.

The original Directive 2008/105/EC included biota standards for mercury, hexachlorobenzene and hexachlorobutadiene. In Directive 2013/39/EU, biota EQS were introduced for three other existing priority substances (fluoranthene, polyaromatic hydrocarbons and brominated diphenylethers), and set for four new priority substances (dicofol, perfluorooctane sulfonic acid and its derivatives, hexabromocyclododecane, and heptachlor/heptachlor epoxide). This guidance document considers analytical methods for all of these, as well as priority substances for which trend monitoring in sediment and/or biota is to be carried out according to Article 3(6).

This document constitutes guidance and Member States are therefore not legally required to follow the recommendations contained in it. Member States are, however, required to use methods compliant with the requirements of the Environmental Quality Standards Directive 2008/105/EC and the Quality Assurance/Quality Control Directive 2009/90/EC.

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List of Abbreviations and Symbols

Throughout this report the following abbreviations and symbols are used:

AAS	atomic absorption spectrometry
AES	atomic emission spectrometry
AFS	Atomic fluorescence spectrometry
AMA	advanced mercury analyzer
ASE	Accelerated solvent extraction
BCR	Bureau Communautaire de Reference
CRM	Certified Reference Material
CV	Cold-Vapour
DDT	1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane
DG	Directorate-General
DL	Dioxin-like
EC	European Commission
EPA	Environment Protection Agency
EQS	environmental quality standards
EU	European Union
FIMS	Flow injection mercury system
FPD	Flame photometric detector
GC	Gas Chromatography
GPC	Gel permeation chromatography
ICP	inductively coupled plasma
IDL	Instrumental Detection Limit
IES	Institute for Environment and Sustainability
IUPAC	International Union for Pure and Applied Chemistry
JRC	Joint Research Centre
LC	Liquid Chromatography
LoD	Limit of Detection
LoQ	Limit of Quantification
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
NCI	Negative chemical ionisation
PAH	PolyAromatic Hydrocarbon
PCB	PolyChlorinated Biphenyls
PFC	Perfluorinated compounds
PFASs	Perfluoroalkyl Substances
PCDD/F	PolyChlorinated Dibenzo-Dioxin/Furane
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonate
POP	Persistent organic pollutant
SLE	Solid liquid extraction
TOF	Time of flight
US	United States of America
WGS84	World Geodetic System 1984

Chemical elements are identified by the respective symbol according IUPAC

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1 Setting the scene

Directive 2008/105/EC on Environmental Quality Standards (EQS) in the field of water policy, as amended by Directive 2013/39/EU, contains in Part I of Annex I a series of EQS for substances in biota, as listed in Table 1 below.

Table 1 – Environmental quality standards for biota in Directive 2013/39/EU

No	Name of substance	CAS number	EQS for Biota (µg/kg)
(5)	Brominated diphenylethers (BDEs)	32534-91-9	0.0085
(15)	Fluoranthene	206-44-0	30
(16)	Hexachlorobenzene	118-74-1	10
(17)	Hexachlorobutadiene	87-68-3	55
(21)	Mercury (Hg) and its compounds	7439-97-6	20
(28)	Benzo(a)pyrene	50-32-8	5
(34)	Dicofol	115-32-3	33
(35)	Perfluorooctane sulfonic acid (PFOS)	1763-23-1	9.1
(37)	Dioxins and dioxin-like polychlorinated biphenyls (PCBs)		0.0065 (sum TEQ)
(43)	Hexabromocyclododecane (HBCD)	25637-99-4; 3194-55-6	167
(44)	Heptachlor and heptachlorepoxide	76-44-8 / 1024-57-3	0.0067

Unless otherwise indicated, the EQS for biota relate to fish, however no recommendation is given as to the species or whether the entire fish or only selected parts of it should be measured.

An alternative biota taxon, or another matrix, may be monitored, as long as the EQS applied provides an equivalent level of protection. For substances numbered 15 (fluoranthene) and 28 (polycyclic aromatic hydrocarbons - PAHs), the EQS for biota refer to crustaceans and molluscs. The monitoring of fluoranthene and PAHs in fish is not appropriate for the purpose of assessing chemical status.

In addition, the Directive stipulates that Member States shall arrange for the long-term trend analysis of concentrations of those priority substances that tend to accumulate in sediment and/or biota, giving particular consideration to the substances numbered 2, 5, 6, 7, 12, 15, 16, 17, 18, 20, 21, 26, 28, 30, 34, 35, 36, 37, 43 and 44 (see Table 2). Member States are also required to take measures aimed at ensuring that such concentrations do not significantly increase in sediment and/or relevant biota.

Member States shall determine the frequency of monitoring sediment and/or biota so as to provide sufficient data for a reliable long-term trend analysis. As a guideline, monitoring exercises should take place every three years, unless technical knowledge and expert judgment justify a different interval.

Article 8a of the Directive states that "*…technical guidelines on monitoring strategies and analytical methods for substances, including sampling and monitoring of biota, shall be developed, to the extent possible, by 22 December 2014, as part of the existing implementation process of Directive 2000/60/EC.*"

Table 2 – Method needs for biota analyses under the Water Framework Directive

No.	Name of substance	CAS number	EQS for Biota (µg/kg)
(2)	Anthracene	120-12-7	
(5)	Brominated diphenylethers (BDEs)	32534-91-9	0.0085
(6)	Cadmium and its compounds	7440-43-9	
(7)	C10-13 Chloroalkanes	85535-84-8	
(12)	Di(2-ethylhexyl)-phthalate (DEHP)	117-81-7	
(15)	Fluoranthene	206-44-0	30
(16)	Hexachlorobenzene	118-74-1	10
(17)	Hexachlorobutadiene	87-68-3	55
(18)	Hexachlorocyclohexane	608-73-1	
(20)	Lead and its compounds	7439-92-1	
(21)	Mercury (Hg) and its compounds	7439-97-6	20
(26)	Pentachlorobenzene	608-93-5	
(28)	Benzo(a)pyrene	50-32-8	5
(30)	Tributyltin compounds (TBTs)	36643-28-4	
(34)	Dicofol	115-32-3	33
(35)	Perfluorooctane sulfonic acid (PFOS)	1763-23-1	9.1
(36)	Quinoxyfen	124495-18-7	
(37)	Dioxins and dioxin-like PCBs		0.0065 (sum TEQ)
(43)	Hexabromocyclododecane (HBCDD)	25637-99-4; 3194-55-6	167
(44)	Heptachlor and heptachlorepoxide	76-44-8 / 1024-57-3	0.0067

A JRC report touched on some of the implied technical aspects (Loos, 2012) and contains some information on the analysis of several priority substances in a biota matrix.

However, Member States were found to need further assistance in the context of the Common Implementation Strategy (CIS), and the JRC was invited to propose further guidance on appropriate analytical methods on the aforementioned EQS for biota.

This document was drafted by the Commission's in-house science service, the Joint Research Centre, and has been edited in response to comments from Member States and other Stakeholders as part of the Common Implementation Strategy process under the Water Framework Directive.

2 Review of available methods

To better assess the current state of the art, existing methods including several standard methods were reviewed. These methods, which were developed mostly for food and feed analyses, are reported below.

The methods are discussed in view of the applicability of EQS for biota for the substances (see Chapter 3) listed in Table 1. The names or titles of methods refer rather to groups of compounds rather than to single substances, e.g. "*Non-fatty foods – Multiresidue methods for the gas chromatographic determination of pesticide residues*". Not all the European standard methods referred to have yet been finalised because of the lengthy standardisation process involved. As the Commission's in-house science service, the JRC has been able to access them in the Commission/CEN Internal database. The US Environment Protection Agency (EPA) methods were retrieved from the Internet. In Chapter 3, the information retrieved is updated with some selected methods published in recent academic and research papers.

2.1 Principles of biota extraction and analysis

Generally, procedures for the analysis of organic contaminants in biota includes homogenisation, lyophilization, extraction with organic solvents, removal or destruction of lipids, clean-up, fractionation and gas or liquid chromatographic (GC or LC) separation and electron capture, fluorescence detection (for PAHs), or mass-spectrometric detection (OSPAR Commission). For each of these steps, different combinations of methods have to be considered.

For the analysis of organic contaminants (such as brominated diphenylethers (BDEs), PAHs, PCBs, or organochlorine pesticides (OCPs)) both high- and low-resolution gas chromatography mass spectrometry (GC-MS) can be used in conjunction with either electron ionisation (EI) or electron capture negative ionisation (ECNI). Although gas chromatography - high-resolution mass spectrometry with electron impact ionisation (GC-HRMS) is the best method to unambiguously identify and quantify PCBs and BDEs in environmental samples, the expense and limited availability means that most laboratories use low-resolution GC-MS, generally in ECNI mode. GC-ECNI-MS is used most frequently for the analysis of BDEs in environmental samples (OSPAR Commission).

Next to conventional GC-MS, the use of the ion-trap in tandem with the MS^2 option – which improves selectivity - is receiving increased attention. The use of GC-ion-trap-MS provides a less expensive alternative to high-resolution mass spectrometry (HRMS), which is commonly used to determine dioxins / furans (PCDD/Fs) and, as such, is also ideally suited for the detection of all PCB groups. Another technique which is becoming more frequently used is time-of-flight (TOF) – MS (OSPAR Commission).

Extraction techniques commonly used are Soxhlet extraction, pressurised liquid extraction (PLE), supercritical fluid extraction (SFE), ultrasonic extraction, or alkaline digestion followed by liquid-liquid extraction with an organic solvent (OSPAR Commission).

Tissue extracts will always contain many compounds other than the target compounds, and a suitable clean-up - usually by deactivated alumina, silica or Florisil adsorption chromatography, or gel permeation chromatography (GPC) and HPLC based methods - is necessary to remove those compounds which may interfere with the subsequent analysis (OSPAR Commission).

Analysis of trace metals in biota generally includes homogenisation, drying, decomposition (digestion), dissolution, matrix separation and detection using element-specific spectrometric instrumental procedures such as atomic absorption spectrometry (AAS - cold vapour flame and/or graphite furnace), inductively coupled plasma-mass spectrometry (ICP-MS), atomic emission (ICP-AES), x-ray fluorescence (XRF), or neutron activation analysis (NAA) (OSPAR Commission). In the case of organo-metallic compounds, the same analytical principles apply as those used for organics.

While the analytical principle in detection and quantification of a given chemical structure is in most cases independent of the matrix of origin, it is legitimate to focus on animal tissue as regards the sample pre-treatment and clean-up prior to analysis. However, information derived from the comparison to non-fatty matrices, e.g. plant tissue, is useful and helps to better understand an applicability of methods. As a matter of fact, while fatty matrices tend to accumulate apolar compounds, e.g. as the investigated PAHs or PBDEs, respective methods for non-fatty matrices have to cope with the challenge of lower concentrations of these analytes. Although these methods cannot be directly used for aquatic fauna, it is justified to include them in this assessment. Adoption can usually be easily obtained by inclusion of fat-removal clean-up steps, e.g. using gel-permeation.

2.2 Individual methods

2.2.1 prEN 16619 "Food analysis - Determination of benzo[a]pyrene, benz[a]anthracene, chrysene and benzo[b]fluoranthene in foodstuffs by gas chromatography mass spectrometry (GC-MS)"

This European Standard specifies a method for the determination of four of the 15+1 EU priority polycyclic aromatic hydrocarbons (PAHs), identified as target PAHs. They are benz[*a*]anthracene (BaA), benzo[*a*]pyrene (BaP), benzo[*b*]fluoranthene (BbF) and chrysene (CHR). The method allows their quantification in the presence of the other 12 EU priority PAHs (benzo[*j*]fluoranthene (BjF), cyclopenta[*cd*]pyrene (CPP), benzo[*k*]fluoranthene (BkF), dibenz[*a,h*]anthracene (DhA), benzo[*c*]fluorene (BcL), dibenzo[*a,e*]pyrene (DeP), benzo[*ghi*]perylene (BgP), dibenzo[*a,h*]pyrene (DhP), dibenzo[*a,i*]pyrene (DiP), dibenzo[*a,l*]pyrene (DlP), indeno[1,2,3-*cd*]pyrene (IcP), 5-methylchrysene (5MC)) in extruded wheat flour, smoked fish, dry infant formula, sausage meat, freeze-dried mussels, edible oil and wheat flour, by gas chromatography mass spectrometry (GC-MS). The extraction of PAHs from solid samples is performed by pressurised liquid extraction (PLE). Soxhlet extraction may be applied as an alternative to PLE. The sample is cleaned up by applying size exclusion chromatography (SEC) followed by solid phase extraction (SPE).

The method has been validated in an interlaboratory study via the analysis of both naturally contaminated and spiked samples, ranging from 0.5 µg/kg to 11.9 µg/kg. However, linearity of the instrument's response was proven for the concentration range 0.5 µg/kg to 20 µg/kg. For the determination of PAHs in edible fats and oils, two other CEN standards are also available: EN ISO 22959 and EN ISO 15753.

Applicability:
Possibly applicable to identify the substances anthracene (2), fluoranthene (15), and Benzo(a)pyrene (28) in biota.

2.2.2 prEN 15637 "Foods of plant origin - Determination of pesticide residues using LC-MS/MS following methanol extraction and clean-up using diatomaceous earth"

This draft European Standard describes a method for the analysis of pesticide residues in foods of plant origin, such as fruits vegetables, cereals, nuts as well as processed products including dried fruits. The method has been collaboratively studied in a large number of commodity/pesticide combinations. The sample is extracted with methanol after addition of some water. After partition into dichloromethane, the organic phase is evaporated and the residue is reconstituted with methanol. Quantification of pesticide residues is performed by liquid chromatography with tandem mass spectrometric detection, using electrospray ionisation. To achieve the required selectivity, the mass spectrometer is operated in the selected reaction monitoring (SRM) mode. Unfortunately, no indication of method sensitivity (i.e. limit of detection, quantification) is provided.

Applicability:
Possibly applicable to identify the substance quinoxyfen (36) in biota.

2.2.3 prCEN/TR 15641 "Food analysis - Determination of pesticide residues by LC-MS/MS - Tandem mass spectrometric parameters"

This Technical Report lists mass spectrometric parameters which are useful for the application of European Standards for the determination of pesticide residues in foods of plant origin that use LC-MS/MS such as the standards:

- prEN 15637 ("Foods of plant origin - Determination of pesticide residues using LC-MS/MS following methanol extraction and clean-up using diatomaceous earth")

  and the standard

- prEN 15662 ("Foods of plant origin - Determination of pesticide residues using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning – QuEChERC-method").

No indication of method sensitivity (i.e. limit of detection, quantification) is reported.

Applicability:
Possibly applicable to identify the substances dicofol (34), quinoxyfen (36), heptachlor and heptachlor-epoxide (44) in biota.

2.2.4 prEN 12393-1 "Non-fatty foods – Multiresidue methods for the gas chromatographic determination of pesticide residues - Part 1: General considerations"

This European Standard gives general considerations for the determination of pesticide residues in non-fatty foods. Each method described in this European Standard is suitable for identifying and quantifying a definite range of those organohalogen, and/or organophosphorus and/or organonitrogen pesticides which occur as residues in foodstuffs of plant origin. This European Standard contains the following methods that have been subjected to interlaboratory studies and/or are adopted throughout Europe:

- method L: Extraction with acetone, liquid-liquid partition with dichloromethane and clean-up on a silica-gel/charcoal column;
- method M: Extraction with acetone and liquid-liquid partition with dichloromethane/light petroleum, if necessary clean-up on Florisil®;
- method N: Extraction with acetone, liquid-liquid partition with dichloromethane and clean-up with gel permeation and silica gel chromatography;
- method O: Extraction with acetonitrile, liquid-liquid partition with light petroleum and clean-up on a Florisil column;
- method P: Extraction of organophosphorus compounds with ethyl acetate and, if necessary, clean-up with gel permeation chromatography.

The conditions for application is given for each of the five methods L to P for residue analysis of organohalogen, organophosphorus and organonitrogen pesticides, respectively.

Gas chromatography (GC) with selective detectors may be used: electron-capture detection (ECD) for organohalogen, thermionic detector (NPD, P-mode or N/P mode) for organophosphorus and organonitrogen compounds and flame-photometric detector (FPD) for organophosphorus and organosulfurous pesticides. Hall detector (ECHD), atomic emission detector (AED) and mass spectrometry (MS) may also be used for a large class of pesticides. Procedures are given to confirm the identity and quantity of observed residues, particularly in those cases where it would appear that the maximum residue limit (MRL) has been exceeded.

No indication of method sensitivity (i.e. limit of detection, quantification) is reported.

Applicability:
Possibly applicable to identify the substances dicofol (34), quinoxyfen (36), heptachlor and heptachlor-epoxide (44) in biota.

2.2.5 prEN 12393-2 "Foods of plant origin - Multiresidue methods for the determination of pesticide residues by GC or LC-MS/MS – Part 2: Methods for extraction and clean-up"

This European Standard specifies methods for the extraction and clean-up of food samples of plant origin for the quantitative determination of pesticide residues. Different solvents can be used for this purpose. These pesticide residues are generally associated with other co-extracted compounds, which would interfere with the analysis. To purify the crude extracts to be analysed, several techniques can be used. This European Standard contains the following extraction and clean-up methods that have been subjected to interlaboratory studies and/or are adopted throughout Europe:

- method M: Extraction with acetone and liquid-liquid partition with dichloromethane/light petroleum, if necessary clean-up on Florisil®;
- method N: Extraction with acetone, liquid-liquid partition with dichloromethane or cyclohexane/ethyl acetate and clean-up with gel permeation and silica gel chromatography;
- method P: Extraction with ethyl acetate, and if necessary, clean-up by gel permeation chromatography.

This European Standard specifies the details of methods M to P for the extraction and the clean-up of food samples of plant origin. Several solvents at different volumes are used for extraction. Clean-up techniques are listed such as liquid-liquid partition, liquid chromatography on various adsorbents and gel permeation chromatography.

A table providing the couples (matrix/pesticide) which have been submitted to collaborative studies and a list of indicative applicability of the method to different pesticides are given for each method, wherever possible.

No indication of method sensitivity (i.e. limit of detection, quantification) is reported.

Applicability:
Possibly applicable to identify the substances dicofol (34), quinoxyfen (36), heptachlor and heptachlor-epoxide (44) in biota.

2.2.6 prEN 13805 "Foodstuffs - Determination of trace elements – Pressure Digestion"

This European Standard specifies a method for the pressure digestion of foodstuffs intended for the determination of trace elements. This method has been collaboratively tested in combination with atomic absorption (flame, furnace, hydride, cold-vapour) techniques and ICP-MS. Other techniques such as e.g. ICPOES, voltammetry or atomic fluorescence can be used in combination with this standard.

The method is a physicochemical pressure digestion method used to mineralise the sample material and to prepare a measurement solution containing trace elements to be determined. The method described here is applied when the measurement has been validated in combination with this digestion method and reference is made to this standard. This procedure will relate to the total element content depending on reagents and determination procedures used. The sample is homogenised, avoiding contamination. Afterwards it is digested with nitric acid (sometimes with the addition of other acids), at high temperatures in a pressure vessel, applying conventional or microwave-assisted heating.

No indication of method sensitivity (i.e. limit of detection, quantification) is reported.

Applicability:
Possibly applicable to identify the substances cadmium and its compounds (6), lead and its compounds (20), mercury and its compounds (21) in biota.

2.2.7 prEN 13806 "Foodstuffs - Determination of trace elements - Determination of mercury by cold-vapour atomic absorption spectrometry (CVAAS) after pressure digestion"

This European Standard specifies a method for the determination of mercury in foodstuffs by cold-vapour atomic absorption spectrometry (CVAAS) after pressure digestion. Specific foodstuffs for which European Standards exist are excluded from the scope of this horizontal European Standard.

The test solution is transferred to the reaction vessel of the mercury analysis unit, and the mercury is reduced with divalent tin or sodium borohydride and flushed into the cuvette of the AAS instrument using a carrier gas stream. The absorption at 253.7 nm (mercury line) is used as a measure of the mercury concentration in the cuvette. If the amounts of mercury in the test solution are very small, it is advisable to enrich the mercury expelled on a gold/platinum gauze (amalgam technique) prior to determination in the cuvette.

The limit of quantification according to prEN 13804 of the measuring solution depends on the following parameters:

- principle of release of mercury (batch- or flow system);
- enrichment (amalgam) or no enrichment;
- in the case of flow systems:
- continuous/discontinuous release of Hg;
- amount of digestion solution used;
- construction of the equipment;
- influences of the matrix.

The limit of quantification is regularly in the range between 0.05 µg/l and 5 µg/L, with regard to the measuring solution. With a test portion of 0.5 g and a final digestion volume of 20 ml, the limit of quantification for the foodstuff will be calculated between 0.002 mg/kg and 0.2 mg/kg.

Applicability:
Possibly applicable to identify the substance mercury and its compounds (21) in biota.

2.2.8 prEN 14082 Foodstuffs - Determination of trace elements - Determination of lead, cadmium, zinc, copper, iron and chromium by atomic absorption spectrometry (AAS) after dry ashing

This draft European Standard specifies a method for the determination of lead, cadmium, zinc, copper, iron and chromium in foodstuffs by atomic absorption spectrometry (AAS) after dry ashing at 450°C. The method is applicable to various types of foodstuffs. It has been successfully tested in an interlaboratory trial in which 16 laboratories participated. Foodstuffs covered by the method include composite diets, cereals, fish, fruit, liver and milk.

The samples are dry ashed under a gradual increase in temperature to 450°C. Hydrochloric acid is added, and the solution obtained evaporated to leave a dry residue. This residue is dissolved in c (0.1 mole/l) nitric acid, and the metal contents are determined using flame or graphite furnace atomic absorption spectrometry-procedures.

No limits of quantification or detection are specified and need to be established by each laboratory using the method.

Applicability:
Possibly applicable to identify the substances cadmium and its compounds (6), lead and its compounds (20) in biota.

2.2.9 prEN 14083 Foodstuffs - Determination of trace elements - Determination of lead, cadmium, chromium and molybdenum by graphite furnace atomic absorption spectrometry (GFAAS) after pressure digestion

This draft European Standard specifies a method for the determination of lead, cadmium, chromium and molybdenum in foodstuffs by graphite furnace atomic absorption spectrometry (GFAAS) after pressure digestion. It describes the determination of the elements in the test solution by graphite furnace atomic absorption spectrometry (GFAAS) after pressure digestion, according to prEN 13805.

Achievable limits of quantification have been set for samples of 0.5 g and 2 g. For lead, a LoQ of 0.16 mg/kg (with 0.5g test portion) and of 0.04 mg/ kg (with 2 g test portion) have been reported in the standard. For cadmium, the LoQ is 0.016 mg/kg (at 0.5 g) and 0.004 mg/kg (at 2 g).

Applicability:
Possibly applicable to identify the substances cadmium and its compounds (6), lead and its compounds (20) in biota.

2.2.10 prEN 14084 Foodstuffs - Determination of trace elements - Determination of lead, cadmium, zinc, copper and iron by atomic absorption spectrometry (AAS) after microwave digestion

This draft European Standard specifies a method for the determination of lead, cadmium, zinc, copper and iron in foodstuffs by atomic absorption spectrometry (AAS) after microwave digestion. The method is applicable to various types of foodstuffs. The method is not applicable to oils, fats and other extremely fatty products. The method has been successfully tested in an interlaboratory trial in which 16 laboratories participated. Foodstuffs covered by the method include composite diets, cereals, fish, beef, milk and fungi.

The samples are digested in closed vessels in a microwave oven in a mixture of nitric acid and hydrogen peroxide. The resulting solution is diluted with water, and the metal contents are determined by flame or graphite furnace atomic absorption spectrometry procedures.

The detection and quantification limits should be estimated for each element according to prEN 13804: 1999, taking into account the SD found in the long-term evaluation. No further information is provided.

Applicability:
Possibly applicable to identify the substances cadmium and its compounds (6), lead and its compounds (20) in biota.

2.2.11 prEN 15763 Foodstuffs - Determination of trace elements - Determination of arsenic, cadmium, mercury and lead in foodstuffs by inductively coupled plasma mass spectrometry (ICP-MS) after pressure digestion

This European Standard specifies a method for the determination of arsenic, cadmium, mercury and lead in foodstuffs by inductively coupled plasma mass spectromerty (ICP-MS). The collaborative study included foodstuffs having an arsenic content ranging from 0.06 mg/kg to 21.5 mg/kg dry matter (d.m.), cadmium ranging from 0.03mg/kg to 28.3mg/kg d.m., mercury ranging from 0.04 mg/kg to 0.56mg/kg d.m., and lead from 0.01 mg/kg to 2.4 mg/kg d.m.

The test solution, obtained by pressure digestion, is nebulised and the aerosol transferred to a high frequency inductively coupled argon plasma (ICP). The high temperature of the plasma is used to dry the aerosol and to atomise and ionise the elements. The ions are extracted from the plasma by a set of sampler and skimmer cones, and transferred to a mass spectrometer where the ions are separated by their mass/charge ratio and determined by a pulse-count and/or analogue detector.

The detection and quantification limits should be estimated for each element according to prEN 13804: 1999, taking into account the SD found in the long-term evaluation. No further information is provided.

Applicability:
Possibly applicable to identify the substances cadmium and its compounds (6), lead and its compounds (20), mercury and its compounds (21) in biota.

2.2.12 prEN 15741 "Animal feedstuffs - Determination of OC-pesticides and PCB's by GC/MS."

This European Standard specifies a gas chromatographic/mass spectrometric method for the determination of organochlorine pesticides (OCs) and polychlorinated biphenyls (PCBs) in animal feedstuffs and oil. The method is applicable to animal feedstuffs with a water content up to about 20 wt%, a fat content up to about 10 wt%, and oil/fatty samples containing residues of one or more of the following OCs and PCBs and some of their isomers and degradation products:

- Aldrin
- Dieldrin
- Chlordane (= sum of Chlordane isomers and Oxychlordane)
- DDT (= sum of isomers op'-DDT, pp'-DDT, pp'-TDE (pp'-DDD), and pp'-DDE)
- Endosulfan (sum of α-/β-isomers and Endosulphanesulphate)
- Endrin
- Heptachlor (= sum of Heptachlor and β-Heptachlorepoxide)
- Hexachlorobenzene (HCB)
- Hexachlorocyclohexane isomers α-HCH (α-BHC), β-HCH (β-BHC), γ-HCH (γ-BHC or lindane)
- PCB 28, 52, 101, 118, 138, 153 and 180 ("Indicator PCBs") and PCB-198, 209.

The method is not yet applicable to Chlorocamphene (Toxaphene), a complex mixture of polychlorinated camphenes. Chlorocamphene has a very distinctive chromatographic profile and is easily recognisable by GC/ECD. Positive identification of the toxaphene isomers can be performed by negative chemical ionisation mass spectrometry (NCI-MS), electron impact tandem mass spectrometry (EI-MSxMS) or electron impact high resolution mass spectrometry (EI-HRMS), which is not within the scope of this method. The limit of

quantification for the organochlorine pesticides and PCBs is 5 ng/g in general. However, 10 ng/g applies for Heptachlor Aldrin, Endrin, Dieldrin, and Endosulfan (α, β and sulphate). Individual laboratories are responsible for ensuring that the equipment they use will yield results within these quantification limits.

A test portion of animal feedstuff is fortified with internal standard (PCB-198), and is extracted with ethyl acetate. The extract is concentrated and subsequently purified by: Gel permeation chromatography (GPC), with cyclohexane/ethyl acetate as eluting solvent and chromatography on partially deactivated silica gel. The collected fraction containing the compounds of interest is concentrated and re-dissolved in a solution containing another internal standard (PCB 209) as a reference standard. After concentration, an aliquot of the extract is injected into a GC-MS, using a splitless injector (an alternative here is PTV injection.

No indication of method sensitivity (i.e. limit of detection, quantification) is reported.

Applicability:
Possibly applicable to identify the substances hexachlorobenzene (16), heptachlor and heptachloreoxide (44), dicofol (34) in biota.

2.2.13 prEN 15742 "Animal feedstuffs - Determination of OC-pesticides and PCB's by GC/ECD"

This European Standard specifies a gas chromatographic method with electron capture detection (ECD) for the determination of organochlorine pesticides (OCs) and polychlorinated biphenyls (PCBs) in animal feedstuffs. The method is applicable to animal feedstuffs with a water content up to about 20 wt%, a fat content up to about 10 wt%, and oil/fatty samples containing residues of one or more of the following OCs, PCBs, toxaphene and some of their isomers and degradation products:

- Aldrin
- Dieldrin
- Chlorocamphene (Toxaphene)
- Chlordane (= sum of Chlordane isomers and Oxychlordane)
- DDT (= sum of isomers op'-DDT, pp'-DDT, pp'-TDE (pp'-DDD), and pp'-DDE)
- Endosulfan (sum of α-/β-isomers and Endosulphanesulphate)
- Endrin
- Heptachlor (= sum of Heptachlor and β-Heptachlorepoxide)
- Hexachlorobenzene (HCB)
- Hexachlorocyclohexane isomers α-HCH (α-BHC), β-HCH (β-BHC), γ -HCH (γ -BHC or lindane) PCB 28, 52, 101, 118, 138, 153 and 180 ("Indicator PCBs") and PCB 198, 209.

A test portion of animal feedstuff is fortified with internal standard (PCB-198), and is extracted with ethyl acetate. The extract is concentrated and subsequently purified by Gel permeation chromatography (GPC), with cyclohexane/ethyl acetate using as eluting solvent and chromatography on partially deactivated silica gel. The collected fraction containing the compounds of interest is concentrated and re-dissolved in a solution containing another internal standard (PCB 209) as a reference standard. After clean-up, the analytes are measured using GC-ECD. Identification is made by comparing retention times on capillary columns of different polarity. Quantification is made using the internal standard method.

The limit of quantification for the mentioned organochlorine pesticides and PCBs is 5 ng/g in general. However 10 ng/g applies for Heptachlor, Aldrin, Endrin, Dieldrin, and Endosulfan (α-

, β- and sulphate). Individual laboratories are responsible for ensuring that the equipment they used will yield results that are within these quantification limits.

Applicability:
Possibly applicable to identify the substances hexachlorobenzene (16), heptachlor and heptachloreoxide (44), dicofol (34) in biota.

2.2.14 EPA Method 1613 "Tetra- through Octa-Chlorinated Dioxins and Furans by Isotope Dilution HRGC/HRMS"

This method is for the determination of tetra- through octa-chlorinated dibenzo-*p*-dioxins (CDDs) and dibenzofurans (CDFs) in water, soil, sediment, sludge, tissue, and other sample matrices by high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS).

2.2.14.1 Solid, semi-solid, and multi-phase samples (excluding tissue)

The labelled compounds are spiked into a sample containing 10 g (dry weight) of solids. Samples containing multiple phases are pressure filtered and any aqueous liquid is discarded. Coarse solids are ground or homogenised. Any non-aqueous liquid from multi-phase samples is combined with the solids and extracted in an SDS extractor. The extract is concentrated for clean-up.

2.2.14.2 Fish and other tissue

The sample is extracted using one of two procedures:

- Soxhlet or SDS extraction—A 20 g aliquot of sample is homogenised, and a 10 g aliquot is spiked with the labelled compounds. The sample is mixed with sodium sulphate, allowed to dry for 12-24 hours, and extracted for 18-24 hours using methylene chloride:hexane (1:1) in a Soxhlet extractor. The extract is evaporated to dryness, and the lipid content is determined.
- HCl digestion—A 20 g aliquot is homogenised, and a 10 g aliquot is placed in a bottle and spiked with the labelled compounds. After equilibration, 200 ml of hydrochloric acid and 200 ml of methylene chloride:hexane (1:1) are added, and the bottle is shaken for 12-24 hours.

The extract is evaporated to dryness, and the lipid content is determined.

After extraction, ^{37}Cl-labelled 2,3,7,8-TCDD is added to each extract to measure the efficiency of the clean-up process. Sample clean-ups may include back-extraction with acid and/or base, gel permeation, alumina, silica gel, Florisil and activated carbon chromatography. High-performance liquid chromatography (HPLC) can be used for further isolation of the 2,3,7,8-isomers or other specific isomers or congeners. Prior to the clean-up procedures cited above, tissue extracts are cleaned up using an anthropogenic isolation column, a batch silica gel adsorption, or sulphuric acid and base back-extraction, depending on the tissue extraction procedure used.

After clean-up, the extract is concentrated to near dryness. Immediately prior to injection, internal standards are added to each extract, and an aliquot of the extract is injected into the gas chromatograph. The analytes are separated by the GC and detected by a high resolution (³10,000) mass spectrometer. Two exact m/z' are monitored for each analyte.

According to the method, the Minimum Levels (ML - defined for each analyte as the level at which the entire analytical system) must give a recognisable signal and accepted calibration point. It is equivalent to the concentration of the lowest calibration standard, assuming that all method-specified sample weights, volumes and clean-up procedures have been employed) are shown below:

Table 3 – Minimum levels established by EPA 1613

Analyte	ML Solid (ng/kg, ppt)	WHO 2005 TEF	WHO 2005 Dioxin estimated ML (ng/kg)
2,3,7,8-TCDD	1	1	1
1,2,3,7,8-PeCDD	5	1	5
1,2,3,4,7,8-HxCDD	5	0.1	0.5
1,2,3,6,7,8-HxCDD	5	0.1	0.5
1,2,3,7,8,9-HxCDD	5	0.1	0.5
1,2,3,4,6,7,8-HpCDD	5	0.01	0.05
OCDD	10	0.0003	0.003
2,3,7,8-TCDF	1	0.1	0.1
1,2,3,7,8-PeCDF	5	0.03	0.15
2,3,4,7,8-PeCDF	5	0.3	1.5
1,2,3,4,7,8-HxCDF	5	0.1	0.5
1,2,3,6,7,8-HxCDF	5	0.1	0.5
1,2,3,7,8,9-HxCDF	5	0.1	0.5
2,3,4,6,7,8-HxCDF	5	0.1	0.5
1,2,3,4,6,7,8-HpCDF	5	0.01	0.05
1,2,3,4,7,8,9-HpCDF	5	0.01	0.05
OCDF	10	0.0003	0.003
		TEQ (µg/kg)	0.011

Applicability:
Possibly applicable to identify the substances dioxins and dioxin-like PCBs (37) in biota

2.2.15 EPA Method 1614 "Brominated Diphenylethers in Water Soil, Sediment and Tissue by HRGC/HRMS"

EPA Method 1614 ("Method 1614"; the "Method") is for the determination of brominated diphenyl ether (BDE) congeners in water, soil, sediment, biosolids, tissue, and other sample matrices by high resolution gas chromatography combined with high resolution mass spectrometry (HRGC/HRMS).

2.2.15.1 Extraction, concentration, and clean-up of solid, semi-solid, and multi-phase samples (excluding tissue)

The labelled compounds are spiked into a sample containing 10 g (dry weight) of solids. Samples containing multiple phases are pressure filtered and any aqueous liquid is discarded. Coarse solids are ground or homogenised. Any non-aqueous liquid from multi-phase samples is combined with the solids and extracted in a Soxhlet/Dean-Stark extractor.

2.2.15.2 Extraction, concentration, and clean-up of Fish and other tissue

A 20-g aliquot of sample is homogenised, and a 10-g aliquot is spiked with the labelled compounds. The sample is mixed with anhydrous sodium sulphate, dried for a minimum of 30 minutes, and extracted for 18-24 hours using methylene chloride in a Soxhlet extractor. The extract is evaporated to dryness, and the lipid content is determined.

After extraction, a labelled clean-up standard is spiked into the extract and the extract is concentrated. Tissue extracts are first cleaned up using an anthropogenic isolation column, and all extracts are cleaned up using back-extraction with sulphuric acid and/or base, gel permeation, silica gel, and/or Florisil or alumina chromatography, as required.

After clean-up, the extract is concentrated to 20 µL and labelled internal standards are injected. An aliquot of the extract is injected into the gas chromatograph (GC). The analytes are separated by the GC and detected by a high-resolution (5,000) mass spectrometer. Two exact m/z_s are monitored at each level of bromination (LOB) throughout a pre-determined retention time window.

2.2.15.3 Performance

According to the method, the LOD and ML are the following:

Table 4 – LOD and ML established for EPA Method 1614

Analyte PBDE N°	LOD (µg/kg)	ML (µg/kg)
28	0.002	0.005
47	0.0025	0.01
99	0.004	0.01
100	0.002	0.005
153	0.002	0.005
154	0.002	0.005

Applicability:
Possibly applicable to determine the existence of brominated diphenylethers (5) in biota.

2.2.16 *EPA Method 1699 "Pesticides in Water, Soil, Sediment, Biosolids, and Tissue by HRGC/HRMS"*

EPA Method 1699 determines organochlorine, organophosphorus, triazine, and pyrethroid pesticides in environmental samples by high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS) using isotope dilution and internal standard quantification techniques. This method has been developed for use with aqueous, solid, tissue and biosolid matrices.

2.2.16.1 Solid, semi-solid, and multi-phase samples (excluding municipal sludge and tissue)

The labelled compounds are spiked into a sample containing 10 g (dry weight) of solids. Samples containing multiple phases are pressure filtered, and any aqueous liquid is discarded. Coarse solids are ground or homogenised. Any non-aqueous liquid from multi-phase samples is combined with the solids and extracted with methylene chloride, methylene chloride:hexane (1:1) or acetone:hexane (1:1) in a Soxhlet extractor or with toluene in a Soxhlet/Dean-Stark (SDS) extractor.

Municipal sludges are homogenised, spiked with labelled compounds, and Soxhlet extracted with dichloromethane.

2.2.16.2 Fish and other tissue

A 20-g aliquot of sample is homogenised, and a 10-g aliquot is spiked with the labelled compounds. The sample is mixed with anhydrous sodium sulphate, allowed to dry for 30 minutes minimum, and extracted for 18 - 24 hours using methylene chloride in a Soxhlet extractor. The extract is evaporated to dryness, and the lipid content is determined.

Extracts are macro-concentrated using rotary evaporation, a heating mantle, or a Kuderna-Danish evaporator. Extracts to be injected into the HRGC/HRMS are concentrated to a final volume of 20 µL using nitrogen evaporation (blowdown).

2.2.16.3 Clean-up

Extracts of aqueous, solid or mixed phase samples are cleaned up using an aminopropyl SPE column followed by a microsilica column. Extracts may be further cleaned up using gel permeation chromatography (GPC) or solid-phase cartridge techniques. Extracts in which only the organo-chlorine pesticides are to be determined may be further cleaned up using silica gel, Florisil, or alumina chromatography. Immediately prior to injection, a labelled injection internal standard is added to each extract and an aliquot of the extract is injected into the gas chromatograph (GC). The analytes are separated by the GC and detected by a high-resolution (≥8,000) mass spectrometer. Two exact m/z'_s for each pesticide are monitored throughout a pre-determined retention time window.

According to the method, the LOD and ML are the following:

Table 5 – LOD and ML established for EPA Method 1699

Analyte	Method Detection Limit (ng/kg)	Minimum Level of Quantification (ng/kg)
Aldrin	0.6	10
DDT o, p	0.3	5
DDT p, p	0.3	5
Dieldrin	0.5	5
Endosulfan-alfa	-	-
Endosulfan-beta	-	-
Endosulfan-sulphate	11	50
Endrin	0.4	5
Heptachlor	-	-
Heptachlor epoxide	0.3	5
Hexachlorobenzene	1.9	5
Chlorpyriphos	2	10
Atrazine	-	-
Cypermethrin	2.4	20

Applicability:
Possibly applicable to identify the substances heptachlor and heptachlorepoxyde (44) in biota.

2.2.17 EPA Method 1668 Revision A "Chlorinated Biphenyl Congeners in Water, Soil, Sediment, and Tissue by HRGC/HRMS"

2.2.17.1 Solid, semi-solid, and multi-phase samples (excluding tissue)

The labelled compounds are spiked into a sample containing 10 g (dry weight) of solids. Samples containing multiple phases are pressure filtered and any aqueous liquid is discarded. Coarse solids are ground or homogenised. Any non-aqueous liquid from multi-phase samples is combined with the solids and extracted in a Soxhlet/Dean-Stark (SDS) extractor. The extract is concentrated for clean-up.

2.2.17.2 Fish and other tissue

A 20-g aliquot of sample is homogenised, and a 10-g aliquot is spiked with the labelled compounds. The sample is mixed with anhydrous sodium sulphate, allowed to dry for 12-24 hours, and extracted for 18-24 hours using methylene chloride:hexane (1:1) in a Soxhlet extractor. The extract is evaporated to dryness, and the lipid content is determined.

After extraction, a labelled clean-up standard is spiked into the extract which is then cleaned up using back-extraction with sulphuric acid and/or base, gel permeation, silica gel, or Florisil chromatography. Activated carbon and high-performance liquid chromatography (HPLC) can be used for further isolation of specific congener groups. Prior to the clean-up procedures cited above, tissue extracts are cleaned up using an anthropogenic isolation column.

After clean-up, the extract is concentrated to 20 µL. Immediately prior to injection, labelled recovery standards are injected into each extract and an aliquot of the extract is injected into the gas chromatograph (GC). The analytes are separated by the GC and detected by a high-resolution mass spectrometer. Two exact m/z's are monitored at each level of chlorination (LOC) throughout a pre-determined retention time window.

Table 6 - – LOD and LOQ established for EPA Method 1668

Analyte	LOD (ng/kg)	LOQ (ng/kg)	WHO 2005 TEF	WHO 2005 DL-PCB estimated LOD (ng/kg)	WHO 2005 DL-PCB estimated LOQ (ng/kg)
PCB 77	17	50	0.0001	0.0017	0.005
PCB 81	18	50	0.0003	0.0054	0.015
PCB 105	11	20	0.00003	0.00033	0.0006
PCB 114	12	50	0.00003	0.00036	0.0015
PCB 118	19	50	0.00003	0.00057	0.0015
PCB 123	15	50	0.00003	0.00045	0.0015
PCB 126	14	50	0.1	1.4	5
PCB 156	13	50	0.00003	0.00039	0.0015
PCB 157	13	50	0.00003	0.00039	0.0015
PCB 167	11	50	0.00003	0.00033	0.0015
PCB 169	16	50	0.03	0.48	1.5

Analyte	LOD (ng/kg)	LOQ (ng/kg)	WHO 2005 TEF	WHO 2005 DL-PCB estimated LOD (ng/kg)	WHO 2005 DL-PCB estimated LOQ (ng/kg)
PCB 189	18	50	0.00003	0.00054	0.0015
			TEQ (µg/kg)	0.002	0.007

Applicability:
Possibly applicable to identify the substances dioxins and dioxin-like PCBs (37) in biota.

2.2.18 EPA Method 8323 "Determination of organotins by micro-liquid chromatography – ion trap mass spectrometry"

This method covers the use of solid-phase extraction (SPE) discs, solvent extractions (for biological tissues), and micro-liquid chromatography (µLC) coupled with electrospray ion trap mass spectrometry (ES-ITMS). This technique would also be applicable to ES-quadrupole mass spectrometry (ES-MS) for the determination of organotins (as the cation) in waters and biological tissues. The following compounds can be determined by this method: Tributyltin chloride, Dibutyltin dichloride, Monobutyltin trichloride, Triphenyltin chloride, Diphenyltin dichloride, Monophenyltin trichloride.

Method 8323 is designed to detect the organotin compounds (as the cation) without the use of hydrolysis and derivatisation in the extraction procedure. The compounds listed in this method were chosen for analysis by µLC-ES-ITMS because they have been designated as problem compounds that are hard to analyse using gas chromatographic methods The sensitivity of this method is dependent upon the level of interference within a given matrix, and varies with compound class and even by compound within a class. Additionally, the sensitivity is dependent upon the mobile phase used with the µLC, as well as the electrospray voltages and tuning parameters used in optimising the ES-ITMS.

This method provides reversed-phase micro-liquid chromatographic (µLC) and electrospray (ES) mass spectrometric (MS) conditions for the detection of the target analytes. Sample extracts can be analysed by direct injection into the electrospray (though interference is possible) or through a liquid chromatographic-electrospray interface. A gradient elution program is used on the chromatograph to separate the compounds.

Quantitative analysis may be performed by µLC-ES-ITMS, using an external standard approach. µ-LC-ES-ITMS detection is to be performed in the positive ionisation mode, with either an ion-trap mass spectrometer or a single-quadrupole mass spectrometer. In some cases, the electrospray interface may introduce variability that leads to less precise quantification.

Prior to analysis, appropriate sample preparation techniques must be used. Tissue samples (e.g. fish, brain), are extracted by using a solvent mixture of hexane:acetic acid:tropolone (99:1:0.1 v/v). After sonication is completed, the sample is adjusted to pH 2 with a small quantity of 12N HCl. The acidified sample is then centrifuged for approximately thirty minutes.

Electrospray ionisation is considered to be a "soft" ionisation technique. Consequently, few ions are produced, usually the molecular ion plus some adduct ion from the mobile phase solutions. Based on the fact that 120Sn - tin has ten isotopes, electrospray ionisation produces a distinctive mass spectral pattern. However, if further confirmatory analysis is warranted, this can then be carried out by performing MS/MS experiments (for those analysts using an ion-trap mass spectrometer) on those compounds of uncertain identity.

With a sample intake of 0.5 g of tissue, 750 pg of TBT can be detected, which corresponds to a limit of detection of 1.56 µg/kg.

Applicability:
Possibly applicable to identify the substance tributyltin compounds (30) in biota

2.2.19 JRC Method "Automated clean-up method for the determination of PCBs and PBDEs in fish"

10g of reference fish were lyophilised and submitted to the extraction process. The extraction was carried out by Soxhlet for 24h with a mixture of acetone/n-hexane 1/1 after spiking with 13C-labelled internal standards (12 Dioxin Like and 7 indicators PCBs and in accordance with IUPAC nomenclature: BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, BDE-183; BDE-197, BDE-207 and BDE-209)

The extract was dried under a nitrogen flow and the lipid content was diluted to 5 ml with a mixture of cyclohexane/ethyl acetate 1/1 and injected into a 5 ml loop of automated GPC system. The GPC column was 2.5 cm x 32 cm filled with BioBeads SX-3 resin working at a flow rate of 5 ml/min. using cyclohexane/ethyl acetate 1/1. The eluate was collected between 23:30 and 45 min. (107 ml). The sample collected was concentrated under nitrogen flow to 0.5 ml and then diluted with n-hexane to 5 ml. These 5 ml were loaded automatically on the acid silica/neutral silica column and eluted with 75 ml of n-hexane at a flow rate of 6 ml/min. This fraction was collected, concentrated to 100 µl, spiked with 13C-labelled recovery standards (PCB-111, PCB-170, BDE-126 and BDE-206) and submitted for instrumental analysis. The instrumental analyses of PBDEs and PCBs were based on isotope dilution using HRGC-HRMS (high resolution gas chromatography – high resolution mass spectrometry) for quantification on the basis of EPA16146 and EPA 16687. PBDEs were analysed on a double HRGC coupled with a DFS high resolution mass spectrometer HRMS operating in the EI-mode at 45 eV with a resolution of >10 000.

Mono-ortho PCBs and Indicator-PCBs were analysed on a GC coupled with a VG Autospec Ultima high resolution mass spectrometer operating in EI-mode at 36 eV with a resolution of >10 000.

Applicability:
Possibly applicable to identify the substances brominated diphenylethers (5), dioxins and dioxin-like PCBs (37) in biota.

Table 7 – Synopsis of methods reviewed.

Compound class	Matrix	Amount - Extraction - Clean Up	Instrument	LOD/LOQ	Reference
Benzo[a]pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene	food, freeze-dried mussels	5 g , Pressurised Liquid Extraction (PLE) with Hexane 100% or cyclohexane 100%; Soxhlet extraction may be applied as alternative to PLE, Size Exclusion chromatography (SEC) followed by SPE	GC-MS	0.5 µg/kg	prEN 16619 (2.2.1)
Pesticides	food of plant origin (fruits vegetables, cereals, nuts, dried fruits)	10 g test portion; 5 g homogenised portion Extraction with Methanol after addition of some water Solid supported liquid extraction; partition into dichloromethane	LC-MS/MS		prEN15637 (2.2.2)
Pesticides, including quinoxyfen	food of plant origin (fruits vegetables, cereals, nuts, dried fruits)	Standard lists only mass spectrometric parameters	LC-MS/MS		peCEN/TR 15641 (2.2.3)
Pesticides including p,p'-DDT, cis-Heptachlor epoxide, trans-Heptachlor epoxide, quinoxyfen	food samples of plant origin	100 g, Extraction with 200 ml Acetone and liquid-liquid partition on 80 ml of extract with 200 ml dichlorometahne/light petroleum if necessary clean-up with Fluorisil	GC-MS LC-MS/MS		prEN 12393-2 (2.2.5)
Pesticides including p,p'-DDT, cis-Heptachlor epoxide, trans-Heptachlor epoxide, quinoxyfen	food samples of plant origin	100 g (relevant if water content of the matrix is greater than 70%, Extraction with 200 ml acetone, liquid-liquid partition on 200 ml extract with 100 ml dichloromethane or cyclohexane/ethyl acetate, clean-up with gel permeation and silica gel	GC-MS, LC-MS/MS		prEN 12393-2 (2.2.5)
Pesticides including p,p'-DDT, cis-Heptachlor epoxide, trans-Heptachlor epoxide, quinoxyfen	food samples of plant origin	10 g Extraction with 20 ml ethyl acetate if necessary clean-up with gel permeation chromatography	GC-MS, LC-MS/MS		prEN 12393-2 (2.2.5)
Organohalogen, organophosphorus and organonitrogen pesticides	Non-fatty food	Extraction with acetone followed by liquid-liquid partition with dichloromethane clean-up upon silica gel/charcoal column	GC-MS		prEN 12393-1 (2.2.4)
Organohalogen, organophosphorus and organonitrogen pesticides	Non-fatty food	Extraction with acetone followed by liquid-liquid partition with dichloromethane/light petroleum if necessary clean-up on Florisil	GC-MS		prEN 12393- 1 (2.2.4)
Organohalogen, organophosphorus and organonitrogen pesticides	Non-fatty food	Extraction with acetone followed by liquid-liquid partition with dichloromethane clean-up with gel permeation and silica gel chromatography	GC-MS		prEN 12393-1 (2.2.4)
Organohalogen, organophosphorus and organonitrogen pesticides	Non-fatty food	Extraction with acetonitrile followed by liquid-liquid partition with light petroleum clean-up on Florisil	GC-MS		prEN 12393-1 (2.2.4)
Organotins	Biological tissues	0.5 – 2. G, Extraction by using a solvent mixture of hexane:acetic acid:tropolone (99:1:0.1 v/v), sonication plus adjustment to pH 2, Centrifugation, solvent reduction, no further clean-up	LC-ITMS	1.56 µg/kg	EPA Method 8323 (2.2.18)
Organohalogen, organophosphorus and organonitrogen pesticides	Non-fatty food	Extraction of organophosphorous compounds with ethyl acetate if necessary clean-up with gel permeation chromatography	GC-MS		prEN 12393-1 (2.2.4)

Table 7 – Synopsis of methods reviewed.

Compound class	Matrix	Amount - Extraction - Clean Up	Instrument	LOD/LOQ	Reference
Trace elements	Foodstuffs	depending on the volume of the digestion vessel and the carbon content of the samples (from 0.2 up to 3 g), Acidic digestion	Not applicable		prEN 13805 (2.2.6)
Trace elements	Foodstuffs	0.5 to 2 g, pressure digestion	AAS		prEN 14082 (2.2.8)
Trace elements	Foodstuffs	0.5 to 2 g, pressure digestion	GF-AAS	Pb: 0.04-0.16 mg/kg Cd: 0.004-0.016 mg/kg	prEN 14083 (2.2.9)
Trace elements	Foodstuffs	0.5 to 2 g, micro-wave assisted digestion	AAS		prEN 14084 (2.2.10)
Trace elements	Foodstuffs	0.5 to 2 g, pressure digestion	ICP-MS		prEN 15763 (2.2.11)
Mercury	Foodstuffs	Pressure digestion	Cold-vapour atomic absorption spectrometry (CVAAS)	Between 0,002 mg/kg and 0,2 mg/kg	prEN 13806 (2.2.7)
PAHs, phthalates, BPA, octylphenol, nonylphenols branched, nonylphenol monoethoxylated, nonylohenol diethoxylated, PBDE, PCB, DDE, DDD	homogenised and freeze-dried mussel samples	1 g freeze dried and homogenised, 2 x 10 ml dichloromethane/hexane and 1 x 10 ml dichloromethane/acetone, Florisil (5g) SPE cartridge: Conditioning: 20 ml hexane/dichlorometahen (1;1, v/v%) and 20 ml hexane. Elution: with 15 ml hexane/dichloromethane (1:1, v/v) and with 15 ml hexane/acetone (1:1, v/v)	GC-EI-MS/MS	B(a)P: 21 µg/kg; DEHP: 16 µg/kg; BDE 28: 3 µg/kg BDE 47: 0.9 µg/kg BDE 99: 1.2 µg/kg BDE 100: 3 µg/kg BDE 153: 2 µg/kg BDE 154: 5 µg/kg PCB 28: 4 µg/kg PCB 52: 13 µg/kg PCB 101: 16 µg/kg PCB 118: 13 µg/kg PCB 138: 19 µg/kg PCB 153: 12 µg/kg PCB 180: 6 µg/kg	Sánchez-Avila *et al.* (2011) (4.2)
PAHs, phthalates, BPA, octylphenol, nonylphenols branched, nonylphenol monoethoxylated, nonylohenol diethoxylated, BDE, PCB, 2,4'-DDE, 4,4'-DDE, 2,4'-DDD	homogenised and freeze-dried mussel samples	1 g freeze dried and homogenised, 2 x 10 ml dichloromethane/hexane and 1 x 10 ml hexane/acetone, Florisil (5g) SPE cartridge: Conditioning: 20 ml hexane/dichlorometahen (1;1, v/v%)and 20 ml hexane. Elution: with 15 ml hexane/dichloromethane (1:1, v/v) and with 15 mL hexane/acetone (1:1, v/v)	GC-EI-MS/MS	B(a)P: 21 µg/kg; DEHP: 16 µg/kg; BDE 28: 3 µg/kg BDE 47: 0.9 µg/kg BDE 99: 1.2 µg/kg BDE 100: 3 µg/kg BDE 153: 2 µg/kg BDE 154: 5 µg/kg PCB 28: 4 µg/kg PCB 52: 13 µg/kg PCB 101: 16 µg/kg PCB 118: 13 µg/kg PCB 138: 19 µg/kg PCB 153: 12 µg/kg PCB 180: 6 µg/kg	Sánchez-Avila *et al.* (2011) (4.2)

Table 7 – Synopsis of methods reviewed.

Compound class	Matrix	Amount - Extraction - Clean Up	Instrument	LOD/LOQ	Reference
PAHs, phthalates, BPA, octylphenol, nonylphenols branched, nonylphenol monoethoxylated, nonylohenol diethoxylated, PBDE, PCB, DDE, DDD	homogenised and freeze-dried mussel samples	1 g freeze dried and homogenised, 1 x 10ml dichloromethane/hexane and 2 x 10 ml hexane/acetone, Florisil (5 g) SPE cartridge: Conditioning: 20 ml hexane/dichlorometahen (1;1, v/v%)and 20 ml hexane. Elution: with 15 ml hexane/dichloromethane (1:1, v/v) and with 15 ml hexane/acetone (1:1, v/v)	GC-EI-MS/MS	B(a)P: 21 µg/kg; DEHP: 16 µg/kg; BDE 28: 3 µg/kg BDE 47: 0.9 µg/kg BDE 99: 1.2 µg/kg BDE 100: 3 µg/kg BDE 153: 2 µg/kg BDE 154: 5 µg/kg PCB 28: 4 µg/kg PCB 52: 13 µg/kg PCB 101: 16 µg/kg PCB 118: 13 µg/kg PCB 138: 19 µg/kg PCB 153: 12 µg/kg PCB 180: 6 µg/kg	Sánchez-Avila *et al.* (2011) (4.2)
Aldrin dieldrin, endrin, isodrin, HCH alfa, beta, delta; a-endosulfan, b-endosulfan, heptachlor, heptachlor epoxide hexachllorobenzene, p,p'-DDT, pentachlorobenzene, chlorfenvinphos, chlorpyrifos, PAHs, BDE 28, 47, 99, 100, 153, 154, PCB 8, 20, 28, 35, 52, 101, 118, 138, 153, 180, alachlor, atrazine, diuron isoproturon, trifluralin	river water	200 ml + 10 g NaCl, Stir bar sorptive extraction (SBSE) with PDMS stir bars	GC-MS/MS	Aldrin: 0.25 ng/L Dieldrin: 0.25 ng/L Endrin: 1.50 ng/L Isodrin: 0.50 ng/L HCHalfa: 0.25 ng/L HCHbeta: 1.50 ng/L HCHdelta: 1.5 ng/L a-endosulfan: 0.25 ng/L b-endosulfan: 1.50 ng/L heptachlor: 0.25 ng/L heptachlor epoxide: 0.25 ng/L hexachlorobenzene: 2.50 ng/L p,p'-DDT: 0.25 ng/L pentachlorobenzene 0.25 ng/L chlorfenvinphos 5 ng/L chlorpyrifos 5 ng/L PAHs (na), BDE 28, 47, 99, 100, 153, 154, (0.14 ng/L) PCB 8, 20, 28, 35, 52, 101, 118, 138, 153, 180 (10 ng/L) alachlor 10 ng/L atrazine 10 ng/L diuron 10 ng/L isoproturon 10 ng/L simazien 10 ng/L trifluralin 10 ng/L	Camino-Sánchez *et al.* (2012) (4.3)
Tetra- through octa-chlorinated dibenzo-p-dioxins (CDDs) and dibenzofurans (CDFs)	Fish and other tissues	10 g solid (dry weight) of solid, Soxhlet or SDS extraction ; HCl digestion, Back-extraction with acid or base and gel permeation, alumina silica gel, Florisil and activated carbon chromatography. Prior to clean up: anthropogenic isolation column, a batch silica gel absorption or sulphuric acid and base back extraction, depending on the tissue extraction procedure.	GC-HRMS	*See* Table 3	EPA 1613 (2.2.14)

Table 7 – Synopsis of methods reviewed.

Compound class	Matrix	Amount - Extraction - Clean Up	Instrument	LOD/LOQ	Reference
PBDE	Water, Soil, Sediment and Tissue	20 g homogenised fish or other tissue, Soxhlet with methylene chloride, Anthropogenic isolation column; back extraction with sulphuric acid and/or base and gel permeation, silica gel and/or Florisiland/or Allumina chromatography	HRGC/HRMS	PBDE 28: 0.002 µg/kg PBDE 47: 0.0025 µg/kg PBDE 99: 0.004 µg/kg PBDE 100: 0.002 µg/kg PBDE 153: 0.002 µg/kg PBDE 154: 0.002 µg/kg	EPA 1614 (2.2.15)
Pesticides (organochlorine, organophosphorous, triazine and pyrethroid pesticides)	Water, Soil, Sediment, Biosolid and Tissue	10 g (dry weight), Soxhlet with methylene chloride, GPC or SPE; if organochlorine persticids only have to be determined: clean up using silica gel , Florisil or Alumina chromatography	HRGC/HRMS	See Table 5	EPA 1699 (2.2.16)
PCBs	Water, Soil, Sediment, Biosolid and Tissue	10 g (dry weight), Soxhlet with methylene chloride:hexane 1:1, back extraction with sulphuric acid and/or base and gel permeation, silica gel and/or Florisil chromatography. Activated carbon can be used for further isolation of specific congener groups.	HRGC/HRMS	See Table 6	EPA 1668 Revision A (2.2.17)
TBT	Water, Soil, Sediment, Biosolid and Tissue	0.5 g of tissue	LC-ES-ITMS	1.56 µg/kg	EPA 8323 (2.2.18)
29 pesticides, indicators and DL-PCBs, PCDD/Fs	fish	10 g, Soxhlet with acetone:hexane 1:1, In line Gel Permeation chromatography, acid silica/neutral silica , basic alumina and active carbon columns clean-up steps	HRGC/HRMS		JRC (G.Mariani *et al.* 2009) (4.1)
PCB and PBDE	fish	10 g, Soxhlet with acetone:hexane 1:1, In line Gel Permeation chromatography, acid silica/neutral silica , basic alumina and active carbon columns clean-up steps	HRGC/HRMS		JRC (G.Mariani *et al.* 2009) (4.1)
Organochlorine pesticides and PCBs (aldrin, dieldrin, Chlordane, DDT, endosulfanm endrinm Heptachlor, Hexachlorobenzene, Hexachlorocyclohexane isomers, PCB 28, 52, 101, 118, 138, 153 and 180, and PCB 198-209	animal feedstuffs and oil	Ethylacetate, GPC with cyclohexane/ethyl acetate and chromatography on partially deactivated silica gel (with 3.5% water)	GC-MS (Splitless injection)	5 ng/g (10 ng/g for Heptachlor, Aldrin, Endrin, Ddieldrin and Endosulfan (α and β and sulphate)	prEn 15741 (2.2.12)
Organochlorine pesticides and PCBs (aldrin, dieldrin, Chlordane, DDT, endosulfanm endrinm Heptachlor, Hexachlorobenzene, Hexachlorocyclohexane isomers, PCB 28, 52, 101, 118, 138, 153 and 180, and PCB 198-209	animal feedstuffs and oil	Ethylacetate, GPC with cyclohexane/ethyl acetate and chromatography on partially deactivated silica gel (with 3.5% water)	GC-ECD	5 ng/g (10 ng/g for Heptachlor, Aldrin, Endrin, Ddieldrin and Endosulfan α and β and and sulphate)	prEN 15742 (2.2.13)

3 Assessment of applicability

Based on the review of the methods mentioned above, this chapter analyses the applicability of appropriate methods for the implementation of the Water Framework Directive (WFD) Environmental Quality Standards (EQS) for biota. Additional methods used in selected research papers are also reviewed. Possible gaps and needs for standardisation are highlighted.

3.1 Cadmium and its compounds

No EQS for biota have been set for cadmium and its compounds, but these are frequently measured in aquatic biota. Some selected examples are shown in Table 8. The same applies to heavy metal analyses in the food and feed sector, usually employing ICP-techniques or AAS after acid digestion.

In most cases, AAS techniques feature lower detection limits than ICP-AES, and offer the advantage of economy and selectivity compared to ICP-MS. On the other hand, ICP techniques allow for multi-parameter measurements.

Table 8 – Selected examples of analytical methods for cadmium and its compounds taken from literature

Analysis principle	Extraction (species)	LOD / LOQ (µg/kg)	Reference
AAS	Microwave digestion (fish)	Not reported	Vieira *et al. (*2011)
AAS	Microwave digestion (fish and shellfish)	0.5	Olmedo *et al.* (2013)
ICP-MS	Microwave digestion (fish)	3.30	Djedjibegovic *et al.* (2012)
AAS	Microwave digestion (mussels)	0.10	Kucuksezgin *et al.* (2013)
AAS	Acid digestion	40	Noël *et al.* (2011)

Conclusion: Considering the availability of both standardised and exploratory methods in the food/feed sector, no further method development is required. However, it is suggested that performance levels of applicable food standard methods (Table 7) be verified.

3.2 Lead and its compounds

As in the case of cadmium, no specific EQS for lead in biota has been established. Many methods are available for analysing lead and its compounds, which usually use ICP or AAS detection principles following acid digestion.

Conclusion: Considering the availability of both standardised and exploratory methods in the food/feed sector, no further method development is required. A verification of performance levels of applicable food standard methods (Table 7) is suggested.

Table 9 – Selected examples of analytical methods for lead and its compounds taken from literature

Analysis principle	Extraction (species)	LOD / LOQ (µg/kg)	Reference
AAS	Microwave digestion (fish)	Not reported	Vieira *et al.* (2011)
ICP-MS	Microwave digestion (fish)	2.70	Djedjibegovic *et al.* (2012)
AAS	Acid digestion	20	Noël *et al.* (2011)
AAS	Microwave digestion (fish and shellfish)	14.5	Olmedo *et al.*(2013)

3.3 Mercury and its compounds

For mercury and its compounds, a EQS for biota of 20 µg/kg has been set. Numerous methods for measuring total mercury and organo-mercury compounds exist. Amongst the most frequently used techniques for total mercury determination, gold amalgamation AAS has to be mentioned. The determination of methyl-mercury in biota, however, remains analytically challenging. Speciation techniques are usually based on hyphenated approaches using LC techniques, but also GC is used for separation prior to injection into an appropriate detector.

From the reviewed literature, it appears that a sufficiently broad range of a variety of analytical procedures exists for aquatic biota, many of which meet the required minimum criteria for LOD established by Directive 2009/90/EC.

prEN 13806 is a potentially applicable standard featuring LOD in the range of 2 to 200 µg/kg. The current state of the art allows for the implementation of the EQS for biota regarding mercury and its compounds, without entailing significant costs.

Table 10 – Selected examples of analytical methods for mercury and its compounds in aquatic biota.

Extraction (species)	Analysis	LOD / LOQ (µg/kg)	Reference
SLE digestion (Hg, methyl-Hg, ethyl-Hg, phenyl-Hg in seafood)	HPLC separation, post column microwave digestion, and cold-vapour atomic fluorescence spectrometry (CVAFS) detection	0.14-0.30	Liang *et al.* (2003)
SLE digestion (Hg, methyl-Hg)	HPLC-ICP-MS	5 (Hg) 7 (methyl-Hg)	Hight and Cheng (2006)
Solvent (fish)	AAS	10	Branco *et al.* (2007)
Solvent (fish)	AAS-FIMS	1	Katner *et al.* (2010)
Digestion (fish)	FIMS mercury analyser	2	Burger and Gochfeld (2011)
SLE digestion; derivatisation with aqueous NaBPr4, headspace solid-phase microextraction (methyl-Hg in fish) **SLE digestion (total Hg in fish)**	Gold amalgamation AAS (total Hg) GC-AFS (methyl-Hg)	0.7 (Hg) 0.13 (methyl-Hg)	Carrasco *et al.* (2011)
Microwave digestion (fish)	ICP-MS	10	Jürgens *et al.* (2013)
Microwave digestion (mussels)	AAS	0.05	Kucuksezgin *et al.* (2013)

Extraction (species)	Analysis	LOD / LOQ (µg/kg)	Reference
Microwave digestion (total Hg and methyl-Hg in fish and shellfish)	AAS (total Hg) HPLC-ICP-MS (methyl-Hg)	0.04 (total Hg)	Olmedo *et al.* (2013)
SLE digestion (methyl-Hg in fish)	Aqueous-phase ethylation purge-and-trap GC with cold vapour atomic fluorescence spectrometer	Not reported	Clayden *et al.*, (2013)
Digestion (fish)	Single gold trap amalgamation technique (total Hg); Aqueous-phase ethylation purge-and-trap GC with cold vapour atomic fluorescence spectrometer (methyl-Hg)	0.2-1.1	Wang *et al.* (2013)
Microwave digestion (fish)	ICP-MS	5.30	Djedjibegovic *et al.* (2012)
Digestion (seafood)	AAS	40	Noël *et al.* (2011)

Conclusion: Considering the availability of both standardised and exploratory methods in the food/feed sector, no need for further method development is identified. A verification of performance levels of applicable food standard methods (Table 7) is suggested.

3.4 Tributyltin (TBT)

No EQS for biota has been set for tributyltin (TBT, Figure 1). Nevertheless, TBT is frequently analysed in sediment and biota due to its eco-toxicological characteristics. Most methods reported in the literature use GC-MS subsequent to derivatisation. While no CEN standard could be found, an EPA Method (EPA 8323) exists. The method, based on LC-MS, features a limit of detection of 1.56 µg/kg at a sample intake of 0.5 g. Use of the derivatisation method employing GC-MS results in comparable limits of performance.

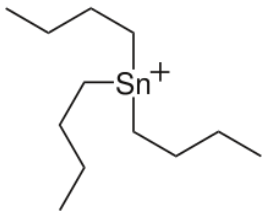

Figure 1 – Structure of tributyltin cation

Table 11 - Selected examples of analytical methods for TBT in aquatic biota.

Extraction (species)	Clean-up	Analysis	LOD / LOQ (µg/kg)	Reference
SLE digestion (fish)	Derivatisation with n-pentylmagnesium bromide	GC-MS	3.36	Hajjaj el Hassania *et al.* (2005)
Digestion (sediment and fish)	Derivatisation with $NaBEt_4$; alumina	GC-FPD	3.82	Lacorte *et al.* (2006)

Extraction (species)	Clean-up	Analysis	LOD / LOQ (µg/kg)	Reference
SLE digestion (fish)	Derivatisation with $NaBEt_4$	GC-HRMS	0.2	Rantakokko *et al.* (2010)
Digestion (mussels)	Derivatisation with $NaBEt_4$	GC-MS-MS	5-10	Fernandez *et al.* (2013)

Conclusion: TBT determination in biota is possible according to the current state of the art. While no urgent need for standardisation is seen by the authors, it is recommend to consider standardisation for a GC-MS approach.

3.5 Polycyclic aromatic hydrocarbons (PAHs) including fluoranthene, anthracene and benzo(a)pyrene

Specific EQS for biota have been set for fluoranthene (30 µg/kg) and benzo(a)pyrene (5 µg/kg), but not for the sum of polycyclic aromatic hydrocarbons (PAHs). The same applies for anthracene. PAH analysis in aquatic biota is well established, and methods exist for both LC and GC approaches. prEN 16619 (for foodstuffs) has been validated for the substances benzo[a]pyrene, benz[a]anthracene, chrysene and benzo[b]fluoranthene and has been shown to detect and quantify these substances at a level of 0.5 µg/kg or above. Unfortunately, the standard was not verified for its applicability to fluoranthene and anthracene.

The literature confirms that PAHs can be monitored in various aquatic biota at levels significantly below the established EQS for biota.

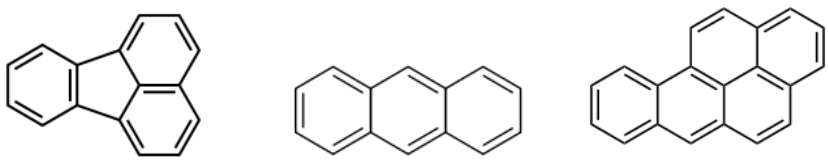

Figure 2 - Structures of fluoranthene, anthracene and benzo(a)pyrene (from left to right)

Table 12 - Selected examples of analytical methods for PAHs in aquatic biota.

Extraction (species)	Clean-up	Analysis	LOD / LOQ (µg/kg)	Reference
Soxhlet (fish)	Alumina	GC-MS	0.008-0.028	Vives *et al.* (2004)
ASE (sediment and fish)	Sulphuric acid	GC-MS	0.96 (sediment)	Lacorte *et al.* (2006)
SLE digestion (fish)	Florisil	HPLC-fluorescence	0.25 (benzo(a)pyrene)	Perugini *et al.* (2007)
Soxhlet (fish)	GPC, silica, alumina	GC-MS	0.010-0.355	Wang *et al.* (2012)
Microwave (mussels)	Silica and alumina	GC-MS	3.97-9.79	Kucuksezgin *et al.* (2013)

Conclusion: PAH determination in biota is possible given the current state of the art. A verification of performance levels of applicable food standard methods (Table 7) for anthracene and fluoranthene is suggested. The authors recommend that PAHs be considered for a multi-residue approach as outlined below.

3.6 Chloroalkanes

C10-13-chloroalkanes are UVCB substances (Substances of Unknown or Variable Composition) with varying chlorine contents (up to around 70% by weight) and carbon chain lengths (between C10 and C13). No EQS for biota have been set for this compound class. No standard method applicable to the determination of chloroalkanes in aquatic biota was found and research data have an exploratory character not necessarily appropriate to implement a routine monitoring of biota under the Water Framework Directive (Table 13). Besides, a well-defined set of indicator substances (e.g. similar to those of other congener classes such as PAHs, PCBs) does not exist, and the available analytical methods do not permit the identification and quantification of single isomers.

An appropriate approach for water analyses has been standardised and is also currently adopted for its application to sediments and particulate matter. This indicates that the approach chosen could also be the basis for developing a standard method applicable for biota.

Table 13 - Selected examples of analytical methods for chloroalkanes in aquatic biota.

Extraction (species)	Clean-up	Analysis	LOD / LOQ (µg/kg)	Reference
ASE (mollusks)	Florisil, silica	GC-NCI-MS-MS	0.1	Yuan *et al.* (2012)
ASE (sediment)	Silica, Florisil	GC-NCI-MS	1	Zeng *et al.* (2013)
Soxhlet (fish and shellfish)	Silica, alumina, carbon	GC-NCI-MS	Not reported	Parera *et al.* (2013)

Conclusion: Methods for chloroalkane determination in biota are still being developed and reported methods have an exploratory character. Ongoing standardisation work for a method for their determination in water is recommended to be extended to apply to biota.

3.7 Polybrominated diphenyl ethers (PBDEs)

The EQS for biota regarding polybrominated diphenyl ethers (PBDEs) has been set at 0.0085 µg/kg. To assess compliance with this EQS, the following congeners are to be measured: PBDE congeners 28, 47, 99, 100, 153 and 154 (Figure 3). No European Standard on PBDE measurement in tissue was found. EPA 1614 and the JRC method use HRGC-HRMS to achieve appropriate detection and quantification limits.

The conclusion that EPA 1614 can reach the required quantification limits is based on the following reasoning: As reported at point 1.5 of the cited EPA Method 1614 "*The laboratory is permitted to omit any step or modify any procedure (e.g., to overcome interferences or lower the cost of measurements), provided that all performance requirements in this method are met. Requirements for establishing equivalency are given in Section 9.1.2*," and in particular: "*9.1.2.1 Each time a modification is made to this Method, the laboratory is required to repeat the procedure in Section 9.2. If the detection limit of the Method will be affected by the change, the laboratory is required to demonstrate that the MDLs (40 CFR*

136, Appendix B) are lower than one-third the regulatory compliance limit or one-third the EMDLs in this Method, whichever are greater."

On the basis of this, one can assume that to extract 20g instead of 10g allows halving the limit of quantification (ML) as reported in Table 14.

Table 14 - LOD and ML established for EPA Method 1614 using 10g of biota tissue and theoretical ML using 20g of biota tissue.

Analyte PBDE N°	10g matrix LOD (µg/kg)	10g Matrix ML (µg/kg)	20g matrix LOD (µg/kg)	20g Matrix ML (µg/kg)
28	0.002	0.005	0.001	0.0025
47	0.0025	0.01	0.00125	0.005
99	0.004	0.01	0.002	0.005
100	0.002	0.005	0.001	0.0025
153	0.002	0.005	0.001	0.0025
154	0.002	0.005	0.001	0.0025

A glance at selected and recent publications shows that low-resolution methods have some difficulties in reaching the minimum requirements of 1/3 EQS established by the QA/QC Directive 2009/90/EC. Nevertheless, the target is reachable. Some successful attempts have been reported to develop multi-residue methods combining analytical determinations of PBDEs, PCDD/Fs and other persistent organic pollutants with similar polarity. For a further in-depth discussion on this topic, the interested reader should refer to chapter 4.

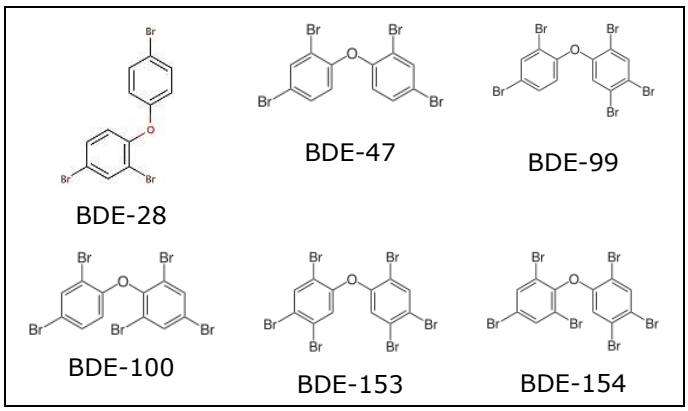

Figure 3 – Structure of PBDE congeners used for PBDE monitoring under the Water Framework Directive

Table 15- Selected examples of analytical methods for PBDE aquatic biota

Extraction (species)	Clean-up	Analysis	LOD / LOQ (µg/kg)	Reference
Soxhlet (fish)	Silica	GC-HRMS	0.010-0.127	Peng *et al.* (2007)
Soxhlet (fish)	GPC	GC-MS	0.02-0.20	Hajslova *et al.* (2007)

SLE (fish)	Silica and alumina	GC-MS-MS	0.001-0.030	Labadie *et al.* (2010)
ASE (sediment and fish)	Sulphuric acid	GC-MS	0.02	Lacorte *et al.* (2006)
ASE (fish)	Silica	GC-MS(-MS)	0.5	Macgregor *et al.* (2010)
ASE (fish)	GPC, silica and alumina	GC-HRMS	0.000002-0.000054	Munschy *et al.* (2011)
Soxhlet (fish)		GC-NCI-MS	0.013-0.016	Montory *et al.* (2012)
Sonication-assisted matrix solid phase dispersion (fish)	Silica and alumina	GC-MS-MS	0.002-0.170	Miège *et al.* (2012)
Soxhlet (fish and shellfish)	Silica	GC-HRMS	ca. 0.002	Parera *et al.* (2013)
Soxhlet (fish)	GPC and silica	GC-MS	0.005-0.031	Jürgens *et al.* (2013)

Conclusion: In summary, it can be concluded that, from a technical point of view, PBDE can be determined at EQS level in biota. An appropriate EPA Method is available, but further vertical standardisation is needed to adopt low-resolution approaches. The authors recommend that a multi-residue approach be considered for PBDEs, as outlined below.

3.8 Dioxins and dioxin-like polychlorinated biphenyls (PCBs)

The Water Framework Directive establishes an EQS for biota regarding dioxins and those PCB congeners that elicit dioxin-like toxicity. It should be noted that the dioxin-like PCB congeners represent only 12 of the 209 possible congeners. The EQS for biota is expressed as the "Sum of PCDD+PCDF+PCB-DL", and is equal to 6.5 10^{-3} µg/kg TEQ (= 0.0065 µg/kg TEQ = 6.5 ng/kg TEQ).

The determination of dioxins and dioxin-like PCBs in biota is usually made using GC-HRMS. Two EPA methods (1613 and 1668-revision A) and one European Standard (prEN 16215:2012) are relevant for this purpose, and are also used in variations in research and academia. The JRC has used a multi-residue method for a series of environmental matrices over many years. No European Standard has yet been developed, and the instrumentation requirements present a challenge.

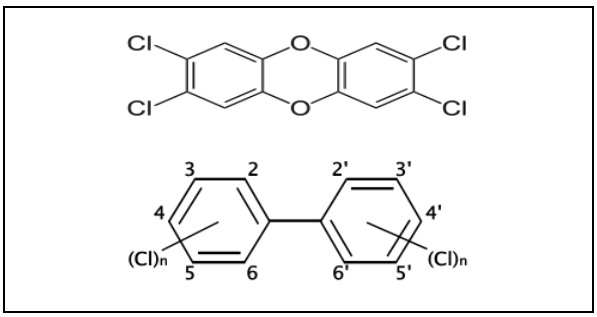

Figure 4 - Structural representation of dioxins and dioxin-like PCBs

Table 16 - Selected examples of analytical methods for dioxins and dioxin-like PCBs

Extraction (species)	Clean-up	Analysis	LOD / LOQ (µg/kg)	Reference
Soxhlet (fish and shellfish)	Silica, alumina, carbon	GC-HRMS	0.000004 (dioxins and furans) 0.0002 (dl-PCBs)	Parera *et al.* (2013)
ASE (mussels)	multilayer silica, alumina and carbon)	GC-HRMS	Not reported	Di Leo *et al.* (2013)
Soxhlet (fish)	GPC, silica, Florisil, carbon, alumina	GC-HRMS	Not reported	Zacs *et al.* (2013)

Conclusion: Dioxins and dioxin-like PCBS can be measured at EQS level in biota. EPA methods are applicable. It is recommended that the integration of the PCDD/Fs and dioxin-like PCBs be considered in a standardised multi-residue method.

3.9 Di(2-ethylhexyl)phthalate (DEHP)

For Di(2-ethylhexyl) phthalate (DEHP) no specific EQS have been set, and uptake by aquatic biota was reported to be low (Swedish EPA Results from the Swedish National Screening Programme 2006. Subreport 1: Phthalates http://www.ivl.se/webdav/files/B-rapporter/B1750.pdf).

No standard method was found that could be applied to aquatic biota. Analytical methods reported in the literature use GC-MS with comparable limits of detection.

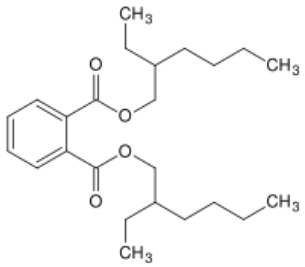

Figure 5 - Structure of Di(2-ethylhexyl) phthalate

Table 17 - Selected examples of analytical methods for DEHP

Extraction (species)	Clean-up	Analysis	LOD / LOQ (µg/kg)	Reference
ASE (fish)		GC-MS	10	Huang *et al.* (2008)
Soxhlet (fish)	Copper, sodium sulphate, Florisil	GC-MS	5	Cheng *et al.* (2013)

Conclusion: A need for a standardised biota method for DEHP has been identified. The authors recommend that DEHP be considered for a multi-residue approach as outlined below.

3.10 Pentachlorobenzene (PCBz) and Hexachlorobenzene (HCB)

For hexachlorobenzene (Figure 6) an EQS for biota of 10 µg/kg has been set.

Appropriate and applicable standard methods identified are EPA 1699, prEN 15742 and prEN15741, all of which require a re-evaluation of detection limits if they are to be applied to aquatic biota. The reported limits of detection and quantification are in line with the minimum requirements of the QA/QC Directive

Reviews were made of papers on the exposure assessment of hexachlorobenzene through food, fish and seafood consumption (Falcó *et al.,* 2004; 2008) and on HCB in the global environment by Barber *et al.* (2005).

While no specific standard method was found for pentachlorobenzene (Figure 6), the abovementioned methods can be used considering the chemical similarity to hexachlorobenzene.

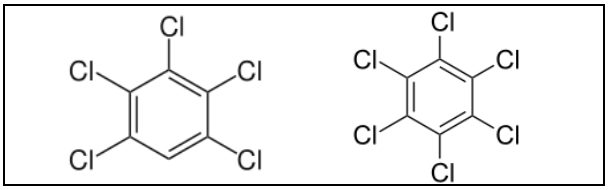

Figure 6 - Structure of penta- and hexachlorobenzene

Table 18 - Selected examples of analytical methods for HCB

Extraction (species)	Clean-up	Analysis	LOD / LOQ (µg/kg)	Reference
SLE (fish)	Alumina	GC-ECD	0.76	Tricklebank *et al.* (2002)
Soxhlet (seafood)	GPC, silica and alumina	GC-HRMS	0.005	Falcó *et al.* (2004)
ASE (sediment and fish)	Sulphuric acid	GC-MS	1.04 (sediment) 0.60 (fish)	Lacorte *et al.* (2006)
Soxhlet (fish; seafood)	GPC	GC-HRMS	0.005	Falcó *et al.* (2008)
ASE (fish)	Silica	GC-MS(-MS)	1	Macgregor *et al.* (2010)
ASE (fish)	Sulphuric acid	GC-ECD or MS	1	Miège *et al.* (2012)
ASE (fish)	Florisil SPE	GC-MS	3.7	Majoros *et al.* (2013) Lava *et al.* (2014)
Soxhlet (fish)	GPC and silica	GC-MS	0.005-0.031	Jürgens *et al.* (2013)

Conclusion: A range of different methods for HCB analyses in biota is available. No urgent need for standardisation was identified. The authors recommend that HCB be considered for a multi-residue approach as outlined below.

3.11 Hexachlorobutadiene (HCBD)

The EQS for biota regarding hexachlorobutadiene (HCBD) has been set at 55 µg/kg, and, as in the case of HCB, no applicable standard method was found. Methods reported in literature feature limits of detection/quantification, which indicate no particular issue for the implementation of the EQS for biota.

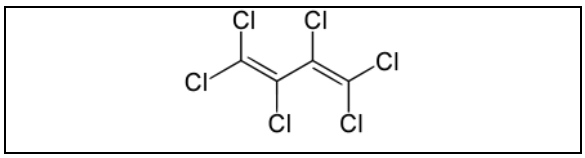

Figure 7 - Structure of hexachlorobutadiene

Table 19 - Selected examples of analytical methods for hexachlorobutadiene

Extraction (species)	Clean-up	Analysis	LOD / LOQ (µg/kg)	Reference
ASE (sediment and fish)	Sulphuric acid	GC-MS	4.02 (sediment) 0.54 (fish)	Lacorte *et al.(*2006)
ASE (fish)	Silica	GC-MS	1	Macgregor *et al.* (2010)
ASE (fish)	Sulphuric acid	GC-ECD or MS	1	Miège *et al.* (2012)
ASE (fish)	Florisil SPE	GC-MS	15.7	Majoros et al., 2013 Lava *et al.* (2014)
Soxhlet (fish)	GPC and silica	GC-MS	0.005-0.031	Jürgens *et al.* (2013)

Conclusion: A range of different methods for HCBD analyses in biota is available. No urgent need for standardisation was identified. The authors recommend that HCBD be considered for a multi-residue approach, as outlined below.

3.12 Heptachlor and Heptachlor epoxide

Heptachlor is a chlorinated insecticide, which is mainly degraded to heptachlor epoxide in the environment. Heptachlor epoxide is resistant to biodegradation and is therefore persistent in the environment. Production and use of heptachlor are regulated globally through the Stockholm Convention on Persistent Organic Pollutants (Vorkamp *et al.*, 2014). An EQS for biota of 0.0067 µg/kg was established.

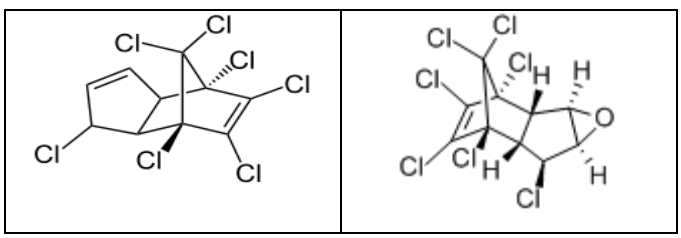

Figure 8 – Structure of heptachlor and heptachlor epoxide

Appropriate and applicable standard methods identified are EPA 1699, prEN 15742 and prEN15741, all of which require a re-evaluation of detection limits if they are to be applied to aquatic biota.

The literature also indicates a good availability of analytical methods, although reported LOD/LOQs may need to be improved (Table 20).

Table 20 - Selected examples of analytical methods for heptachlor and heptachlor epoxide

Extraction (species)	Clean-up	Analysis	LOD / LOQ (µg/kg)	Reference
SLE (mussels, crabs, fish)	Florisil	GC-MS	0.07 (Heptachlor) 0.04 (Heptachlor epoxide)	Falandysz *et al.* (2001)
Soxhlet (fish)	Silica	GC-ECD	0.10-0.60	Zhou *et al.* (2007)
SLE (sediment)		GC-ECD	1	Poolpak *et al.* (2008)
Soxhlet or cryogenic extraction (fish)	C18 and florisil SPE	GC-ECD	0.1	Thomas *et al.* (2012)
Microwave SLE (mussels)	Alumina	GC-MS	0.01-0.08	Fernandez *et al.* (2013)
Microwave (mussels)	Silica and alumina	GC-MS	0.00010-0.00057	Kucuksezgin *et al.* (2013)
Soxhlet (fish)	GPC and silica	GC-HRMS	0.0007 (Heptachlor) 0.0016 (Heptachlor epoxide)	Vorkamp *et al.* (2014)

Conclusion: Standardised methods exist and require re-evaluation for applicability to aquatic biota. No urgent standardisation need is identified. The authors recommend that heptachlor and heptachlor epoxide be considered for a multi-residue approach, as outlined below.

3.13 Hexabromocyclododecane (HBCD)

HBCD is a brominated flame retardant that is mainly used in polystyrene material. The technical product consists of 16 possible stereo-isomers with different biological activities, the most abundant of which are α-, β- and γ-HBCD. While γ-HBCD dominates the technical product, α-HBCD accumulates in the food chain. HBCD has been detected in marine and freshwater fish from Europe (e.g. Janák *et al.*, 2005), but little information is available on HBCD occurrence in surface waters (Harrad *et al.*, 2009). Given scientific evidence of bioaccumulation and long-range transport, HBCD is an official candidate for the Stockholm Convention (Vorkamp *et al.*, 2014). The EQS for biota is set at 167 µg/kg.

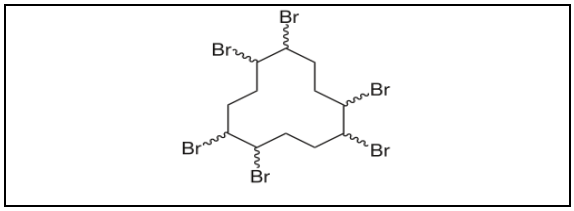

Figure 9 – Structure of hexabromocyclododecane

HBCD can be analysed by GC- and LC-MS techniques (Haug *et al.*, 2008). More LC-MS methods are reported in the literature, because LC achieves the separation of α-, β-, and γ-isomers; GC can only report total HBCD concentrations. Some examples are given here for biota analysis. No directly applicable standardised method was found. Reviewed methods are sufficiently sensitive.

Table 21 - Selected examples of analytical methods for hexabromocyclododecane

Extraction (species)	Clean-up	Analysis	LOD / LOQ (µg/kg)	Reference
Soxhlet (fish)	Silica	LC-MS-MS	0.02-0.075	Janak *et al.* (2005)
Soxhlet (biota)	GPC	LC-MS	1.2	Morris *et al.* (2006)
Soxhlet (fish)	GPC	GC-MS	0.02-0.2	Hajslova *et al.* (2007)
Cold column extraction (sediment and biota)	Silica	LC-MS	0.05-0.15	Haukas *et al.* (2009)
ASE (fish)	GPC and florisil	LC-MS-MS	0.006-0.021	Köppen *et al.* (2010)
Soxhlet (fish)	Alumina and silica gel	LC-MS-MS	0.11-0.24	Miège *et al.* (2012)
Soxhlet (fish)	Alumina and silica gel	LC-MS-MS	0.005	Vorkamp *et al.* (2014)

Conclusion: There is a need for standardisation, but no urgency is identified given the sufficient availability of methods reported in the literature. The authors recommend that HBCD be considered for a multi-residue approach as outlined below.

3.14 Dicofol

Dicofol is an organochlorine pesticide (arcaricide; miticide) that is chemically related to dichlorodiphenyltrichloroethane (DDT) (replacement of hydrogen at the C-1 by a hydroxyl functional group). The technical products may contain DDTs as impurities, so that dicofol is a possible source of DDT (Qiu *et al.*, 2005). The EQS for biota is set at 33 µg/kg. Despite the similarity to DDT, no directly available standard methods were found, and a re-evaluation of DDT-relevant standard methods may be needed. This concerns prEN12393, prEN15741 and prEN15741.

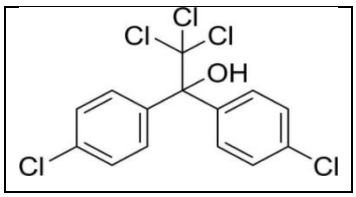

Figure 10 – Structure of dicofol

Only a few recent examples of methods were found in the literature, which is mainly due to the fact that dicofol is not of interest to researchers. Analytical method performance is reported to be sufficient to ensure the implementation of EQS for biota.

Table 22 - Selected examples of analytical methods for dicofol

Extraction (species)	Clean-up	Analysis	LOD / LOQ (µg/kg)	Reference
LLE (human breast milk)	GPC, silica	GC-MS	0.2	Fujii *et al.* (2011)
Ultrasonic extraction (fish)	C8 SPE	GC-ECD	3	El-Amrani *et al.* (2012)

Conclusion: No specific directly applicable standard exists. Standards developed for other organo-chlorine pestides in feed and food matrices should be re-evaluated. The authors recommend that dicofol be considered for a multi-residue approach, as outlined below.

3.15 Quinoxyfen

Quinoxyfen is a fungicide used for protection against powdery mildew diseases on a variety of crops. Quinoxyfen can bioaccumulate in fish, and may also present a threat to aquatic invertebrates and algae. No EQS for biota have been set, and no specific standard method has been developed, although prEN15641 might be applicable. Several multi-residue LC-MS-MS analytical methods are also available for the analysis of pesticides (including quinoxyfen) in food samples (e.g. Hengel and Miller, 2008; Kmellar *et al.*, 2008; Wang *et al.*, 2010). Other methods are reported in the table below.

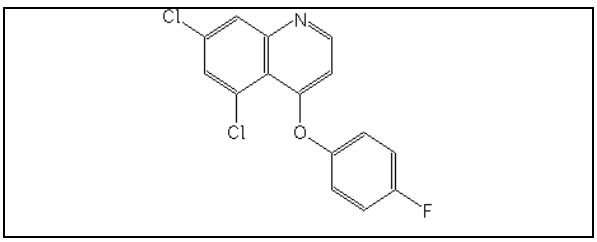

Figure 11 – Structure of quinoxyfen

Table 23 - Selected examples of analytical methods for quinoxyfen

Extraction (species)	Clean-up	Analysis	LOD / LOQ (µg/kg)	Reference
SLE (honeybees)	n.a.	GC-MS-MS	Not reported	Walorczyk and Gnusowski (2009)
SLE (fish; sediment)	n.a.	LC-MS	0.54	Merli *et al.* (2010)

Conclusion: No specific directly applicable standard exists. Standards developed for other pesticides, which are determined by LC-MS in feed and food matrices, should be re-evaluated. The authors recommend that quinoxyfen be considered for a multi-residue approach, as outlined below.

3.16 Perfluorooctane sulphonic acid (PFOS)

The International Organization for Standardization (ISO) has already promoted a standard for the determination of perfluorooctane sulphonic acid (PFOS) in water (ISO, 2009), but at present no standard is available for the analysis of biota samples. Several publications on biota analysis of PFOS[1] are available (e.g. Jahnke and Berger, 2009), and the performance of different methods has already been compared in international interlaboratory studies (Van Leeuwen *et al.*, 2009; 2011).

The EQS for biota has been set at 9.1 µg/kg.

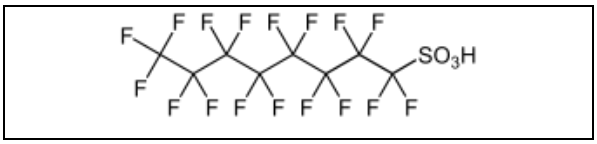

Figure 12 – Structure of PFOS

Several methods to determine perfluorinated compounds (PFCs) in biota tissue are applied in various laboratories; they generally apply extraction with medium polar organic solvents, clean-up steps and liquid-chromatography (LC) with mass spectrometric detection (MS).

Three methods are commonly used for the extraction of PFCs in biota. That published by Hansen *et al.* (2001) uses an ion pair extraction method with tetrabutylammonium (TBA) and the extraction solvent methyl tert-butyl ether (MTBE). The second method, described by Powley *et al.* (2005; 2008), uses ultrasonic extraction with a subsequent graphitised carbon adsorbent (e.g. ENVI-Carb™) clean-up. The third method, described by So *et al.* (2006), includes alkaline digestion followed by solid phase extraction (SPE) on WAX™ cartridges (OSPAR Commission).

Because of the matrix effects on ionisation enhancement/suppression in electrospray tandem mass spectrometry (ESI-MS-MS), a clean-up of the extracts is recommended (the ion pair extraction is usually performed without clean-up). Different methods can be used, either separately or in combination, depending on the biota tissue, extraction solvent and concentration level. Gel permeation chromatography (GPC) for lipid removal is not advisable because lipids are poorly separated from some target compounds (with long chain lengths (>8)) (OSPAR Commission).

LC coupled with a tandem mass spectrometer and interfaced with an electrospray ionisation source in a negative-ion mode (LC-(-)ESI-MS/MS) or LC coupled with an (-)ESI time-of-flight mass spectrometer (LC-ESI-QTOF-MS) is the presumed best choice for PFC analysis.

The review article by Jahnke and Berger (2009) gives an overview on the performance of analytical methods for perfluoroalkyl substances (PFASs) in different matrices, and Valsecchi *et al.* (2013) have recently published a review on the determination of PFCs in aquatic organisms. The results of three international interlaboratory studies on the analysis of PFASs show that analytical methods for PFASs in water and fish have improved considerably (Van Leeuwen *et al.*, 2009; 2011). For the extraction of fish, most laboratories use solid-liquid extraction (SLE) followed by ENVI-Carb™ clean-up, first described by Powely and co-workers

[1] The term "PFOS monitoring" refers commonly to the measurement of the respective anions.

(Powley *et al.*, 2005). This clean-up method has become very popular. Houde *et al.* (2006; 2011) review the presence of perfluorinated compounds in aquatic biota. Reiner *et al.* (2012) summarise the determination of PFASs in standard biological reference materials.

Table 24 - Selected examples of analytical methods for PFOS

Extraction (species)	Clean-up	Analysis	LOD / LOQ (µg/kg)	Reference
Ion pair extraction (biological matrices)	n.a.	LC-MS-MS	8.5	Hansen *et al.* (2001)
Ultrasonic SLE (soil, sediment, sludge, biota)	ENVI-Carb™	LC-MS-MS	0.2	Powley *et al.* (2005; 2008)
Ion pair extraction (fish)	n.a.	LC-MS-MS	1.5	Corsolini *et al.* (2008)
Ultrasonic SLE (fish)	ENVI-Carb™	LC-MS-MS or Q-TOF	0.25	Berger *et al.* (2009)
Ion pair extraction (fish)	n.a.	LC-MS-MS	0.063	Schuetze *et al.* (2010)
Alkaline digestion (fish)	Strata X-AW and Envi-Carb™	LC-MS-MS	0.09	Labadie and Chevreuil (2011)
Ion pair extraction (fish)	n.a.	LC-MS-MS	0.2	Hölzer *et al.* (2011)
Ion pair extraction (dietary food samples including fish)	SPE on florisil and graphitized carbon	LC-MS-MS	0.0017	Vestergren *et al.* (2012)
Ultrasonic SLE (fish)	ENVI-Carb™	LC-MS-MS	0.1	Roland *et al.* (2014)
SLE (fish)	Oasis™ WAX SPE	LC-MS-MS	0.7	Squadrone *et al.* (2014)

Conclusion: PFOS in aquatic biota needs to be standardised. In addition, the authors recommend that PFOS be considered for a multi-residue approach, as outlined below.

4 Towards a multi-residue approach for biota measurements

The availability of sufficiently performing analytical methods is a limiting factor in enforcing EQS for biota. The logistical challenges associated and the sheer volume of work could overwhelm available resources.

Progress has been made in recent years to overcome this limitation by developing rapid multi-residue analysis methods. These techniques quickly analyse many compounds, leading to more samples being examined for a larger number of compounds.

The benefits of multi-residue methods to address the challenges of processing a very large number of samples for a variety of chemical compounds have quickly been recognised, and the implementation of these types of techniques into regular testing programs has become more widespread in recent years. Some examples are highlighted in the following sections, at the end of which a multi-residue approach for biota measurements under the Common Implementation Strategy of the Water Framework Directive is suggested.

4.1 JRC Method "HRGC-HRMS multi-residual POPs analysis method on a novel automated clean up system"

This method used extraction carried out by Soxhlet for 24h using an intake of 10 g of lyophilised fish with a mixture of acetone/n-hexane 1/1, after spiking with internal standards (16 PCDD/Fs ^{13}C-labelled 2,3,7,8-chlorine-substituited congeners with 400 pg each, except OCDD with 800 pg and 12 DL-PCBs and 7 indicators - PCBs ^{13}C-labelled with 2 000 pg each) and 50 ng of 19 labelled OCPs. The extract was dried under nitrogen flow and the lipid content was determined gravimetrically.

The lipid sample was diluted to 5 ml with a mixture of cyclohexane/ethyl acetate 1/1 and injected into a 5-ml loop of automated GPC system. The GPC column was 2.5 cm x 32 cm filled with BioBeads SX-3 resin working at a flow rate of 5 ml/min. using cyclohexane/ethyl acetate 1/1. The eluate was collected between 23:30 and 45 min. (107 ml).

10% of the collected sample was concentrated under nitrogen flow and spiked with OCPs recovery standards. The final volume of 100 µl was submitted to instrumental analysis for OCPs.

The remaining 90% was concentrated under nitrogen flow to 0.5 ml and then diluted with n-hexane to 5 ml. These 5 ml were submitted for an automated clean-up using acid silica/neutral silica, basic alumina and active carbon columns.

The sample was loaded on acid silica/neutral silica connected to a basic alumina column and eluted with 75 ml of n-hexane. This fraction was discharged. The basic alumina column was eluted with 60 ml of 98/2 n-hexane/dichloromethane, and this fraction was collected for PCB analysis. The basic alumina was then connected to an active carbon column, which was eluted with 120 ml of a mixture of 50/50 n-hexane/dichloromethane. The carbon column was eluted beforehand with 4 ml of a mixture of 50/50 ethyl acetate/toluene and then with 10 ml of n-hexane. The last three fractions were collected and added to the PCB fraction in order to improve PCB recovery. Finally, the carbon column was eluted in reverse flow with 75 ml of toluene and collected for non-ortho PCBs and PCDDs/Fs.

PCBs and PCDDs/Fs fractions were concentrated to 100 µl and 30 µl respectively, spiked with their recovery standards, and submitted to instrumental analysis. The flow rate used for all columns was 6 ml/min.

The system, which runs in sequential mode up to nine samples, was equipped with an autosampler that automatically managed the sample loading during the different clean-up steps.

Although the system was equipped with automated evaporator in-line system, it was not used during this study, for which all solvent concentrations were performed by TurboVap. The instrumental analysis of PCDD/Fs, and PCBs were based on isotope dilution using HRGC-

HRMS (high resolution gas chromatography – high resolution mass spectrometry) for quantification on the basis of EPA1613 and EPA 1668.

OCPs were quantified using isotope dilution with HRGC-HRMS on the basis of an in-house method which adheres to the QA/QC criteria laid down in the methods mentioned above.

Non-ortho PCBs, PCDD/Fs, and OCPs were analysed on double HRGC (Thermo Trace GC Ultra, Thermo Electron, Bremen, Germany) coupled with a DFS high-resolution mass spectrometer (HRMS, Thermo Electron, Bremen, Germany) operating in EI-mode at 45 eV with a resolution of >10 000. For Non-ortho PCBs, PCDD/Fs the two most abundant ions of the isotopic molecular cluster were recorded for both native and labelled congeners.

For OCPs, we selected two ions of the isotopic cluster coming from the fragmentation and selected on the basis of close elution of different OCPs and the dynamic mass range of the HRMS. The compounds were identified through a comparison of retention times of the corresponding standard and the isotopic ratio of the two ions recorded.

Mono-ortho PCBs and Indicator-PCBs were analysed on a GC coupled with a VG Autospec Ultima HRMS operating in EI-mode at 34 eV with a resolution of >10 000. The two most abundant ions of the isotopic molecular cluster were recorded for both native and labelled congeners.

4.2 Example of a multi-residue method from the literature – Case I

Sánchez-Avila *et al.* (2011) describe a multi-residue method based on gas chromatography–electron ionisation-tandem mass spectrometry (GC–EI-MS/MS) for the detection of sixteen polycyclic aromatic hydrocarbons (PAHs), five phthalate esters (PEs), seven polychlorinated biphenyls (PCBs), six polybrominated diphenyl ethers (PBDEs), six alkylphenols (APs), three organochlorine pesticides and their isomers or degradation products (OCPs) and bisphenol A in seawater, river water, wastewater treatment plant (WWTP) effluents, sediments and mussels. Solid phase extraction (SPE) was used for the extraction of target analytes in aqueous samples, and ultrasound-assisted extraction for solid samples. GC–EI-MS/MS acquisition conditions in selected reaction monitoring (SRM) using two transitions per compound were optimised.

One gramme of freeze-dried and homogenised mussel samples (*Mytilus galloprovincialis*) were spiked with surrogate standards to get a final concentration of 200 ng/g. One gramme of freeze-dried and homogenised <120 µm particle sediment samples were weighted, and the surrogate standards were added to a final concentration of 50 ng/g. Samples were homogenised and kept at 4°C overnight, and the liquid–solid was subsequently extracted by sonication (10 min.) using different solvents:

- method 1 extraction was performed with 2 × 10 ml of dichloromethane/hexane and 1 × 10 ml of dichloromethane/acetone;
- method 2 with 2 × 10 ml dichloromethane/hexane and 1 × 10 ml of hexane/acetone;
- method 3 with 1 × 10 ml of dichloromethane/hexane and 2 × 10 ml of hexane/acetone.

After each extraction step, samples were centrifuged for 10 min. at 2 500 rpm. Extracts were combined and concentrated to approximately 1 ml under a nitrogen current using a TurboVap LV at 25°C.

Extracts were subsequently cleaned up using Florisil (5 g) SPE cartridges, previously conditioned with 20 ml of hexane/dichloromethane (1:1, v/v) and 20 ml of hexane/acetone (1:1, v/v).

The sample extract was eluted with 15 ml of hexane/dichloromethane (1:1, v/v) and 15 ml of hexane/acetone (1:1, v/v). The eluent was evaporated it was almost dry under a nitrogen

current at room temperature and reconstituted with ethyl acetate to a final volume of 200 µL for mussels and 500 µL for sediments. Internal standard anthracene d_{10} was added at a concentration of 1ngµL^{-1}.

IDLs were in the range of 0.3–20 pg injected, which ensured the detection of low level target compounds.

4.3 Example of a multi-residue method from the literature – Case II

Camino-Sánchez *et al.* (2012) described a multi-residue method for the analysis of 77 semi-volatile organic pollutants in inland groundwater (river water) at ultra-trace levels in compliance with the European Water Framework Directive (WFD). The method uses stir bar sorptive extraction (SBSE) and thermal desorption coupled with gas chromatography–triple quadrupole mass spectrometry (SBSE-TD–GC–MS/MS (QqQ)). The method includes various families of compounds included in the WFD and other compounds listed as persistent organic pollutants that are banned under the Stockholm Convention of Persistent Organic Pollutants, such as polychlorinated biphenyls, polycyclic aromatics hydrocarbons, and other pesticides not included in the WFD. The method can also be applied for compliance with regional environmental laws.

The quantification limits (LOQs) obtained ranged from 0.14 to 10 ng/l, and comply with the requirement for analytical methods to be used in the analysis of the compounds included in the WFD.

4.4 prEN 12393-1 "Non-fatty foods – Multi-residue methods for the gas chromatographic determination of pesticide residues - Part 1: General considerations"

This European Standard gives general considerations for the determination of pesticide residues in non-fatty foods. Each method described in this European Standard is suitable for identifying and quantifying a definite range of those organohalogen, and/or organophosphorus and/or organonitrogen pesticides which occur as residues in foodstuffs of plant origin. This European Standard contains the following methods that have been subjected to interlaboratory studies and/or are adopted throughout Europe:

- method L: Extraction with acetone, liquid-liquid partition with dichloromethane and clean-up on a silica-gel/charcoal column;
- method M: Extraction with acetone and liquid-liquid partition with dichloromethane/light petroleum, if necessary clean-up on Florisil® ;
- method N: Extraction with acetone, liquid-liquid partition with dichloromethane and clean-up with gel permeation and silica gel chromatography;
- method O: Extraction with acetonitrile, liquid-liquid partition with light petroleum and clean-up on a Florisil column;
- method P: Extraction of organophosphorus compounds with ethyl acetate and, if necessary, clean-up with gel permeation chromatography.

The applicability of the five methods L to P for residue analysis of organohalogen, organophosphorus and organonitrogen pesticides is given for each method.

Gas chromatography (GC) with selective detectors may be used: electron-capture detection (ECD) for organohalogen, thermionic detector (NPD, P-mode or N/P mode) for

organophosphorus and organonitrogen compounds, and flame-photometric detector (FPD) for organophosphorus and organosuphfurous pesticides. Hall detector (ECHD), atomic emission detector (AED) and mass spectrometry (MS) may also be used for a large class of pesticides. Procedures are used to confirm the identity and quantity of observed residues, particularly in those cases where it would appear that the maximum residue limit (MRL) has been exceeded.

No indication of method sensitivity (i.e. limit of detection, quantification) is reported.

4.5 Proposal for the development of a multi-residue method for Biota-EQS implementation

As highlighted in this report, one of the larger challenges related to the implementation of biota monitoring programmes consists in the cost of analyses, especially when the programme encloses different typologies of chemicals, a large number of samples and complex matrices, such as aquatic biota. The documentation reviewed above shows that different official and validated methods exist for almost each single or family of the organic compounds in question. Frequently, each method suggests its own extraction, clean-up and type of instrumental analysis method, each with considerable implementation costs.

Making a careful analysis of the various methods and scientific publications consulted in this report and the improvements made in instrumental analytical techniques in recent years, it is possible to develop a versatile multi-residual method that can allow for the detection of a maximum amount of compounds in an as small a number of analyses as possible. This approach has already been successfully employed in the food/feed sector, and has also been streamlined into a format which allows for standardisation.

Presuming that the 17 compounds of interest should be analysed in the same biota tissue (fish tissue/mussel, etc.) and looking into the respective methods consulted, it can be observed that similar solvents and extraction methods are often used. Frequently, the clean-up step used employs Gel Permeation Chromatography (GPC) and the instrumental analysis is performed by either LC-MS/MS for polar or thermo-labile compounds, or GC-MS and GC-MS/MS for apolar compounds.

To facilitate the challenging implementation of EQS for biota, on the basis of this assessment we proposed to develop and validate **one** multi-residual method in which a common extraction step is performed for all compounds. This will be done by exploiting the characteristics of Gel Permeation Chromatography (GPC), which is a type of size exclusion chromatography that separates analytes on the basis of size. This peculiarity of GPC allows for the separation of all analytes from animal/vegetable fat (which interferes with the quantification step), independently from the chemical property (polar/apolar). The technique is applicable for most compounds ranging in molecular weight from 200 to 900 daltons. Another advantage of this approach is that it can integrate new compounds with similar characteristics in the future.

MS/MS-technology based on a coupling of the triple-quadrupole mass spectrometer to LC and/or GC devices will be used. Recent developments of this technology allow for the analysis of hundreds of compounds in a single run, while preserving good specificity and sensitivity. As a matter of fact, as from 2014 a very restrictive EU-Regulation that regulates the controls of dioxins and dioxin-like PCBs in foods accepts the GC-MS/MS as a confirmation method (Commission Regulation (EU) No 589/2014 of 2 June 2014).

Triple-quadrupole mass spectrometry has become a widely diffused technique in the analytical laboratories that engage in chemical monitoring. It is known for its good performance, flexibility and for its moderate cost compared to other types of mass spectrometer (HRMS, Q-TOF).

Figure 13 illustrates the general principle of the analytical methods, where after clean-up the sample is split into two or more fractions that are subsequently submitted to LC-MS/MS analysis for the polar compounds and to GC-MS/MS (Triple-Quadrupole) analysis for the

most apolar compounds. It must be stressed that this is done for each separation technique in a single instrumental run. Based on the complexity of the compound, e.g. dioxins and dioxin-like PCBs, different levels of clean-up are required and the quantification step could be separated.

The JRC method envisages a pre-normative input based on Solid Liquid Extraction (SLE) of the lyophilised matrix followed by sequential clean-up steps and different mass spectrometric analysis. During the development phase, the SLE conditions must be optimised in order to allow the extraction of analytes with very different polarities, and it can be performed by means of ultra-sonication, Accelerated Solvent Extraction (ASE), and/or Soxhlet apparatus.

By means of Gel Permeation Chromatography (GPC), fat content is eliminated and the resulting extracts may be directly be analysed. Using GC-MS/MS, the following analytes could be determined: Anthracene Fluorantene Hexachlorobenzene Hexachlorobutadiene Hexachlorocyclohexane Pentachlorobenzene Benzo (a)pyrene DEHP Quinoxyfen HBCDD Heptachlor and Heptachlorepoxide. The analytes Dicofol, Quinoxifen and HBCDD could be determined using LC-MS/MS.

No evidence is available regarding the suitability or non-appropriateness of GPC for PFOS determination. Further investigations into PFOS are necessary. For dioxins, PCBs and PBDEs, a further clean-up step is necessary. Silica clean-up on the GCP extract produce a fraction in which dioxins and planar PCBs could be analysed using GC-MS/MS. Clean-up using carbon produces a fraction in which it is possible to determine other PCBs and PBDEs by GC-MS/MS.

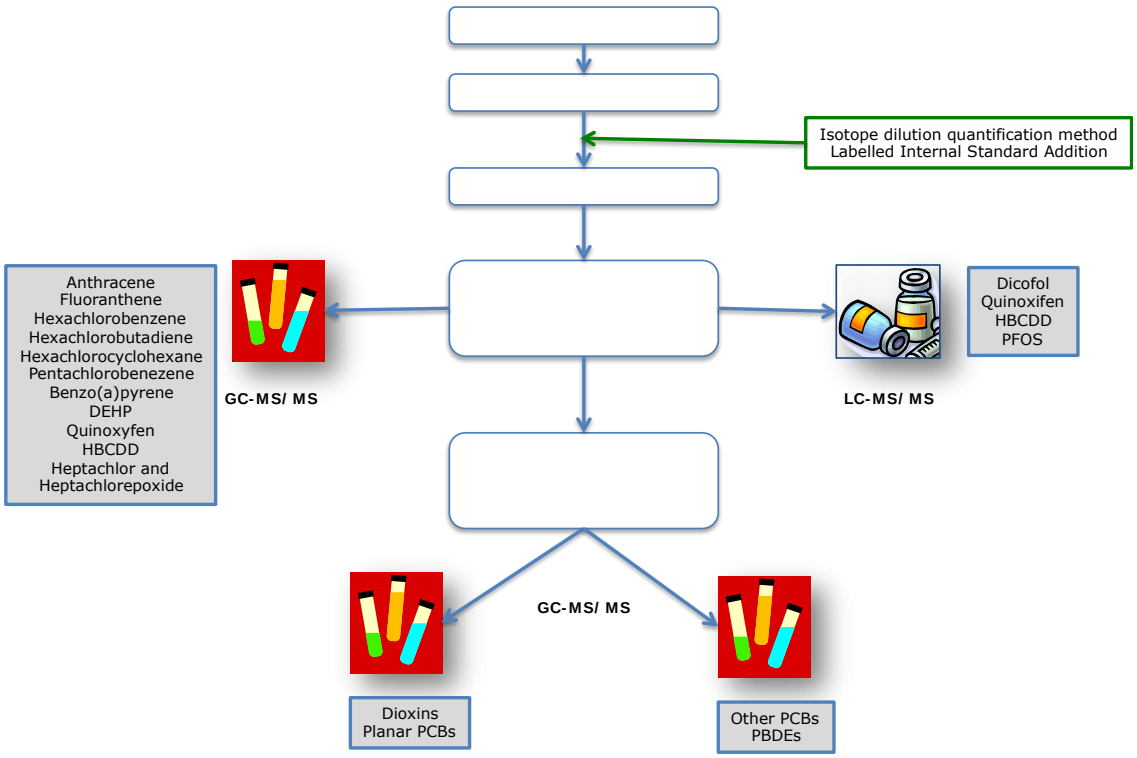

Figure 13 - Schematic outline of an adaptable, multi-residue method for aquatic biota

It is expected that such a multi-residue method can meet the requirements of the EQS for biota without entailing significant costs compared to other approaches. It is expected that the cost of analyses will be cheaper and less time consuming with a multi-residue method as compared to one-off targeted methods.

5 Conclusions

1. For the implementation of EQS for inorganic parameters in biota, the available standards should be re-evaluated regarding the limit of detection and quantification for application in specific aquatic biota. This is part of normal practice when implementing a standard method in a laboratory. However, no imminent need is identified for further standardisation.

2. For Tributyltin (TBT), the reviewed EPA method is fit-for-purpose and should be implemented for biota samples.

3. For organic parameters, the performance of the standard methods applicable to food/feed should be re-evaluated as outlined under 1.) for application to aquatic biota.

4. It should be acknowledged that there is a variety of approaches to reporting performance limits for methods including standard methods, and direct comparison may be difficult. Thus, statements made on PBDE, for instance, rely on expert evaluation and the conversion of Minimum Detection Limits into Limits of Quantification as indicated in the QA/QC Directive, Article 2.

5. In the case of chloroparaffins, no appropriate method was found. It is recommended that an appropriate standard be developed building upon the ongoing standardisation work being carried out for a water method. That method needs to be complemented with steps for sample extraction and the necessary clean-up prior to the quantification step.

6. Given the current state of the art, it is recommended that a multi-residue method based on GPC be designed and validated. The necessary pre-normative work should be mandated to the Commission's in-house science service, and the method should be tested in collaboration with Member States' Laboratories

7. In this report, reviewed methods applicable to the analysis of vegetable samples or similar plant matrices are not intended for application to aquatic fauna, but may be useful if aquatic plants are analysed.

In summary it is concluded that with the exception of an appropriate approach for the extraction and clean-up of chloroparaffins, no need for pre- or co-normative activity is identified and that the reviewed analytical methods available as standards or cited in literature allow the implementation of the biota-EQS or trend monitoring in biota as relevant.

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