

CEMP Guidelines for Monitoring Contaminants in Biota

# (OSPAR Agreement 1999-02) **[[1]](#footnote-1)**

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CEMP Guidelines for Monitoring Contaminants in Biota   
(Agreement 1999-02, revised 2018)

# 1. Introduction

1. These guidelines concern the sampling and analysis of contaminants in fish, shellfish and seabird eggs. They are suitable for hazardous substances: trace metals and organic compounds including chlorinated compounds (such as chlorobiphenyls, DDT and metabolites, HCH isomers, HCB and dieldrin), parent and alkylated PAHs, brominated flame retardants such as polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCD), perfluorinated compounds (PFCs), organotin compounds (TBT and its breakdown products), dioxins, furans and dioxin-like PCBs*.* Technical details relating to sampling, analysis, QA and reporting are given in Technical Annexes 1 and 3-9 (organic contaminants) and Technical Annex 2 (metals).

# 2. Purposes

2. Monitoring of contaminants in marine biota in the North-east Atlantic Ocean is performed within the framework of OSPAR as the regional convention for the protection of the marine environment of this area. The objectives of monitoring and assessment are described in the Joint Assessment and Monitoring Programme (JAMP) under the Hazardous Substances Strategy, providing the basis for the monitoring programme of chemicals for priority action, and hazardous substances in general, and addressing the following issues (see JAMP Theme H):

1. What are the concentrations of hazardous substances in the marine environment? Are those hazardous substances monitored at, or approaching, background levels for naturally occurring substances and close to zero for man-made substances? How are the concentrations changing over time? Are the concentrations of either individual substances or mixture of substances such that they are not giving rise to pollution effects?
2. What are the sources, what are the levels of discharges, emissions and losses and what are the pathways to the marine environment for individual OSPAR chemicals for priority action and other hazardous substances listed by e.g. the Stockholm Convention and the MSFD? Are the discharges, emissions and losses from sources of these substances to the marine environment continuously decreasing, and are they moving towards the target of cessation?

3. The existing level of marine contamination in different parts of the convention area can be assessed by spatial distribution monitoring. Monitoring contaminant concentrations in fish, shellfish and seabird eggs can be used to indicate large-scale regional differences in contamination.

4. The measured levels can be compared to background or close to background reference conditions as well as to levels describing the thresholds above which negative effects on living resources and marine life are expected. OSPAR monitoring can assist member states of the European Union to fulfil their obligations under relevant EU-directives, namely the Marine Strategy Framework Directive (MSFD, 2008/56/EC) and the Water Framework Directive (WFD, 2000/60/EC) and related directives like the WFD daughter directive on Environmental Quality Standards in the field of water policy (2008/105/EC), to assess whether certain regions or sub-regions, have reached or failed to reach Good Environmental Status.

5. The effectiveness of measures taken for the reduction of marine contamination can be assessed by performing trend monitoring. Changes in contaminant inputs are reflected in the concentrations of contaminants in biota over time. The statistical assessment of a trend over a longer period also supplies a more reliable assessment for the status within a certain period or the last measured year and therefore also for the assessment of the actual status, as the within and between year variability is thereby taken into account.

6. An integrated approach is needed to assess harm to living resources and marine life. The role of chemical measurements in integrated chemical and biological effects monitoring programmes is:

i. to identify sites where contaminant-specific biological effects programmes should be applied;

ii. to investigate the chemical cause of observed biological effects;

iii. How to improve and extend OSPAR’s monitoring framework and better link it with the understanding of biological effects and ecological impacts of individual substances and the cumulative impacts of mixtures of substances.

# 3. Quantitative objectives

## 3.1 Temporal trends

7. Before starting to interpret results from statistical time series analyses it is essential to know with what power temporal changes in concentration could be detected (*i.e.* the chances of revealing true trends in concentration within the matrices investigated). When no trend is found, it is essential to know whether this indicates a stable situation or that the sampling strategy is too poor to detect even major changes in the contaminant load to the environment. One approach for solving this problem would be to estimate the power of the time series based on the ‘random’ between-year variation. Alternatively the lowest detectable trend could be estimated at a fixed power to represent the sensitiveness of the time series. It should be stressed that the power estimate must be interpreted with great caution. A matrix showing a very high power is not necessarily a good matrix for monitoring. If the matrix analysed does not respond to the environmental changes being monitored, the between-year variation would probably be low and consequently the power high. Another problem is that a single outlier could ruin an estimate of the between-year variation. Bearing these difficulties in mind, and as an example for the purpose of trend monitoring, the quantified objective could be stated, including the following information:

* the annual change which the programme should be able to detect
* the time period
* the power at a set significance level (α) with a one-sided test.

A typical example which has been used previously is the ability to detect an annual change of 5% within a period of 10 years at a power of 90% at a significance level (α) of 5% with a one-sided test. For many areas, however, due to the decline of contaminant concentrations which has been observed this approach is no longer realistic, so that the annual change to be detected should be lower and the time period longer, e.g. a 2% fall over a time period of 30 years.

8. The necessary or possible power of a monitoring programme will vary with the purpose of the investigation and with the contaminant, matrix and area being investigated. It is thus not possible to give fixed values for all situations. It is the duty of the programme manager to specify the size of the changes the monitoring programme is expected to identify and at what power, or for the programme executor to estimate what it is possible to achieve. It is, however, essential that the quantitative objectives are determined before any monitoring programme is started.

9. Due to the decrease of concentrations of many substances in the last two decades, for certain substances and areas it is not any longer possible to detect significant changes which can be associated with a trend. Monitoring is serving in this case for the assessment of status and to detect any deterioration. Depending on the magnitude of natural variability, it may also be possible to reduce the monitoring effort and to change from annual sampling to longer intervals without loss of relevant information.

## 3.2 Spatial distribution

10. A spatial distribution monitoring programme should enable Contracting Parties to determine the representativeness of their monitoring stations with regard to spatial variability in contaminant concentrations. This would include a definition of the monitoring area and some understanding of the randomness of the monitoring programme. It can also deliver information useful to distinguish between areas of different character and to define water bodies or areas which should be assessed separately. The purpose and quantitative objectives could be expressed as follows, for example:

* Purpose: to identify whether an area has elevated contaminant concentrations, possibly due to anthropogenic inputs.
* Quantitative Objective: to detect a difference of 10 μg/kg between the average contaminant concentrations in area A and the average contaminant concentrations in control area B with a power of 90%.

or

* Purpose: to map the spatial distribution of contaminants.
* Quantitative Objective: for the precision of an interpolated point on the map to be at worst 10%.

or

* Purpose: to locate “hot spots”.
* Quantitative Objective: for the probability of missing a circular “hot spot” of radius 0.5 km to be no greater than 5%.

11. For more detailed information about statistical analyses of monitoring data see Nicholson *et al*., (1997).

12. Spatial distribution monitoring is supplying relevant information for assessing different water bodies and areas both for the purposes of the EU-MSFD and the EU-WFD. Following the sampling and analytical techniques as described in this guideline and its technical annexes will assist in avoiding significantly deviating monitoring results for neighbouring regions with comparable conditions, which consequently affects the assessment of the (good) environmental status.

## 3.3 Qualitative objectives

13. The quantitative results will be used to perform the assessment of the status of the marine environment with regard to hazardous substances. Further information on the assessment procedure, the classification schemes and the threshold values used for distinguishing between the different classes from e.g. unacceptable to excellent or Good Environmental Status (WFD, MSFD) being achieved or not, can be found in the relevant OSPAR agreements and EC Directives and Decisions.

# 4. Sampling strategy

## 4.1 General

14. The sampling strategy should take into account the specific objectives of the monitoring programme, including the quantitative objectives. Natural variability between the samples should be reduced by an appropriate sampling design and the performance of the analytical procedures (*i.e.* the accuracy and precision) must be adequate to meet the objectives. A preliminary/exploratory sampling programme will provide useful information prior to designing the final programme. Statistical procedures must be taken into account to estimate the number of samples and sampling sites required to achieve a satisfactory level of confidence. More guidance on this topic is given by Gilbert (1987).

15. In more exploratory studies, data may be statistically analysed in several ways for several purposes. However there should still be a clear understanding of what must be measured from what population and how the samples are to be selected. The sampling strategy is an intrinsic component of the data, and may limit their use and interpretation. Quantitative objectives for a selected primary purpose should also be established for exploratory studies.

16. When conducting an integrated chemical and biological effects sampling programme, the purpose of the chemical measurements is both to be assessed against limit values and to aid the interpretation of the biological effects measurements in terms of identifying the chemical causes of the biological effects and establishing concentration responses. In such cases, the sampling strategies used should comply with those in the biological effects monitoring guidelines and the monitoring guidelines for the relevant chemical determinands. The analytical methods used should be as specified in the relevant chemical guidelines.

17. With regard to the choice of monitoring parameters, the sampling strategy should cover the demands of as many purposes as possible for both OSPAR and the EU-MFSD, and in particular the compounds determined should address the indicators under descriptor 8 of the MSFD, the species and tissues and, where possible, the selection of sampling sites in coastal areas should also meet the requirements of the EU Water Framework Directive.

### 4.1.1 Species

18. Prior to monitoring, it is important to be clear about:

* the target population (e.g. cod from a specified length‑range caught in a specified area at a specified time);
* the sampled population, if this differs from the target population (e.g. if fishing is restricted within particular areas);
* the sampling unit (e.g. an individual fish or pooled samples);
* the observed variable (e.g. mercury concentration on a wet weight basis in a subsample of tissue from individual fish muscle).

19. When selecting the species to be monitored for chemical contaminants, some basic prerequisites should be considered. Where possible the organisms should:

* reflect changes in the concentration of contaminants in the surrounding environment;
* for a given species, have similar bioconcentration factors throughout the Maritime Area;
* accumulate the contaminant without being seriously affected by the concentrations encountered in the marine environment;
* be representative of the study area;
* be abundant throughout the study area;
* be of reasonable size, giving adequate amounts of tissue for chemical, biochemical and physiological analyses; restrictions to this may occur on different preconditions for performing the different tests and analytical methods;

in particular for shellfish and for investigations exceeding the demands of “routine” monitoring:

* be easy to sample and hardy enough to survive in the laboratory, thus allowing:

- defecation before analysis (if desired);

- laboratory studies of contaminant uptake;

- studies verifying biological field observations.

## 4.2 Sampling strategy for temporal trend monitoring

### 4.2.1 Species and sampling

20. The species of interest can only be selected in the light of information on the fish and shellfish stock and on the seabird population composition and migration pattern.

21. For fish and shellfish, sampling to minimise natural variability is the preferred strategy, see table 1. Length-stratified sampling may be maintained where it has been successfully applied in the past. Recommendations for species, size etc. are given in Table 2. Where conditions have changed such that length stratified sampling cannot be sustained any longer, or the indicator species has to be changed due to changing abundance, it will be appropriate to sample with a view to minimising natural variability within the sample.

For shellfish, a sample should be collected with the number of individuals large enough to be divided into at least 3 equal pools with each pool consisting of at least 20 animals and enough soft tissue for all analyses. The length of the individuals collected should to the extent possible, be constant from year to year at each station, or should at least fall within a very narrow range, e.g. within 5 mm. To reflect recent levels of contamination, young individuals should be chosen. In selecting the sample, care should be taken that it is representative of the population and that it can be obtained annually. Recommendations for sampling to minimise natural variability are given in Table 1. If a Contracting Party decides to change its sampling strategy, data from the old and new programmes should not be compared without first checking the compatibility of the two approaches. More detailed information about length-stratified sampling and sampling to minimise natural variability is given in Technical Annexes 1 and 2 (Agreement 1999-2).

**Table 1**:Sampling to minimise natural variability

| **Species** | **Number** | **Size**[[2]](#footnote-2) | **Age** | **Sex**[[3]](#footnote-3) | **Tissue** |
| --- | --- | --- | --- | --- | --- |
| **Shellfish** |  |  |  |  |  |
| Mussel |  |  |  | - |  |
| *Mytilus edulis* or  *M. galloprovincialis* | 3 pools of 20 | Narrow length range | 1-2 years |  | Whole soft body |
| Pacific oyster |  |  |  |  |  |
| *Crassostrea gigas* |  | Narrow length range | 2 years | - | Whole soft body |
| **Flatfish** |  |  |  |  |  |
| Dab |  |  |  |  |  |
| *Limanda limanda* | At least 12 | Narrow length range | 1-3 years | Single sex, females\* | Muscle for Hg.  Liver for all other determinands |
| Flounder |  |  |  |  |  |
| *Platichthys flesus* | At least 12 | Narrow length range | 1-3 years | Single sex, females\* | Muscle for Hg.  Liver for all other determinands |
| Plaice |  |  |  |  |  |
| *Pleuronectes platessa* | At least 12 | Narrow length range | 1-3 years | Single sex, females\* | Muscle for Hg.  Liver for all other determinands |
| **Roundfish** |  |  |  |  |  |
| Cod |  |  |  |  |  |
| *Gadus morhua* | At least 12 | Narrow length range | Preferably 1-3years\*\* |  | Muscle for Hg.  Liver for all other determinands |
| Whiting |  |  |  |  |  |
| *Merlangius merlangus* | At least 12 | Narrow length range | 2-3 years | Single sex, preferably females | Muscle for Hg.  Liver for all other determinands |
| Hake |  |  |  |  |  |
| *Merluccius merluccius* | At least 12 | Narrow length range | 2-3 years | Single sex, preferably females | Muscle for Hg.  Liver for all other determinands |
| Herring |  |  |  |  |  |
| *Clupea harengus* | At least 12 | Narrow length range | 1-2 years |  | Muscle for organic contaminants and Hg.  Liver for other trace metals. |
| Eel pout |  |  |  |  |  |
| *Zoarces viviparus* | At least 12 | Narrow length range | 2-3 years | Single sex, preferably females | Muscle for Hg.  Liver for all other determinands |
| **Seabird eggs** |  |  |  |  |  |
| Common tern  *Sterna hirunda* | 10 footnote [[4]](#footnote-4) | - | 1-5 days incubation | - | Whole egg content |
| Oyster catcher  *Haematopus ostralegus* | 10 footnote [[5]](#footnote-5) | - | 1-5 days incubation | - | Whole egg content |
| Guillemot  *Uria aalge* | 10 footnote5 | - | 1-5 days incubation |  | Whole egg content |

\* As for flatfish sex can be determined easily. If possible, only females should be chosen, as males show higher variation in age distribution and contaminant concentrations at comparable length

\*\* Smaller fish should, if possible be selected to reflect recent influence and reduce the effects of sex, as age determination without dissection is not possible, When the amount of tissue(s) needed for all investigations within an integrated chemical and biological effects monitoring programme is not sufficient, selection of larger fish may be appropriate.

**Table 2**: Length-stratified sampling

|  |  |  |  |
| --- | --- | --- | --- |
| **Species** | **Number** | **Size (cm)** | **Tissue** |
| **Shellfish** |  |  |  |
| Mussel |  |  |  |
| *Mytilus edulis* or  *M. galloprovincialis* | 3 pools of 20 | 3-6 | Whole soft body |
| Pacific oyster |  |  |  |
| *Crassostrea gigas* | 10 ± 10% | 9-14 (2 years of age) | Whole soft body |
| **Flatfish** |  |  |  |
| Dab |  |  |  |
| *Limanda limanda* | 25 to 20 individuals, or 15 if justified, or 5 batches of 5 individuals | 18-30 | Muscle for Hg. Liver for all other determinands |
| Plaice |  |  |  |
| *Pleuronectes platessa* | 25 to 20 individuals, or 15 if justified, or 5 batches of 5 individuals | 20-30 | Muscle for Hg. Liver for all other determinands |
| Flounder |  |  |  |
| *Platichthys flesus* | 25 to 20 individuals, or 15 if justified, or 5 batches of 5 individuals | 15-35 | Muscle for Hg. Liver for all other determinands |
| **Roundfish** |  |  |  |
| Cod |  |  |  |
| *Gadus morhua* | 25 to 20 individuals, or 15 if justified, or 5 batches of 5 individuals | 25-40 | Muscle for Hg. Liver for all other determinands |
| Whiting |  |  |  |
| *Merlangius merlangus* | 25 to 20 individuals, or 15 if justified, or 5 batches of 5 individuals | 20-35 | Muscle for Hg. Liver for all other determinands |
| Hake |  |  |  |
| *Merluccius merluccius* | 25 to 20 individuals, or 15 if justified, or 5 batches of 5 individuals | 20-35 | Muscle for Hg. Liver for all other determinands |

4.2.2 Sampling area

#### Fish

22. To improve the power of the programme, samples should be collected from areas characterised by relatively low natural variability. The spatial representativeness of the area should be known.

#### Shellfish

23. The spatial representativeness of the area should be known. Samples should preferably be collected from sub-tidal regions, otherwise as near to the low water spring tide level as possible. They should be collected as near to the same depth and exposure (i.e. in terms of light and wave action) as possible in order to reduce variability in contaminant uptake. The boundary of the sampling site must be specified. At locations where suitable natural populations are not available, caged mussels may be used.

#### Seabird eggs

24. Sampling sites should reflect important breeding areas. To collect the necessary number of eggs over the period of the monitoring programme sampling sites should be chosen where sufficient numbers of pairs of birds can be expected to breed for the required number of years.

### 4.2.3 Sampling frequency

25. Sampling should be annual. In cases where no trend can still be observed, no local source is influencing the sampling site and natural variability is the dominant reason for variations in concentrations, it may also be possible to reduce the monitoring effort and to change from annual sampling to longer intervals.

### 4.2.4 Sampling period

#### Fish

26. Sampling should take place when fish are in a stable physiological state and, in any case, outside the period of spawning. See Table 3 for further guidance. Sampling should take place within a fixed time span each year (e.g. mid August-mid October for fish in the southern North Sea).

#### Shellfish

27. Sampling should take place during late autumn/early winter, when mussels are in a more stable physiological status, and in any case during a period before spawning. Gametogenesis and spawning generally occur in late spring to early summer, when individuals may lose up to 50% of their soft tissue weight. Table 3 give guidance on spawning periods.

#### Seabird eggs

28. Eggs should be sampled during the species-specific, year-specific and site-specific peak of the first laying cycle within the year. This generally occurs in May/June. Only fresh eggs should be taken from full clutches. For each species, site and year, 10 eggs should be sampled with one egg taken randomly from each of 10 clutches from the first laying cycle within the year.

## 4.3 Sampling strategy for spatial distribution monitoring

29. For each spatial distribution programme, the species and sampling strategy, including quantitative objectives, should be clearly defined to ensure that the purpose of the programme is fulfilled.

### 4.3.1 Species and sampling

30. Table 4 gives the recommended species and number of fish, shellfish and seabird eggs, the size of individual fish and shellfish and the tissue type. However, the number of fish and the number of stations as well as whether individuals or pooled samples should be analysed will depend on the specific objectives of the monitoring programme. In order to reduce the number of analyses which must be performed, pooled samples may be used. Additional, more specific, guidelines on the treatment of samples may need to be prepared by the programme managers.

**Table 3:** Time of spawning season. Spawning season varies regionally due to climate conditions and in the case of fish it is recommended to use FishBase (<http://www.fishbase.org/search.php>) in order to find the specific spawning time for a particular sea area.

|  |  |  |
| --- | --- | --- |
| **Species** | **Spawning season** | **Reference** |
| **Shellfish** |  |  |
| Mussel |  |  |
| *Mytilus edulis*  *M. galloprovincialis* | Spawning throughout the year but normally peaks in springtime and autumn | <http://www.ukmarinesac.org.uk/communities/biogenic-reefs/br4_4.htm> |
| Pacific oyster | Summer months (above 20 °C) | <http://www.fao.org/fishery/culturedspecies/Crassostrea_gigas/en> |
| *Crassostrea gigas* |  |  |
| **Flatfish** |  |  |
| Dab |  |  |
| *Limanda limanda* | January-August | <http://www.fishbase.org/Reproduction/SpawningList.php?ID=695&GenusName=Limanda&SpeciesName=limanda&fc=440&StockCode=711> |
| Plaice |  |  |
| *Pleuronectes platessa* | January-June | <http://www.fishbase.org/Reproduction/SpawningList.php?ID=1342&GenusName=Pleuronectes&SpeciesName=platessa&fc=440&StockCode=1360> |
| Flounder |  |  |
| *Platichthys flesus* | January-June | <http://www.fishbase.org/Reproduction/SpawningList.php?ID=1341&GenusName=Platichthys&SpeciesName=flesus&fc=440&StockCode=1359> |
| **Roundfish** |  |  |
| Cod |  |  |
| *Gadus morhua* | [[6]](#footnote-6) | <http://www.fishbase.org/Reproduction/SpawningList.php?ID=69&GenusName=Gadus&SpeciesName=morhua&fc=183&StockCode=79> |
| Whiting |  |  |
| *Merlangius merlangus* | January-September | <http://www.fishbase.org/Reproduction/SpawningList.php?ID=29&GenusName=Merlangius&SpeciesName=merlangus&fc=183&StockCode=39> |
| Hake |  |  |
| *Merluccius merluccius* | December-August | <http://www.fishbase.org/Reproduction/SpawningList.php?ID=30&GenusName=Merluccius&SpeciesName=merluccius&fc=184&StockCode=40> |

**Table 4:** Spatial distribution sampling

When monitoring for trends at various sites, refer to tables 1 and 2

|  |  |  |  |
| --- | --- | --- | --- |
| **Species** | **Recommended Number** | **Size (cm)** | **Tissue** |
| **Shellfish** |  |  |  |
| First choice |  |  |  |
| Mussel |  |  |  |
| *Mytilus edulis* or  *M. galloprovincialis* | 50footnote 4 ± 10% | 3-6 | Whole soft body |
| Second choice1 |  |  |  |
| Pacific oyster |  |  |  |
| *Crassostrea gigas* | 10footnote 4 ± 10% | 9-14 (2 years of age) | Whole soft body |
| **Flatfish** |  |  |  |
| First choice |  |  |  |
| Dab |  |  |  |
| *Limanda limanda* | 20 to 25 individuals, or 15 if justified, or 5 batches of 5 individuals | 18-30 | Muscle for Hg. Liver for all other determinands |
| Second choice1 |  |  |  |
| Flounder |  |  |  |
| *Platichthys flesus* | 20 to 25 individuals, or 15 if justified, or 5 batches of 5 individuals | 15-35 | Muscle for Hg. Liver for all other determinands |
| **Roundfish** |  |  |  |
| First choice |  |  |  |
| Cod  *Gadus morhua* | 20 to 25 individuals, or 15 if justified, or 5 batches of 5 individuals | >20 | Muscle for Hg. Liver for all other determinands |
| Second choice1 |  |  |  |
| Whiting  *Merlangius merlangus* | 20 to 25 individuals, or 15 if justified, or 5 batches of 5 individuals | 20-35 | Muscle for Hg. Liver for all other determinands |
| Hake  *Merluccius merluccius* | 20 to 25 individuals, or 15 if justified, or 5 batches of 5 individuals | 20-35 | Muscle for Hg. Liver for all other determinands |
| **Seabird eggs** |  |  |  |
| Common tern  *Sterna hirunda* | 10 footnote 2 | - | Whole egg content |
| Oyster catcher  *Haematopus ostralegus* | 10 footnote 2 | - | Whole egg content |
| Guillemot  *Uria aalge* | 10 footnote 3 | - | Whole egg content |

1 Where first choice species is not available.

2 One egg taken randomly from each of 10 clutches.

3 The eggs should be collected as early as possible to avoid collecting replacement eggs.

4 The number of specimens can be adjusted upwards to assure ample sample material for the expected analysis, depending on the actual size class available.

### 4.3.2 Sampling area

#### Fish

31. Samples should be collected from as many locations as necessary to fulfil the objectives of the programme, taking into account the representativeness of the area with regard to spatial variability in contaminant concentrations.

#### Shellfish

32. Samples should be collected from as many locations as necessary to fulfil the objectives of the programme, taking into account the representativeness of the area with regard to spatial variability in contaminant concentrations. Samples should preferably be collected from sub-tidal regions, otherwise as near to the low water spring tide level as possible. They should be collected as near to the same depth and exposure (i.e. in terms of light and wave action) as possible in order to reduce variability in contaminant uptake. The boundary of the sampling site must be specified. At those locations where suitable natural populations are not available, caged mussels may be used.

#### Seabird eggs

33. Sampling sites should reflect important breeding areas. To collect the necessary number of eggs over the period of the monitoring programme sampling sites should be chosen where sufficient numbers of pairs of birds can be expected to breed for the required number of years. Both coastal sites adjacent to the open sea and known “hot spots” such as estuaries should be included.

### 4.3.3 Sampling period

#### Fish

34. Sampling should take place when fish are in a stable physiological state and, in any case, outside the period of spawning. See Table 3 for further guidance.

#### Shellfish

35. Sampling should take place during late autumn/early winter when mussels are in a more stable physiological state and, in any case, during a period before spawning. Gametogenesis and spawning generally occur in late spring to early summer, when individuals may lose up to 50% of their soft tissue weight. Table 3 give guidance on spawning periods.

#### Seabird eggs

36. Eggs should be sampled during the species-specific, year-specific and site-specific peak of the first laying cycle within the year. This generally occurs in May/June. Only fresh eggs should be taken from full clutches. For each species, site and year, 10 eggs should be sampled with one egg taken randomly from each of 10 clutches from the first laying cycle within the year.

## 4.4 Sampling strategy for biological effects monitoring

37. The sampling strategy will, in all cases, depend on the biological effect(s) to be studied. No general guidelines can therefore be given. For more details see specific guidelines (References: OSPAR Guidelines for General Biological Effects Monitoring. OSPAR Ref. No. 1997-7 and Guidelines for contaminant specific biological effects monitoring. OSPAR Ref. No. 2008-9).

# 5. Field sampling and sampling equipment

## 5.1 Fish

38. Fish can be sampled from either research vessels or commercial vessels. The former is the preferred option, since research vessels are likely to have better facilities for processing and storing scientific samples. In both cases, the following precautions must be taken when selecting samples from the trawl catch to ensure that contamination is kept to a minimum:

1. trained personnel must be present when a trawl comes on board to ensure that the sample can be isolated from possible sources of contamination during the release of fish from the net;
2. fish which are visibly damaged or in bad condition must not be selected;
3. clean containers should be available on deck to hold the samples temporarily before they are taken to the ship’s laboratory. Containers used for holding fish collected from the ship’s normal trawling operations must not be used;
4. personnel must wear clean gloves when the samples are taken from the net. The samples should be transferred to the ships laboratory as quickly as possible and rinsed with clean sea water to remove any material adhering to the surface;
5. equivalent precautions should be taken on modern fisheries research vessels, when the catch is released from the net directly into facilities below deck;
6. only material suitable for the subsequent analyses should be retained for storage (see Technical Annexes 1 and 2 for guidance on appropriate storage containers).

39. Suitable fishing gear should be used to ensure that the catch reflects the target population. The trawling time should not exceed one hour and the trawling speed should be as slow as possible to reduce damage and stress to the fish. Details of the requirements for recording the relevant sampling parameters are given in Technical Annexes 1 and 2.

## 5.2 Shellfish

40. Only those individuals that are free of fouling and bored shells should be sampled. When collecting mussels by ship, a commercial mussel dredge can be used. When collecting mussels by hand, personnel should wear gloves. Clean containers consisting of material suitable for the subsequent analyses should be used for transportation. Details of the requirements for recording the relevant sampling parameters and information on sampling methods are given in Technical Annexes 1 and 2.

## 5.3 Seabird eggs

41. The equipment required, details of the requirements for recording the relevant sampling parameters, and information on sampling methods are specified in Technical Annexes 1 and 2.

# 6. Storage and pre-treatment

42. Samples should be analysed as soon as practicable after sampling in order to obtain reliable results. Experience has shown that freezing will degrade soft tissues. Long-term storage and samples for biological effects studies therefore require special conditions. Further advice on archiving and storage techniques used in maintaining biological tissues and other environmental samples for future contaminant analyses can be found in Technical Annexes 1 and 2 and in Tema Nord (1995). Details of the requirements for recording the storage and pre-treatment parameters are given in Technical Annexes 1 and 2.

## 6.1 Fish

43. If conditions allow, samples should be dissected immediately after collection; sub-samples of particular tissue should be removed and deep-frozen. Freezing undissected fish, particularly large ones, may cause soft tissues to degrade and may result in uncontrollable losses of the determinands in the tissue or cross-contamination from other deteriorating tissues. When there are no shipboard laboratories suitable for processing work, warranting the necessary precautionary conditions or personnel on board are not trained for such work, samples of ungutted fish should be preserved by deep freezing, preferably shock freezing to ‑20°C or lower as soon as practicable after collection. Sub-samples for enzymatic tests to be performed in parallel with contaminant analysis, must be stored in liquid nitrogen and analysed as soon as possible after the cruise. Only materials appropriate for the intended analytical techniques should be retained for storage (see Technical Annexes 1 and 2).

44. When pooling samples, an equivalent quantity of tissue must be taken from each fish, *e.g.* a whole fillet from every fish. If the total quantity of tissue obtained would be too large to be handled conveniently, the tissue may be sub-sampled, but a fixed proportion of each tissue must then be taken, *e.g.* 10% of the whole fish for muscle or 10% of each whole liver. This may cause an increase in the inter-individual variability, as contaminants are not equally distributed across the entire tissue. So the sub-sample should be taken from the same part of the organ/muscle of each individual.

## 6.2 Shellfish

45. Mussels should be depurated prior to preservation and analysis. This is to facilitate the discharge of unassimilated particles in the mantle cavity or the gut that might contaminate the sample. This is especially important for mussels collected in water with high turbidity or on silt/clay bottoms. Whether or not the sample has been depurated prior to storage and analysis should be reported. Mussels should be shucked while still alive and opened with minimum tissue damage. The soft tissue samples should be analysed immediately or stored at temperatures below -20°C.

## 6.3 Seabird eggs

46. To avoid deterioration, eggs should be frozen soon after collection and transported frozen to the laboratory. Details of the preparation of the eggs for subsequent chemical analysis are described in Technical Annexes 1 and 2.

# 7. Analytical procedures

47. Details of the requirements for recording the relevant parameters are given in Technical Annexes 1 and 2.

## 7.1 Organic contaminants

48. Procedures for the analysis of organic contaminants in biota include homogenisation, drying, extraction with organic solvents, removal of lipids, clean up, fractionation, followed by separation and detection of single compounds by means of gas chromatography with electron capture (GC-ECD) or mass-spectrometry (GC-MS, GC-MSn) or lipid chromatography coupled with mass-spectrometry (LC-MS, LC-MSn). The total fat weight should be determined, where sufficient material is available. The extractable lipid weight should also be determined on the extract used for organohalogen compound analyses. For tissue containing more than 10-15% of lipids, the results of both total fat and extractable lipid weight are comparable within acceptable limits. Particularly for small amounts of tissue available for analysis, sharing the sample for separate fat determination may result in an insufficient amount for the determination of the analytes and so should be avoided.

Results should be reported on a wet weight basis, along with the total fat and/or extractable lipid weight (in percentage). This will make it possible to recalculate values on both fat and lipid bases. Detailed information is given in Technical Annex 1.

## 7.2 Metals

49. Analysis of trace metals in biota generally includes homogenisation, drying, decomposition, dissolution, matrix separation and detection using element‑specific spectrometric instrumental procedures (*e.g.* AAS, ICP-OES, ICP-MS, TXRF,). Recently developed spectrometric devices aim to reduce the often costly and time consuming sample preparation by applying direct methods without preceding matrix separation and decomposition steps. The results should be reported on a wet weight basis along with the dry weight percentage. Detailed information is given in Technical Annex 2.

# 8. Quality assurance

50. Quality assurance (QA) is the relevant part of the work related to all procedures from sampling to the final instrumental analytical measurement, within a quality management system required to ensure the consistent delivery of quality controlled information. All procedures must be evaluated and controlled on a regular basis. For this purpose a QA scheme must be established in each laboratory. This includes participation in inter laboratory proficiency testing schemes, preferably at an international level, to ensure the long-term stability of the laboratory’s performance, the use of reference materials and the maintenance of all required documentation.

51. To minimise the risk of contamination or the loss of determinands during sampling, storage, pre-treatment or analysis (and so to avoid the generation of false data) QA measures should be applied to the sample from first contact to final measurement and data reporting. All detailed QA data should be recorded in accordance with the QA procedures laid down in the relevant documents. Laboratories should work according to EN 17025.

# 9. Data reporting

52. Data reporting, including QA information, should be in accordance with the requirements set by the relevant OSPAR bodies to ensure that all information for the assessment procedure to be applied are available, and using the latest ICES reporting formats to ensure an efficient and controlled data storage and processing procedure. Information on the ICES data base is available via the ICES-Website (see references).

# 10. References

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Technical Annex 1: Analysis of organic contaminants in biota

This annex is intended as a supplement to the general OSPAR CEMP guidelines. The Annex applies to analysis of all types of organic contaminants but is an overview and is not intended as a complete description or a substitute for detailed instructions. Advice and recommendations given in documents prepared through the QUASIMEME project (Quality Assurance of Information for Marine Environmental Monitoring in Europe) are frequently cited.

# 1. Species

## 1.1 Fish and shellfish

### 1.1.1 Criteria for the selection of species for temporal trend monitoring

Species for temporal trend monitoring can only be selected in the light of information on fishstock composition and history. It is essential that long time series with one species are obtained. Care should be taken that the sample is representative of the population and can be repeated annually. Fish and shellfish species currently used for trend monitoring are listed in Tables 1 and 2 of the main guidelines.

### 1.1.2 Criteria for the selection of species for spatial distribution monitoring

In order to standardise results the first choice species *Limanda limanda*, *Gadus morhua* and *Mytilus edulis* or *Mytilus galloprovincialis* should be used whenever possible. The second choice species *Merlangius merlangus, Merluccius merluccius, Platichthys flesus* and *Crassostrea gigas* should only be used when none of the first choice species are available.

First choice species

*Limanda limanda* **(dab)**

Dab is a ground dwelling species confined to the shelf seas. It has replaced the previously recommended plaice and flounder for the following reasons:

* its migration is less pronounced, thus it is more likely to represent the area in which it is caught;
* it has been used successfully in disease studies, thus complementary information from such studies would be available (in fish disease studies a length range for individual fish of 20‑25 cm is used).

The southern distribution limit of dab is the north coast of Spain.

*Gadus morhua* **(cod)**

Cod normally live near the seabed but may also be pelagic. Cod occur in coastal areas and to 600 m depth. Cod may also be found in the open ocean and so may also be used for monitoring oceanic regions of the Maritime Area. The southern distribution limit of cod is at 45°N. A sampling size range of 30‑45 cm is specified because cod of that size and age tend to feed on a fairly uniform diet.

*Mytilus sp***. (mussel)**

*Mytilus edulis* occurs in shallow waters along almost all coasts of the Contracting Parties. It is therefore suitable for monitoring in nearshore waters. No distinction is made between *Mytilus edulis*, *Mytilus Trossulus* and *Mytilus galloprovincialis* along the Atlantic coasts but their hybridisation could potentially influence the capacity of bioaccumulation and biotransformation of the chemical contaminants. It seems relevant to develop the measure of this parameter by means of genetic markers to integrate these parameters into monitoring where it may occur. A sampling size range of 3‑6 cm is specified to ensure availability throughout the whole maritime area.

**Second choice species**

*Platichthys flesus* (flounder)

The distribution of flounder extends further south than that of dab and might therefore represent the flatfish of choice for certain Portuguese coastal areas and Spain’s northwestern coastal areas. Flounder is not suitable for monitoring in open sea areas due to its migration pattern. A sampling size range of 15‑35 cm ensures individuals of the 2‑3 year age class.

*Merlangius merlangus* (whiting)

Whiting can be caught in coastal waters and up to 200 m depth. Its distribution is from Portugal to Iceland and Norway, thus covering all the maritime area subject to monitoring by Contracting Parties. It is a suitable substitute for Cod. The sampling size range, 20‑35 cm, may need adjustment in the light of future experience.

*Merluccius merluccius* (hake)

Hake live at 100‑300 m along the shelf margins. The sampling size range is 20‑35 cm. The sampling size interval suggested is arbitrary and may need adjustment in the light of future experience.

*Crassostrea gigas* (Pacific oyster)

The Pacific oyster should be sampled in areas where *Mytilus sp.* is not available. The sampling size should be within the length range 9‑14 cm to ensure individuals of the 2 year age class.

## 1.2 Seabirds

Relevant references concerning the use of seabirds in contaminant monitoring programmes include Gilbertson, 1987; Becker, 1989 and 1991; Becker *et al.*, 1991 and 1992; Walker, 1992 and Bignert *et al.*, 1995.

*Sterna hirundo* (common tern)

The common tern is widely distributed over the European and North American Atlantic coasts as well as the Baltic Sea, but does not occur in Iceland. It feeds in marine, brackish and fresh waters.

*Haematopus ostralegus* (oystercatcher)

The oystercatcher is widely distributed along the coasts of the North-west Atlantic, including Iceland, and also occurs in the Baltic Sea. The species is not strictly marine as it also feeds inland. It feeds on benthos. In contrast to other seabirds, nest sites are accessible and the eggs within reach.

*Uria aalge* (guillemot)

The guillemot feeds in the open sea and nests on the coasts of northern Europe, in the Baltic Sea and on the North American coast.

# 2. Sampling

Two alternative sampling strategies are described: sampling to minimise natural variability and length-stratified sampling. References of relevance to sampling and statistics include Gilbert, 1987; Bignert *et al.*, 1993 and 1994; Tema Nord, 1995 and Nicholson *et al.*, 1996 and 1997.

## 2.1 Sampling to minimise natural variability

Gain in precision of the contaminant data can be obtained by minimising variance from the biological covariables. For fish, this can be achieved by sampling and analysing individually at least 12 young fish of the same sex, *e.g.* 2-3 year old female fish. For shellfish, a sample should be collected with the number of individuals large enough to be divided into at least 3 equal pools with each pool consisting of at least 20 animals and enough soft tissue for all analyses. The length of the individuals collected should be constant from year to year at each station, or should at least fall within a very narrow range, *e.g.* within 5 mm. To reflect recent levels of contamination, young individuals should be chosen. In selecting the sample, care should be taken to ensure that it is representative of the population and that it can be obtained annually.

## 2.2 Length-stratified sampling

Where successfully ongoing, length-stratified time series should be continued.

### 2.2.1 Fish

Gain in precision of the contaminant data can also be obtained from stratification using biological variables. Although several biological parameters are appropriate, length appears to be the parameter which can most easily be applied onshore and at sea and which has also been shown to be significant in many analyses. Much discussion has been devoted as to whether simple linear or log-linear (multiplicative) models give the better fit. General experience with other fish and other types of data indicate a preference for the log-normal model at least for the present. As the length dependence of the contaminant concentration is not well understood, sampling should keep the length/contaminant relationship under constant surveillance, *i.e.* the entire length range should be covered evenly. Care should be taken that the samples are not unduly clustered within a particular length interval. More length intervals could be used and the test of the hypothesised contaminant/length relationship becomes stronger if the lengths are evenly distributed. It is essential to keep the length stratification identical from one year to the next. The length range should be defined on the basis of practical considerations. For fish, the upper limit should be chosen in such a way that at least 5 fish in the largest length interval can easily be found. The length stratification should be determined in such a way that it can be maintained over many years. The length interval should be at least 2 cm in size. The length range should be split into 5 length intervals, which are of equal size after log transformation. For example, if the length range is 18‑36 cm, then the interval boundaries could be (rounded to 01 cm) as follows:

18 – 20.7 20.8-23.8 23.9-27.3 27.4-31.3 31.4-36.0 cm.

### 2.2.2 Shellfish

For shellfish, the upper limit should be chosen in such a way that at least 20 mussels in the largest length interval can easily be found. The length stratification should be determined in such a way that it can be maintained over many years. The length interval should be at least 5 mm in size. The length range should be split into at least 3 length intervals (small, medium and large) which are of equal size after log transformation. For example, if the length range is 40‑70 mm, then the interval boundaries could be (rounded to 1 mm) as follows:

5 intervals: 40 - 45 46 - 50 51 - 56 57 - 63 64 - 70

3 intervals: 40 - 48 49 - 58 59 – 70

## 2.3 Seabird eggs

### 2.3.1 Permission

Permission to collect the eggs must be received from the appropriate national authorities.

### 2.3.2 Sampling period and frequency

Eggs should be sampled annually at each site in May or June. Only clutches from the first laying cycle within a single year should be selected.

### 2.3.3 Number of eggs and sampling procedure

Eggs should only be taken from full clutches (*i.e.* common tern 3 eggs, oystercatcher 3‑4 eggs). Eggs should not be taken from abandoned clutches. Only one egg should be taken from each clutch. Ten eggs should be selected in total (*i.e.* one egg from 10 separate clutches) and it is important to choose the egg from each clutch randomly. As the eggs must be fresh (*i.e.* between 1‑5 days incubation) information about the incubation stage of each egg is required. Two methods are recommended for determining incubation stage:

* locate 12‑15 clutches containing one egg only and mark these by placing a peg about 1 m from the nest. Check the clutches every other day until they are complete. Take one egg randomly from the completed clutch;
* fill a 1 litre plastic beaker with water and place the egg in the water:
* fresh eggs (*i.e.* of 1‑2 days incubation) will lie on the bottom with the long axis parallel to the bottom;
* eggs of 3‑6 days incubation will rest with the small end on the bottom of the beaker and the long axis forming an angle of 30-45°;
* eggs which float or stand vertically with the small end on the bottom are of more than 7 days incubation and should not be selected.

Each nest from which an egg has been taken should be marked, using a peg or some other type of marker, to ensure that a second egg is not taken. While still in the field the egg selected should be put into a numbered plastic egg box. The number of the box should be written on the shell of the egg in soft pencil. The clutch size from which the egg was taken, the nest number and the sampling date should be recorded.

### 2.3.4 Materials

For each species, area and year the following are required:

* nest pegs;
* a non-toxic, waterproof marker;
* a 1 litre plastic beaker;
* numbered egg boxes (*e.g.* for oystercatcher: 100 ml, polypropylene polyethylene, ø 55\*73 mm, and for common tern: 50 ml, polystyrol/ polyethylene, ø 41\*49 mm).

# 3. Transportation

## 3.1 Fish and shellfish

Samples should be kept cool and frozen at < -20°C as soon as possible after collection. Length and weight should be determined before freezing. Live mussels should be transported in closed containers at temperatures between 5 ‑ 15°C, preferably < 10°C. Frozen samples should be transported in closed containers at temperatures < -20°C. More rigorous conditions will be necessary for samples taken for biological effects monitoring, *e.g.* storage in liquid nitrogen.

## 3.2 Seabird eggs

Eggs should be kept cool and frozen at -18°C as soon as possible after collection.

# 4. Pre-treatment and storage

## 4.1 Contamination

Sample contamination may occur during sampling, sample handling, pre-treatment and analysis (Oehlenschläger, 1994), due to the environment, the containers or packing material used, the instruments used during sample preparation or from the chemical reagents used during the analytical procedures. The risk of contamination is particularly high for dioxin-like PCBs which occur at ultra-trace levels in the environment. Controlled conditions are therefore required for all procedures, including the dissection of fish organs on board ship. One way of minimising the risk is to conduct dissection in a clean area, such as within a laminar-flow hood away from the deck areas of the vessel.

## 4.2 Fish

### 4.2.1 Dissection and storage

Ungutted fish should be wrapped separately in suitable material (*e.g.* pre-cleaned aluminium foil) and frozen. Plastic materials, except polyethylene or polytetrafluorethylene, must not be used (*cf.* Smedes and de Boer, 1994). The frozen samples should be stored in suitable containers to avoid damage. Sub-samples (*e.g.* of liver) should be stored in pre-cleaned containers made of glass (*e.g.* borosilicate glass), stainless steel or aluminium, or should be wrapped in pre-cleaned aluminium foil and frozen quickly in liquid nitrogen or a blast freezer. The individual samples should be clearly labelled and stored together in a suitable container placed in a deep freeze at < -20°C until analysis. Sub-samples for enzyme tests should be stored in vials suitable for storage in liquid nitrogen, labelled clearly and stored in liquid nitrogen until analysis.

When samples are processed at sea the dissection must be done by trained personnel preferably on a clean bench wearing clean gloves and using clean stainless steel knives. Stainless steel tweezers are recommended for holding tissues during dissection. After each sample has been prepared, the tools should be cleaned. Washing in acetone or alcohol and high purity water is the procedure recommended.

### 4.2.2 Sub-sampling

To sample fish muscle, care should be taken to avoid including any epidermis or subcutaneous fatty tissue in the sample. Samples should be taken underneath the red muscle layer. In order to ensure uniformity, the right side dorso-lateral muscle should be sampled. If possible, the entire right dorsal lateral filet should be homogenised and sub-samples taken for replicate dry weight and contaminant determinations. If however the amount of material to be homogenised would be too large, a specific portion of the dorsal musculature should be chosen. It is recommended that the portion of the muscle lying directly under the first dorsal fin be used in this case. As both fat and water content vary significantly in the muscle tissue from the anterior to the caudal muscle of the fish, in order to ensure comparability it is important to obtain the same portion of the muscle tissue for each sample (see Oehlenschläger, 1994).

When dissecting the liver, care should be taken to avoid contamination from other organs. The whole liver should be homogenised or freeze-dried. If however the amount of material homogenised was too large, a specific portion of the liver should be chosen in order to ensure comparability.

Where pooling of tissues is necessary, an equivalent quantity of tissue must be taken from each fish, *e.g.* a whole fillet from every fish. If the total quantity of tissue so yielded would be too large to be handled conveniently, the tissue may be sub-sampled, but a fixed proportion of each tissue must then be taken, *e.g.* 10% of each whole fillet or 10% of each whole liver or for muscle tissue 10% of the fish.

Personnel must be capable of identifying and removing the desired organs according to the requirements of the investigations.

## 4.3 Shellfish

### 4.3.1 Depuration

Mussels should be placed on a polyethylene tray elevated above the bottom of a glass aquarium. The aquarium should be filled with filtered sub-surface sea water collected from the same site as the samples and which has not been subject to contamination from point sources if possible. The aquarium should be aerated and the mussels left for 20‑24 hours at water temperatures and salinity close to those from which the samples were removed.

### 4.3.2 Opening of the shells

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

### 4.3.3 Dissection and storage

The soft tissues should be removed and deep frozen (< -20°C) as soon as possible in containers appropriate to the intended analysis. The dissection of the soft tissue must be done under clean conditions on a clean bench by trained personnel, wearing clean gloves and using clean stainless steel knives. After each sample has been prepared, the tools should be cleaned. Washing in acetone or alcohol and high purity water is recommended. When the analysis is eventually undertaken, all fluids that may initially separate on thawing should be included with the materials homogenised. Homogenisation should be performed immediately prior to any sub-dividing of the sample.

## 4.4 Seabird eggs

Before thawing, each egg should be placed in a previously weighed goblet. The weight of the egg (to the nearest 0.1 g inclusive of shell), the length of the egg between poles and the breadth of the egg at the equator (to the nearest 0.1 mm using callipers) should be recorded. The egg should then be opened (if this has not already happened during thawing) and the content carefully separated from the shell. If the egg contains an embryo, the eye diameter or the “crown-tail” length of the embryo should be measured (to the nearest 0.1 mm using callipers). The content of the egg (*i.e.* the albumen and yolk) should be weighed (to the nearest 0.1 g) and homogenised in the same goblet for each egg (*e.g.* by an Ultra Turrax). Any sub-sampling should be done immediately after homogenisation. The samples can then be analysed or deep frozen for later analysis. The shell (including the shell-skin) should be washed with water and dried in laboratory air for at least a week before weighing (to the nearest 0.01 g). The shell thickness should be measured at three points along the egg equator.

Technical Annex 2: Metals

This annex is intended as a supplement to the general guidelines. It is not a complete description or a substitute for detailed analytical instructions. Advice and recommendations given in documents prepared through the QUASIMEME project (Quality Assurance of Information for Marine Environmental Monitoring in Europe) are frequently cited.

# 1. Species

## 1.1 Fish and shellfish

### 1.1.1 Criteria for the selection of species for temporal trend monitoring

Species for temporal trend monitoring can only be selected in the light of information on fish stock composition and history. It is essential that long time series with one species are obtained. Care should be taken that the sample is representative of the population and can be repeated annually. Fish and shellfish species currently used for trend monitoring are listed in Tables 1 and 2 of the main guidelines.

### 1.1.2 Criteria for the selection of species for spatial distribution monitoring

To standardise results the first choice species Limanda limanda, Gadus morhua and Mytilus edulis or M. galloprovincialis should be used if possible. The second choice species Merlangius merlangus, Merluccius merluccius, Platichthys flesus and Crassostrea gigas should only be used when none of the first choice species are available.

**First choice species**

*Limanda limanda (dab)*

Dab is a ground dwelling species confined to the shelf seas. It has replaced the previously recommended plaice and flounder for the following reasons:

a. its migration is less pronounced, thus it is more likely to represent the area in which it is caught;

b. it has been used successfully in disease studies, thus complementary information from such studies would be available (in fish disease studies a length range for individual fish of 20‑25 cm is used).

The southern distribution limit of dab is the north coast of Spain.

*Gadus morhua (cod)*

Cod normally live near the seabed but may also be pelagic. Cod occur in coastal areas and to 600 m depth. Cod may also be found in the open ocean and so may also be used for monitoring oceanic regions of the Maritime Area. The southern distribution limit of cod is at 45°N. A sampling size range of 30‑45 cm is specified because cod of that size and age tend to feed on a fairly uniform diet.

*Mytilus sp*. (mussel)

*Mytilus edulis* occurs in shallow waters along almost all coasts of the Contracting Parties. It is therefore suitable for monitoring in nearshore waters. No distinction is made between M. edulis and M. galloprovincialis because the latter, which may occur along Spanish and Portuguese coasts, cannot easily be discerned from *M. edulis*. A sampling size range of 3‑6 cm is specified to ensure availability throughout the whole maritime area. For monitoring in polluted areas, mussels may be transplanted from an unpolluted area and then left in the polluted area (Benedicto et al., 2011; Søndergaard et al., 2011) for e.g. one year before sampling and analyses. The results will reflect the last years contamination in contrast to resident mussels that will reflect several years of contamination.

**Second choice species**

*Platichthys flesus* (flounder)

The distribution of flounder extends further south than that of dab and might therefore represent the flatfish of choice for certain Portuguese coastal areas and Spain’s northwestern coastal areas. Flounder is not suitable for monitoring in open sea areas due to its migration pattern. A sampling size range of 15‑35 cm ensures individuals of the 2-year age class.

*Merlangius merlangus* (whiting)

Whiting can be caught in coastal waters and up to 200 m depth. Its distribution is from Portugal to Iceland and Norway, thus covering all the maritime area subject to monitoring by Contracting Parties. It is a suitable substitute for cod. The sampling size range, 20‑35 cm, may need adjustment in the light of future experience.

*Merluccius merluccius* (hake)

Hake live at 100‑300 m along the shelf margins. The sampling size range is 20‑35 cm. The sampling size interval suggested is arbitrary and may need adjustment in the light of future experience.

*Crassostrea gigas* (Pacific oyster)

The Pacific oyster should be sampled in areas where Mytilus sp. is not available. The sampling size should be within the length range 9‑14 cm to ensure individuals of the 2 year age class.

## 1.2 Seabirds

Relevant references concerning the use of seabirds in contaminant monitoring programmes include Gilbertson (1987), Becker (1989; 1991); Becker et al. (1991; 1992), Walker (1992), Herzke et al. (2009), Miljeteig et al. (2009) and Dittmann et al. (2012).

*Sterna hirundo* (common tern)

The common tern is widely distributed over the European and North American Atlantic coasts as well as the Baltic Sea, but does not occur in Iceland. It feeds in marine, brackish, and fresh waters.

*Haematopus ostralegus* (oystercatcher)

The oystercatcher is widely distributed along the coasts of the North-west Atlantic, including Iceland, and also occurs in the Baltic Sea. The species is not strictly marine as it also feeds inland. It feeds on benthos. In contrast to other seabirds, nest sites are accessible and the eggs within reach.

*Uria aalge* (guillemot)

The guillemot feeds in the open sea and nests on the coasts of northern Europe, in the Baltic Sea and on the North American coast.

# 2. Sampling

Two alternative sampling strategies are described: sampling to minimise natural variability and length-stratified sampling.

## 2.1 Sampling to minimise natural variability

Gain in precision of the contaminant data can be obtained by minimising variance from the biological covariables. For fish, this can be achieved by sampling and analysing individually at least 12 young fish of the same sex, *e.g.* 2-3 year old female fish. To assist the selection of the relevant length range in order to find individuals of the recommended age, it is advised to produce specific species and region related correlation graphs by use of existing data from the respective monitoring data base. An example is given in Appendix 1.

For shellfish, a sample should be collected with the number of individuals large enough to be divided into at least 3 equal pools with each pool consisting of at least 20 animals and enough soft tissue for all analyses. The length of the individuals collected should be constant from year to year at each station, or should at least fall within a very narrow range, *e.g.* within 5 mm. To reflect recent levels of contamination, young individuals should be chosen. In selecting the sample, care should be taken to ensure that it is representative of the population and that it can be obtained annually.

## 2.2 Length-stratified sampling

Where successfully ongoing, length-stratified time series should be continued.

### 2.2.1 Fish

Gain in precision of the contaminant data can also be obtained from stratification using biological variables. Although several biological parameters are appropriate length appears to be the parameter which can most easily be applied onshore and at sea and which has also been shown to be significant in many analyses. Much discussion has been devoted as to whether simple linear or log-linear (multiplicative) models give the better fit. General experience with other fish and other types of data indicate a preference for the log-normal model at least for the present.

As the length dependence of the contaminant concentration is not well understood, sampling should keep the length/contaminant relationship under constant surveillance, i.e. the entire length range should be covered evenly. Care should be taken that the samples are not unduly clustered within a particular length‑interval. More length intervals could be used and the test of the hypothesised contaminant/length relationship becomes stronger if the lengths are evenly distributed. It is essential to keep the length stratification identical from one year to the next. The length range should be defined on the basis of practical considerations. For fish, the upper limit should be chosen in such a way that at least 5 fish in the largest length interval can easily be found. The length stratification should be determined in such a way that it can be maintained over many years. The length interval should be at least 2 cm in size. The length range should be split into 5 length intervals, which are of equal size after log transformation. For example, if the length range is 18‑36 cm, then the interval boundaries could be (rounded to 0.1 cm) as follows:

18 – 20.7 20.8-23.8 23.9-27.3 27.4-31.3 31.4-36 cm.

### 2.2.2 Shellfish

For shellfish, the upper limit should be chosen in such a way that at least 20 mussels in the largest length interval can easily be found. The length stratification should be determined in such a way that it can be maintained over many years. The length interval should be at least 5 mm in size. The length range should be split into at least 3 length intervals (small, medium and large) which are of equal size after log transformation. For example, if the length range is 40‑70 mm, then the interval boundaries could be (rounded to 1 mm) as follows:

a. 5 intervals: 40 - 45 46 - 50 51 - 56 57 - 63 64 - 70

b. 3 intervals: 40 - 48 49 - 58 59 – 70.

## 2.3 Seabird eggs

### 2.3.1 Permission

Permission to collect the eggs must be received from the appropriate national authorities.

### 2.3.2 Sampling period and frequency

Eggs should be sampled annually at each site in May or June. Only clutches from the first laying cycle within a single year should be selected.

### 2.3.3 Number of eggs and sampling procedure

Eggs should only be taken from full clutches (i.e. common tern 3 eggs, oystercatcher 3‑4 eggs). Eggs should not be taken from abandoned clutches. Only one egg should be taken from each clutch. Ten eggs should be selected in total (i.e. one egg from 10 separate clutches) and it is important to choose the egg from each clutch randomly. As the eggs must be fresh (i.e. between 1‑5 days incubation) information about the incubation stage of each egg is required. Two methods are recommended for determining incubation stage:

a. locate 12‑15 clutches containing one egg only and mark these by placing a peg about 1 m from the nest. Check the clutches every other day until they are complete. Take one egg randomly from the completed clutch;

b. fill a 1 litre plastic beaker with water and place the egg in the water:

i. fresh eggs (*i.e.* of 1‑2 days incubation) will lie on the bottom with the long axis parallel to the bottom;

ii. eggs of 3‑6 days incubation will rest with the small end on the bottom of the beaker and the long axis forming an angle of 30-45°;

iii. eggs which float or stand vertically with the small end on the bottom are of more than 7 days incubation and should not be selected.

Each nest from which an egg has been taken should be marked, using a peg or some other type of marker, to ensure that a second egg is not taken. While still in the field the egg selected should be put into a numbered plastic egg box. The number of the box should be written on the shell of the egg in soft pencil. The clutch size from which the egg was taken, the nest number and the sampling date should be recorded.

### 2.3.4 Materials

For each species, area and year the following are required:

1. nest pegs;
2. a non-toxic, waterproof marker;
3. a 1 litre plastic beaker;
4. numbered egg boxes (e.g. for oystercatcher: 100 ml, polypropylene polyethylene, ø 55\*73 mm, and for common tern: 50 ml, polystyrol/polyethylene, ø 41\*49 mm).

# 3. Transportation

## 3.1 Fish and shellfish

Samples should be kept cool and frozen at below -20°C as soon as possible after collection. Length and weight should be determined before freezing. Live mussels should be transported in closed containers at temperatures between 5‑15°C, preferably below 10°C. Frozen samples should be transported in closed containers at temperatures below -20°C. More rigorous conditions will be necessary for samples for biological effects monitoring, *e.g.* storage in liquid nitrogen.

### 3.2 Seabird eggs

Eggs should be kept cool and frozen at -18°C as soon as possible after collection.

## 4. Pre-treatment and storage

### 4.1 Contamination

Sample contamination may occur during sampling, sample handling, pre-treatment and analysis (Oehlenschläger, 1994a), due to the environment, the containers or packing material used, the instruments used during sample preparation or from the chemical reagents used during the analytical procedures. Controlled conditions are therefore required for all procedures, including the dissection of fish organs on board ship. Relevant references concerning clean laboratories include Moody (1982), Mitchell (1982a), Boutron (1990) and Schmidt and Gerwinski (1994).

## 4.2 Fish

### 4.2.1 Dissection, storage and drying

Ungutted fish should be wrapped separately in suitable material (e.g. polyethylene or polytetrafluorethylene) and frozen. The frozen samples should be stored in suitable containers to avoid damage. Sub-samples (e.g. of liver) should be stored in a suitable acid-cleaned container, preferably glass, and frozen or freeze-dried immediately. During freeze-drying, sample temperature should be maintained below 0°C to avoid the loss of volatile compounds. The individual samples should be clearly labelled and stored together in a suitable container. The frozen samples should be maintained at below ‑20°C until analysis. Freeze-dried samples should be stored in a dessicator. Sub-samples for enzyme tests should be stored in vials suitable for storage in liquid nitrogen, labelled clearly and stored in liquid nitrogen until analysis.

The dissection must always be done by trained personnel on a clean bench, wearing clean gloves and using clean stainless steel knives which may be equipped with blades made of ceramics or titanium to reduce the risk of Cr and Ni contamination. Colourless polyethylene tweezers are recommended for holding tissues during dissection. After each sample has been prepared, the tools should be cleaned regularly. The following procedure is recommended:

* wash in acetone or alcohol and high purity water;
* wash non-stainless steel tools in HNO3 p.a./high purity water diluted 1+1, (for tweezers diluted 1+6);
* rinse with high purity water.

### 4.2.2 Sub-sampling

To sample fish muscle, care should be taken to avoid including any epidermis or subcutaneous fatty tissue in the sample. Samples should be taken underneath the red muscle layer. In order to ensure uniformity the right side dorso-lateral muscle should be sampled. If possible, the entire right dorsal filet should be homogenised or freeze-dried and sub-samples taken for replicate dry weight and contaminant determinations. If however the amount of material to be homogenised would be too large, a specific portion of the dorsal musculature should be chosen. It is recommended that the portion of the muscle lying directly under the first dorsal fin be used in this case. As both fat and water content vary significantly in the muscle tissue from the anterior to the caudal muscle of the fish, in order to ensure comparability it is important to obtain the same portion of the muscle tissue for each sample (see Oehlenschläger, 1994b).

When dissecting the liver, care should be taken to avoid contamination from other organs (Viñas et al., 2012). The whole liver should be homogenised or freeze-dried. If however the amount of material homogenised would be too large, a specific portion of the liver should be chosen. In order to ensure comparability, this should always be the same part of the liver, preferably the middle part. Liver samples can be freeze-dried. However, for very fatty samples, freeze-drying can be difficult. In this case, the lipids can be extracted prior to freeze-drying. It has to be ensured that no metals are extracted together with the lipids.

Where pooling of tissues is necessary, an equivalent quantity of tissue must be taken from each fish, *e.g.* a whole fillet from every fish. If the total quantity of tissue so yielded would be too large to be handled conveniently, the tissue may be sub-sampled, but a fixed proportion of each tissue must then be taken, *e.g.* 10% of each whole fillet or 10% of each whole liver or for muscle tissue 10% of the fish.

Personnel must be capable of identifying and removing the desired organs according to the requirements of the investigations.

## 4.3 Shellfish

### 4.3.1 Depuration

Mussels should be placed on a polyethylene tray elevated above the bottom of a glass aquarium. The aquarium should be filled with sub-surface sea water collected from the same site as the samples and which has not been subject to contamination from point sources if possible. The aquarium should be aerated and the mussels left for 20‑24 hours at water temperatures and salinity close to those from which the samples were removed.

### 4.3.2 Opening of the shells

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

### 4.3.3 Dissection and storage

The soft tissues should be removed and deep frozen (-20°C) as soon as possible in containers appropriate to the intended analysis. The dissection must always be done by trained personnel on a clean bench wearing clean gloves and using clean stainless steel knives which may be equipped with blades made of ceramics or titanium to reduce the risk of Cr and Ni contamination. Colourless polyethylene tweezers are recommended for holding tissues during dissection. After each sample has been prepared, the tools should be cleaned regularly. The following procedure is recommended:

* wash in acetone or alcohol and high purity water;
* wash non-stainless steel tools in HNO3 p.a./high purity water 1+1 (for tweezers 1+6);
* rinse with high purity water.

## 4.4 Seabird eggs

Before thawing, each egg should be placed in a previously weighed goblet. The weight of the egg (to the nearest 0.1 g inclusive of shell), the length of the egg between poles and the breadth of the egg at the equator (to the nearest 0.1 mm using callipers) should be recorded. The egg should then be opened (if this has not already happened during thawing) and the content carefully separated from the shell. If the egg contains an embryo, the eye diameter or the “crown-tail” length of the embryo should be measured (to the nearest 0.1 mm using callipers). The content of the egg (*i.e.* the albumen and yolk) should be weighed (to the nearest 0.1 g) and homogenised in the same goblet for each egg (*e.g.* by an Ultra Turrax). Any sub-sampling should be done immediately after homogenisation. The samples can then be analysed or deep frozen for later analysis. The shell (including the shell-skin) should be washed with water and dried in laboratory air for at least a week before weighing (to the nearest 0.01 g). The shell thickness should be measured at three points along the egg equator.

# 5. Analysis

## 5.1 Preparation of equipment and reagents

Glassware and Teflon equipment should be washed extensively with dilute nitric acid, distilled water and acidified metal-free deionised water, and should be rinsed immediately before use with the acids or solvents used according to the following procedure. The blank from all plastic and glassware after the purification procedure should be controlled. Acids, solvents, chemicals and adsorption materials should be free of trace metals or organometallic compounds. If not they should be purified by appropriate methods. Acids should be checked by measuring blanks using the analytical procedure applied to the samples. If necessary, the acids should be purified by distillation, preferably under sub-boiling point conditions in a quartz distillation apparatus. If appropriate, chemicals and adsorption materials should be purified by exhaustive extraction with the solvents used for extraction of the metal compounds. Care should be taken to avoid contamination from laboratory air dust particles. Relevant references concerning reagents and materials Moody et al. (1982; 1989); Tschöpel et al. (1980), Kosta (1982), Mitchell (1982b), Paulsen et al. (1989) and Luque de Castro and Luque García (2002).

## 5.2 Dry weight determination

Dry weight determinations should be carried out by air-drying homogenised sub-samples of the material to be analysed to constant weight at 105°C. Freeze‑drying could also be used for the dry weight determination.

## 5.3 Determination of metals

### 5.3.1 Homogenisation and drying

When the analysis is undertaken, all fluids that may initially separate on thawing should be included with the materials homogenised. Wet or freeze-dried tissues should be homogenised. Homogenisation of wet tissues should be performed immediately prior to any subdividing of the sample. Fresh tissue should be thoroughly homogenised to include any moisture and lipids that may have separated from the solid parts of the sample. Aliquots should be taken as soon as possible, either for direct analysis or for drying. When grinding samples after drying, classical techniques using a ball mill made of different materials should be used. Relevant references concerning homogenisation include Iyengar, 1976; Iyengar et al., 1977 and Klussmann et al., 1985. References concerning sample pre-treatment include Klussmann et al. (1985), Luque de Castro and Luque García (2002) and Larsen et al. (2011).

### 5.3.2 Digestion

The minimum requirements for the digestion procedure are the following:

* complete destruction of all organic material and mineralisation of the sample;
* avoidance of loss of the elements to be determined;
* avoidance of contamination;
* a sampling size of minimum 200 mg dry material

The following aspects should be considered as well:

• Digestion methods will be favoured with use only small amounts of ultra-pure reagents and chemicals;

• The method should be safe to handle (e.g. avoiding hydroperchloric acid);

• Some methods analyse directly and dissolution is not necessary e.g. AMA-

254 for mercury analyses.

• A microwave digestion closed system is preferred for biota samples.

• The use of automated procedures is preferred.

Trace element analysis in biological tissues normally involves digestion of the sample with acids. Very pure acids are essential to ensure acceptable blanks. If “matrix-effects” prevail after sample digestion, three strategies may be followed:

* standard addition for calibration;
* chemical separation procedures;
* matrix modifiers.

### 5.3.3 Instrumental determination

The appropriate instrumental equipment has to be chosen with regard to (i) the elements to be analysed (ii) the concentration levels to be detected (iii) the matrix and the sampling processing prior to the measurement (e.g. digestion, pre-cleaning), but for economic reasons also taken into account (iv) the typical throughput number of samples and (v) investigation and operational costs.

For marine biota samples, all relevant monitoring programmes include mercury, cadmium and lead as mandatory parameters. For analysing Cd and Pb from open sea samples, e.g. flatfish liver of dab and plaice, Graphite Furnace Atomic Absorption Spectometry (GFAAS) and ICP-MS are appropriate. For higher concentrated metals such as Cu and Zn, Flame-AAS, ICP-AES or ICP-OES (weak for Pb) and Total Refection X-Ray Spectrometry (TXRF, weak for Cd) may also be used, but are not suitable to cover all obligating measurements in the required concentration range at very low concentrations without additional preconcentrating procedures.

For mercury cold vapour AAS-systems are commonly used, as stand-alone device or addition to AAS-systems. In recent years, direct measuring systems for analysing mercury from liquid and solid samples without any preceding digestion have become available (e.g. AMA, PE SMS 100 and MLS DMA-80), which have been proven to produce accurate and reliable results. Also a GFAAS-system equipped with a solid sample (autosampler) device for direct measuring and a high-resolution continuum source has become available, which reduces the pretreatment of the samples and has only one source for all elements. Direct methods for analysing mercury using pyrolysis combined with a gold trap and fluorescence or atomic absorption detection are sensitive enough to measure biota sample directly (Carbonell et al., 2009; Maggi et al., 2009; Torres et al., 2012). For the detection of hydride forming elements, such as arsenic, selenium or antimony, nearly all manufacturers of AAS offer additional hydride add-on devices.

# 6. Analytical quality assurance

The programme planners must decide on the accuracy, precision, repeatability, limit of detection and limit of determination required for each specific programme. Achievable limits of determination are as follows:

Cd 5 μg/kg wet weight;

Hg 10 μg/kg wet weight;

Pb 20 μg/kg wet weight;

Cu 200 μg/kg wet weight.

Relevant references concerning QA include HELCOM (1988), QUASIMEME (1992), Harms (1994) and ICES (1995).

## 6.1 Calibration and preparation of calibrands

For calibration purposes, single element standard stock solutions at a concentration of 1000 mg/l are commercially available or can be prepared from the highest quality elements available (generally 99,999% purity) dissolved in high purity acid (usually 1 molar nitric acid). Single or mixed working element standard solutions for calibration purposes are prepared by taking aliquots of the standard stock solutions which are diluted using diluted acid as required. Both standard stock and working solutions are stored in polyethylene, borosilicate or silica volumetric flasks. Borosilicate flasks must not be cleaned with alkaline solutions or heated above 70°C.

Working standard solutions at concentrations less than 100 µg/l should be prepared immediately before use. The actual concentration of the element should be stated on the label together with the date of the preparation of all standard solutions. The calibration procedure must meet some basic criteria or assumptions in order to give a best estimate of the true (but unknown) element content of the sample analysed. These are as follows:

* the masses or concentrations of standards for the establishment of the calibration function must be prepared without bias;
* the chemical and physical properties of the calibration standards must closely resemble those of the sample under investigation;
* sample and calibration standard must be subject to the same operational steps of the analytical procedure;
* signals of repeatedly analysed calibration standards must be randomly distributed on either side of the calibration line.

Application of chemical separation procedures. Although relatively simple standards with a minimum of matrix matching are required, separation procedures that consist of several stages are prone to systematic errors due to both uncontrollable contamination and analyte losses, respectively.

## 6.2 Blanks

A procedural blank should be measured for each sample series and should be prepared simultaneously using the same chemicals and solvents as for the samples. Its purpose is to indicate sample contamination by interfering compounds, which will lead to errors in quantification. Detailed information how to reduce and control contamination is given by ICES (1995).

## 6.3 Accuracy and precision

A laboratory reference material (LRM), preferably a Certified Reference Material (CRM), should be included in the analyses, at least one LRM/CRM sample for each series of identically prepared samples.

The LRM must be homogeneous, well characterised for the determinands in question and stability tests must have shown that it produces consistent results over time. The LRM should be of the same type of matrix (*e.g.* liver, muscle tissue, fat or lean fish) as the samples, and the determinand concentrations should occur in a comparable range to those of the samples. If the range of determinand concentrations in the sample is large (> factor of 5) two reference materials should be included in each batch of analyses to cover the lower and upper concentrations. It is good practice to run duplicate analyses of a reference material to check within-batch analytical variability. The use of a freeze-dried LRM is a practicable alternative to a homogenised and frozen LRM. However, the efficiency of the preceding steps such as homogenisation and drying cannot be checked. A quality control chart should be recorded for each metal. When introducing a new LRM or when it is suspected from the control chart, that there is a systematic error possibly due to an alteration of the LRM, another LRM (preferably a CRM) with a matrix as close as possible to the material analysed, should be used to check the reference material. Table 1 contains information on CRMs commercially available for use in marine monitoring.

**Table 1**: Certified Reference Materials for metals in marine organisms.

|  |  |  |
| --- | --- | --- |
| **Code** | **Organization** | **Matrix** |
| ERM-CE278k | IRMM1 | Mussel tissue |
| ERM-BB422 | IRMM | Fish muscle |
| BCR-463 | IRMM | Tuna fish |
| DOLT-4 | NRC2 | Dogfish liver |
| DORM-4 | NRC | Fish |
| LUTS-1 | NRC | Non defatted lobster hepatopancreas |
| TORT-3 | NRC | Lobster hepatopancreas |
| SRM 2976 | NIST3 | Mussel tissue |
| SRM 1946 | NIST | Lake fish tissue |

1) IRMM: Institute for Reference Materials and Measurements (Europe)

2) NRC: National Research Council (Canada)

3) NIST: The National Institute of Standards and Technology (USA)

Additionally a duplicate of at least one sample should be run with every batch of samples. Each laboratory should participate in inter laboratory comparison studies and proficiency testing schemes on a regular basis, preferably at an international level.

## 6.4 Data collection and transfer

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

# 7. Data recording and reporting parameters

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

The following parameters should be recorded although they may serve different purposes, e.g. internal sampling protocols, and QA or requirements of the database of the assessing body:

## 7.1 Sampling and biological parameters

|  |
| --- |
| Fish   1. location of catch (name, latitude, longitude); 2. date of collection and time (start and end time of trawling operations, GMT); 3. mean trawling depth; 4. type of gear; 5. irregularities and unusual conditions; 6. name and institution of sampling personnel; 7. for each individual:   - the species, its length, total weight, sex, age, reproductive status (GSI);  - sample type (*e.g.* muscle, liver);  - total tissue weight of the dissected organ;   * the number of individuals and data specified for pooled samples. |

|  |
| --- |
| Shellfish   1. location of sampling site (name, latitude and longitude); 2. date and time of sampling (GMT); 3. sampling depth with respect to low tide (for sub-tidal sites only); 4. irregularities and unusual conditions; 5. name and institution of sampling personnel; 6. number of pooled samples; 7. number of individuals in pool; 8. mean, minimum and maximum length and standard deviation; 9. mean dry shell weight; 10. mean soft tissue weight (wet weight); 11. condition index.   Seabird eggs   1. location of sampling site (name, latitude, longitude); 2. species; 3. estimated number of pairs of the species breeding in the sampling area; 4. date of collection; 5. estimated or known laying date; 6. size of clutch from which the egg was taken; 7. number of eggs in the sample from the site; 8. irregularities and unusual conditions; 9. name and institution of sampling personnel; 10. for each egg:   - weight (to the nearest 0.1g);  - length and breadth (between poles and the equator to the nearest 0.1mm);  - content weight exclusive of shell (to the nearest 0.1g);  - shell thickness (to the nearest 5 μm) taking the mean of triplicate measurements with a micrometer;  - shell weight (to the nearest 0.01g);  - embryo length (to the nearest 0.1mm) or eye diameter of the embryo (to the nearest 0.1mm). |

## 7.2 Analytical and quality assurance parameters

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| 1. LRM and CRM results for the metals listed in section 7.3 reported on a wet weight basis; 2. Uncertainty as U2 in the units of the result for use in the OSPAR assessment tool 3. mean soft tissue dry weight and method of determining water content if this differs from air drying to constant weight at 105°C; 4. descriptions of the digestion and instrumental determination methods used; 5. the determination limit for each element. The limits should not exceed the values in Section 6; 6. the relevant QA information according to the requirements specified in the programme;  * the mean tissue lipid weight and method of extraction could also provide valuable information. |

## 7.3 Parameters

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| --- |
| 1. Elements of interest for monitoring programmes for which these guidelines apply:   - cadmium (total);  - mercury (total);  - lead (total);  - zinc;  - copper. |

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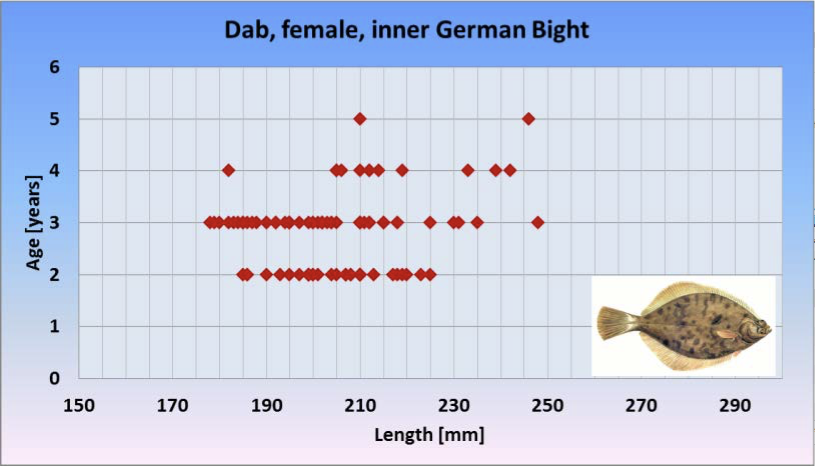
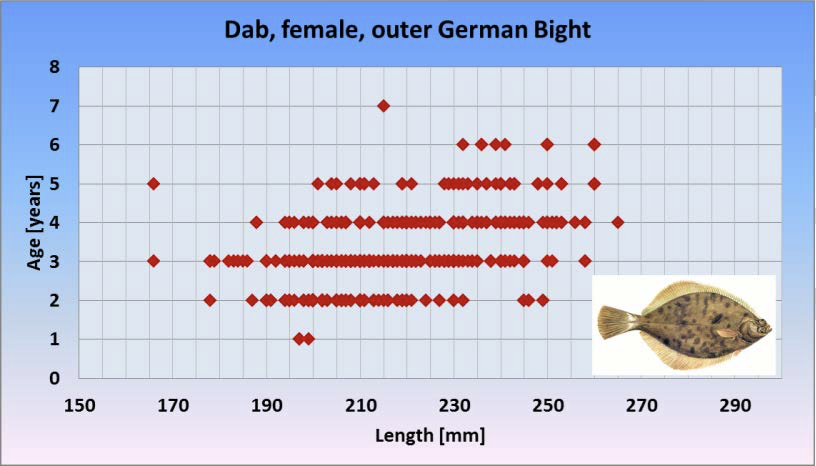
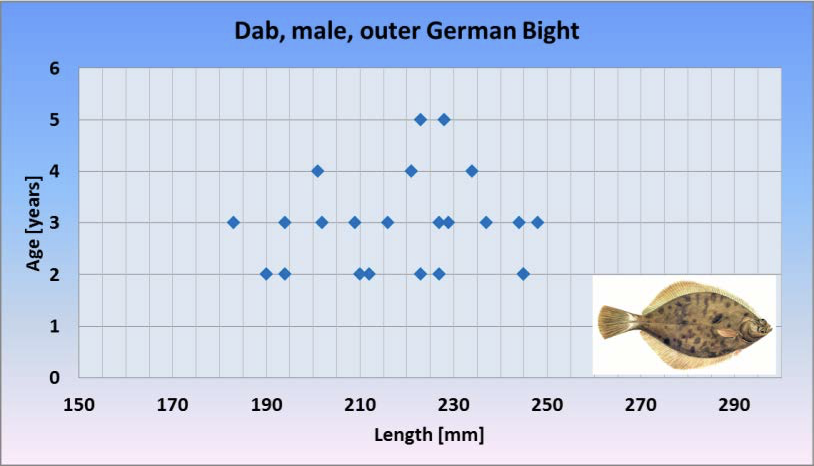
# Appendix

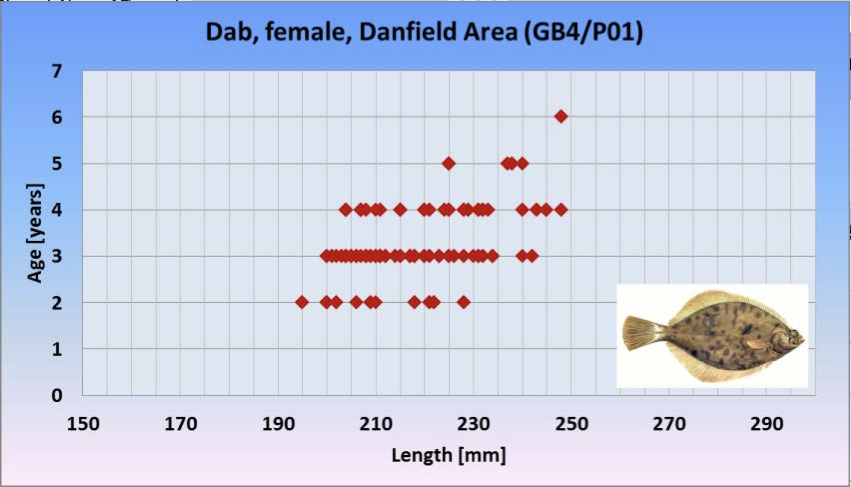
Examples of length-age relations of fish for different species and regions.

These relationships can be used to select individuals of the recommended age by using the length measurement when sampling onboard ship.

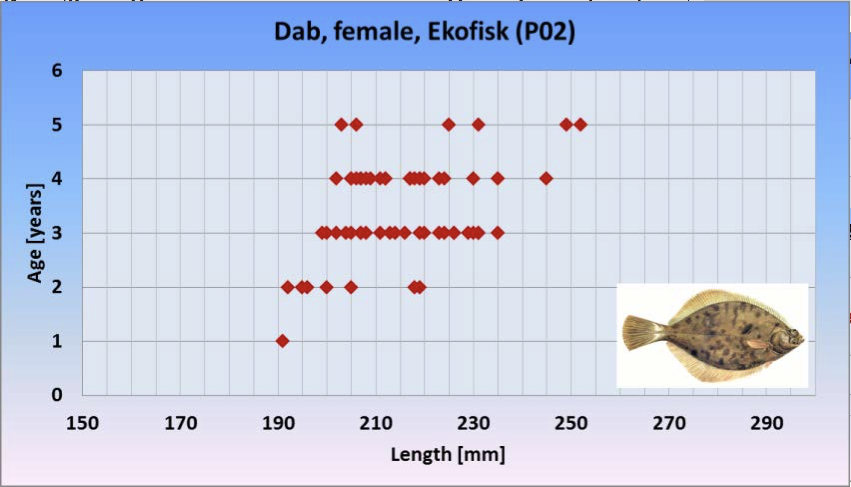
The laboratories of the contracting parties performing metal analysis in biota should use their databases to produce comparable information for their specific species and regions.

Dab, Southern North Sea

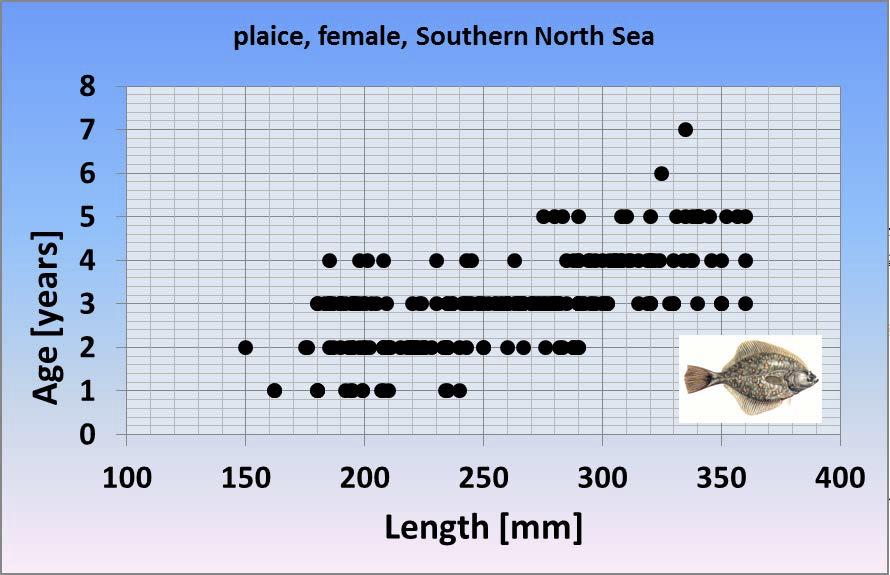
  

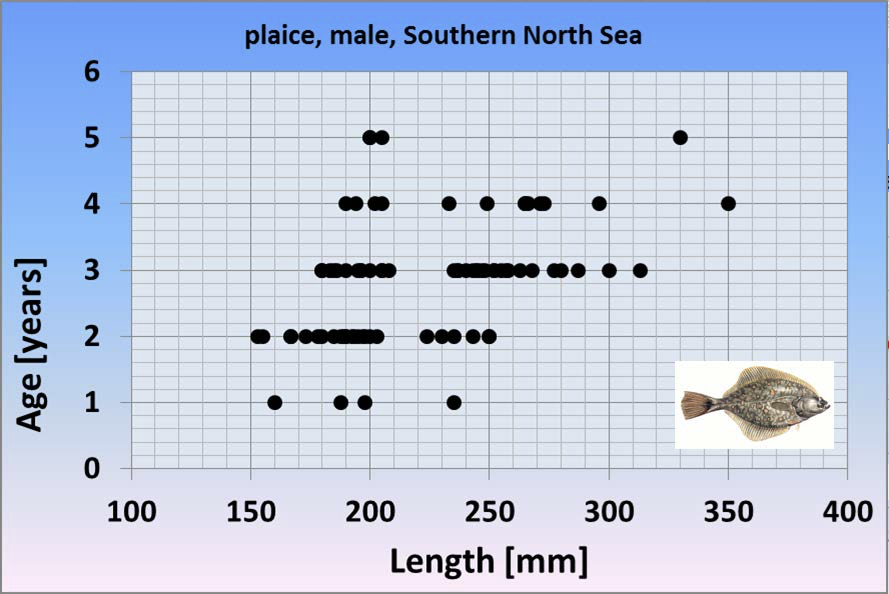


Dab, Central North Sea



Plaice, Southern North Sea, outer German Bight





Technical Annex 3: Determination of parent and alkylated PAHs in biological materials

# 1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) consist of a variable number of fused benzene rings. By definition, PAHs contain at least two fused rings. PAHs arise from incomplete combustion processes and from both natural and anthropogenic sources, although the latter generally predominate. PAHs are also found in oil and oil products, and these include a wide range of alkylated PAHs formed as a result of diagenetic processes, whereas PAHs from combustion sources comprise mainly parent (non-alkylated) compounds. PAHs are of concern in the marine environment for two main reasons: firstly, low molecular weight (MW) PAHs can cause tainting of fish and shellfish and render them unfit for sale; secondly, metabolites of some of the high MW PAHs are potent animal and human carcinogens — benzo[*a*]pyrene is the prime example. Carcinogenic activity is closely related to structure. Benzo[*e*]pyrene and the four benzofluoranthene isomers all have a molecular weight of 252 Da; however, they are much less potent than benzo[*a*]pyrene. Less is known about toxicity of alkylated PAHs. However, one study has demonstrated that alkylated PAHs may have increased toxicity compared to the parent compound (Marvanova *et al.,* 2008).

PAHs are readily taken up by marine animals both across gill surfaces (lower MW PAHs) and from their diet. They may bioaccumulate, particularly in shellfish. Filter-feeding organisms such as bivalve molluscs can accumulate high concentrations of PAHs, both from chronic discharges to the sea (e.g., of sewage) and following oil spills. Fish are exposed to PAHs both via uptake across gill surfaces and from their diet, but do not generally accumulate high concentrations of PAHs as they possess an effective mixed-function oxygenase (MFO) system which allows them to metabolise PAHs and to excrete them in bile. Other marine vertebrate and marine mammals also metabolise PAHs efficiently. An assessment of the exposure of fish to PAHs therefore requires the determination of PAH metabolite concentrations in bile, as turnover times can be extremely rapid.

There are marked differences in the behaviour of PAHs in the aquatic environment between the low MW compounds (such as naphthalene; 128 Da) and the high MW compounds (such as benzo[*ghi*]perylene; 276 Da) as a consequence of their differing physico-chemical properties. The low MW compounds are appreciably water soluble (e.g. naphthalene) and can be bioaccumulated from the dissolved phase by transfer across gill surfaces, whereas the high MW compounds are relatively insoluble and hydrophobic, and can attach to both organic and inorganic particulates within the water column. PAHs derived from combustion sources may actually be deposited to the sea already adsorbed to atmospheric particulates, such as soot particles. The sediment will act as a sink for PAHs in the marine environment.

# 2. Appropriate species for analysis of parent and alkylated PAHs

## 2.1 Benthic fish and shellfish

Guidance on the selection of appropriate species for contaminant monitoring is given in the OSPAR Joint Assessment and Monitoring Programme guidelines. All teleost fish have the capacity for rapid metabolism of PAHs, thereby limiting their usefulness for monitoring temporal or spatial trends of PAHs. Shellfish (particularly molluscs) generally have a lesser metabolic capacity towards PAHs, and so they are preferred because PAH concentrations are generally higher in their tissues. The blue mussel (*Mytilus edulis*) occurs in shallow waters along almost all coasts of the Northeast Atlantic. It is therefore suitable for monitoring in near shore waters. No distinction is made between *M. edulis* and *M. galloprovincialis* because the latter species, which may occur along Spanish and Portuguese coasts, fills a similar ecological niche. A sampling size range of 30–70 mm shell length is specified to ensure availability throughout the whole maritime area. In some areas (e.g., the Barents Sea), other species may be considered. Recent monitoring studies have indicated a seasonal cycle in PAH concentrations (particularly for combustion-derived PAHs) in mussels, with maximum concentrations in the winter prior to spawning and minimum concentrations in the summer. It is particularly important, therefore, that samples selected for trend monitoring and spatial comparisons are collected at the same time of year, and preferably in the first months of the year prior to spawning.

For the purposes of temporal trend monitoring, it is essential that long time-series with either a single species or a limited number of species be obtained. Care should be taken that the sample is representative of the population and that it can be sampled annually. There are advantages in the use of molluscs for this purpose as they are sessile, and so reflect the degree of contamination in the local area to a greater degree than fish which are mobile and metabolise PAHs relatively efficiently. The analysis of fish tissues is often undertaken in conjunction with biomarker and disease studies, and associations have been shown between the incidence of some diseases (e.g., liver neoplasia) in flatfish and the concentrations of PAHs in the sediments over which they live and feed (Malins *et al*., 1988; Vethaak and Rheinallt, 1992). The exposure of fish to PAHs can be assessed by the analysis of PAH metabolites in bile, and by measuring the induction of mixed-function oxygenase enzymes which catalyse the formation of these metabolites.

# 3. Transportation

Live biota should be transported in closed containers at temperatures between 5°C and 10°C. For live animals it is important that the transport time is short and controlled (e.g., maximum of 24 hours). If biomarker determinations are to be made, then it will be necessary to store tissue samples at lower temperatures, for example, in liquid nitrogen at -196°C.

# 4. Pre-treatment and storage

## 4.1 Contamination

Sample contamination may occur during sampling, sample handling, pre-treatment, and analysis, due to the environment, the containers or packing materials used, the instruments used during sample preparation, and from the solvents and reagents used during the analytical procedures. Controlled conditions are therefore required for all procedures. In the case of PAHs, particular care must be taken to avoid contamination at sea. On ships there are multiple sources of PAHs, such as the oils used for fuel and lubrication, and the exhaust from the ship’s engines. It is important that the likely sources of contamination are identified and steps taken to preclude sample handling in areas where contamination can occur. A ship is a working vessel and there can always be procedures occurring as a result of the day-to-day operations (deck cleaning, automatic overboard bilge discharges, etc.) which could affect the sampling process. One way of minimizing the risk is to conduct dissection in a clean area, such as within a laminar-flow hood away from the deck areas of the vessel. It is also advisable to collect samples of the ship’s fuel, bilge water, and oils and greases used on winches, etc., which can be used as fingerprinting samples at a later date, if there are suspicions of contamination in particular instances.

Freeze-drying of tissue samples may be a source of contamination due to the back-streaming of oil vapours from the rotary vacuum pumps. Furthermore, drying the samples may result in losses of the lower molecular weight and more volatile PAHs through evaporation (Law and Biscaya, 1994).

## 4.2 Shellfish

4.2.1 Depuration  
Depending upon the situation, it may be desirable to depurate shellfish so as to void the gut contents and any associated contaminants before freezing or sample preparation. This is usually applied close to point sources, where the gut contents may contain significant quantities of PAHs associated with food and sediment particles which are not truly assimilated into the tissues of the mussels. Depuration should be undertaken in controlled conditions and in clean seawater; depuration over a period of 24 hours is usually sufficient. The aquarium should be aerated and the temperature and salinity of the water should be similar to that from which the animals were removed.

### 4.2.2 Dissection and storage

Mussels should be shucked live and opened with minimal tissue damage by detaching the adductor muscles from the interior of at least one valve. The soft tissues should be removed and homogenised as soon as possible, and frozen in glass jars or aluminium cans at –20°C until analysis. Plastic materials must not be used for sampling and storage owing to possible adsorption of the PAHs onto the container material. As PAHs are sensitive to photo-degradation, exposure to direct sunlight or other strong light must be avoided during storage of the samples as well as during all steps of sample preparation, including extraction and storage of the extracts (Law and Biscaya, 1994). The use of amber glassware is strongly recommended.

When samples are processed, both at sea and onshore, the dissection must be undertaken by trained personnel on a clean bench wearing clean gloves and using PAH-free stainless steel knives and scalpels. Stainless steel tweezers are recommended for holding tissues during dissection. After each sample has been prepared, all tools and equipment (such as homogenisers) should be cleaned by wiping with tissue and rinsing with solvent.

# 5. Analysis

## 5.1 Preparation of materials

Solvents and adsorptive materials must all be checked for the presence of PAHs and other interfering compounds. If found then the solvents, reagents, and adsorptive materials must be purified or cleaned using appropriate methods. Absorptive materials should be cleaned by solvent extraction and/or by heating in a muffle oven as appropriate. Glass fibre materials (e.g. Soxhlet thimbles and filter papers used in pressurised liquid extraction (PLE)) should be cleaned by solvent extraction or pre-baked at 450°C overnight. It should be borne in mind that clean materials can be re-contaminated by exposure to laboratory air, particularly in urban locations, and so the method of storage after cleaning is of critical importance. Ideally, materials should be prepared immediately before use, but if they are to be stored, then the conditions should be considered critically. All containers which come into contact with the sample should be made of glass or aluminium, and should be pre-cleaned before use. Appropriate cleaning methods would include washing with detergents, rinsing with water of known quality, and finally solvent rinsing immediately before use.

## 5.2 Lipid determination

Although PAH data are not usually expressed on a lipid basis, the determination of the lipid content of tissues can be of use in characterising the samples. This will enable reporting concentrations on a wet weight or lipid weight basis. The lipid content should be determined on a separate subsample of the tissue homogenate, as some of the extraction techniques used routinely for PAHs determination (e.g., PLE with fat retainers, alkaline saponification) destroy or remove lipid materials. The total lipid content of fish or shellfish should be determined using the method of Bligh and Dyer (1959) as modified by Hanson and Olley (1963) or an equivalent method such as Smedes (1999). Extractable lipid may be used, particularly if the sample size is small and lipid content is high. It has been shown that if the lipid content is high (>5%) then extractable lipid will be comparable to the total lipid.

## 5.3 Extraction

PAHs are lipophilic and so are concentrated in the lipids of an organism, and a number of methods have been described for PAH extraction (Ehrhardt *et al*., 1991). These methods generally utilise either Soxhlet extraction, or alkaline digestion followed by liquid-liquid extraction with an organic solvent. In the case of Soxhlet extraction, the wet tissue must be dried by mixing with a chemical drying agent (e.g., anhydrous sodium sulphate), in which case a time period of several hours is required between mixing and extraction in order to allow complete binding of the water in the sample. Samples are spiked with recovery standard and should be left overnight to equilibrate. Alkaline digestion is conducted on wet tissue samples, so this procedure is unnecessary.

Apolar solvents alone will not effectively extract all the PAHs from tissues when using Soxhlet extraction, and mixtures such as hexane/dichloromethane may be effective in place of solvents such as benzene and toluene, used historically for this purpose. Alkaline digestion has been extensively used in the determination of PAHs and hydrocarbons and is well documented. It is usually conducted in alcohol (methanol or ethanol), which should contain at least 10% water, and combines disruption of the cellular matrix, lipid extraction and saponification within a single procedure, thereby reducing sample handling and treatment. Solvents used for liquid-liquid extraction of the homogenate are usually apolar, such as pentane or hexane, and they will effectively extract all PAHs.

Alternatively extraction of wet or dry samples of biota may be carried out by pressurised liquid extraction (PLE). This is a more recent method, requiring less solvent and time for the extraction process. The wet biota sample is dried by mixing with sufficient anhydrous sodium sulphate to form a free flowing mixture and is packed into stainless steel extraction cells containing a glass fibre filter and sodium sulphate or glass powder to fill the cell. To ensure a better recovery samples may be extracted twice and extractions are performed at elevated temperatures and pressure.

## 5.4 Clean-up

Tissue extracts will always contain many compounds other than PAHs, and a suitable clean-up is necessary to remove those compounds which may interfere with the subsequent analysis. Different techniques may be used, either singly or in combination, and the choice will be influenced by the selectivity and sensitivity of the final measurement technique and also by the extraction method employed. If Soxhlet extraction was used, then there is a much greater quantity of residual lipid to be removed before the analytical determination can be made than in the case of alkaline digestion. An additional clean-up stage may therefore be necessary. The most commonly used clean-up methods involve the use of deactivated alumina or silica adsorption chromatography. When applying fractionation, the elution pattern has to be checked frequently. This should be carried out in the presence of sample matrix, as that can partially deactivate the clean-up column, resulting in earlier elution of the PAHs than in a standard solution.

Gel permeation chromatography (GPC) and high performance liquid chromatography (HPLC) based methods are also employed (Nondek *et al.,* 1993; Nyman *et al.,* 1993; Perfetti *et al.*, 1992). The major advantages of using HPLC-based clean-up methods are their ease of automation and reproducibility.

Isocratic HPLC fractionation of the extract can be used to give separate aliphatic and aromatic fractions (Webster *et al*., 2002). A metal-free silica column is used for the clean-up/fractionation as dibenzothiophene (DBT) can be retained on ordinary silica columns. The split time is determined by injection of a solution containing representative aliphatic and PAH standards. The silica column is regenerated by a cleaning cycle after a set number of samples. If PAHs are to be analysed by HPLC and there are significant amounts of alkylated PAHs present then the removal of the alkylated PAHs may be difficult.

## 5.5 Pre-concentration

In the methods suggested above, all result in an extract in which non-polar solvents are dominant. The sample volume should be 2 ml or greater to avoid errors when transferring solvents during the clean-up stages. Syncore parallel evaporators can be used with careful optimisation of the evaporation parameters. Evaporation of solvents using a rotary-film evaporator should be performed at low temperature (water bath temperature of 30°C or lower) and under controlled pressure conditions, in order to prevent losses of the more volatile PAHs such as naphthalenes. For the same reasons, evaporation to dryness must be avoided. When reducing the sample to final volume, solvents can be removed by a stream of clean nitrogen gas. Suitable solvents for injection into the GC-MS include pentane, hexane, heptane, *iso*-hexane and *iso*-octane.

## 5.6 Selection of PAHs to be determined

The choice of PAHs to be analysed is not straightforward, both because of differences in the range of PAH compounds resulting from combustion processes and from oil and oil products, and also because the aims of specific monitoring programmes can require the analysis of different representative groups of compounds. PAHs arising from combustion processes are predominantly parent (unsubstituted) compounds, whereas oil and its products contain a much wider range of alkylated compounds in addition to the parent PAHs. This has implications for the analytical determination, as both HPLC-based and GC-based techniques are adequate for the determination of a limited range of parent PAHs in samples influenced by combustion processes, whereas in areas of significant oil contamination and following oil spills only GC-MS has sufficient selectivity to determine the full range of PAHs present. The availability of pure individual PAHs for the preparation of standards is problematic and limits both the choice of determinands and, to some degree, the quantification procedures which can be used. The availability of reference materials certified for PAHs is also rather limited. A list of target parent and alkylated PAHs suitable for environmental monitoring is given in Table A1.1. This differs both from the list previously developed within ICES specifically for intercomparison purposes, and the historic list of Borneff. In both cases, the lists were concentrated on a subset of parent (predominantly combustion-derived) PAHs due to analytical limitations. This approach completely neglects the determination of alkylated PAHs, which allows the interpretation of PAH accumulation from multiple sources including those due to oil inputs. It will not be necessary for all of these PAH compounds and groups to be analysed in all cases, but an appropriate selection can be made from this list depending on the specific aims of the monitoring programme to be undertaken.

**Table A1.1**:Compounds of interest for environmental monitoring for which the guidelines apply. For compounds in italics standards are not available for any isomers in this group.

|  |  |  |  |
| --- | --- | --- | --- |
| **Compound** | **MW** | **Compound** | **MW** |
| Naphthalene | 128 | 2, 3d-benzonapthothiophene | 234 |
| C1-Naphthalenes | 142 | C1-234 | 248 |
| C2-Naphthalenes | 156 | C2-Fluoranthenes/Pyrenes | 230 |
| C3-Naphthalenes | 170 | Benz[*a*]anthracene | 228 |
| *C4-Naphthalenes* | 184 | Chrysene | 228 |
| Acenaphthylene | 152 | 2,3-Benzanthracene | 228 |
| Acenaphthene | 154 | C1- Benz[*a*]anthracene/ Chrysene | 242 |
| Biphenyl | 154 | C2- Benz[*a*]anthracene/ Chrysene | 256 |
| Fluorene | 166 | C3- Benz[*a*]anthracene/ Chrysene | 270 |
| C1-Fluorenes | 180 | Benzo[*a*]fluoranthene | 252 |
| *C2-Fluorenes* | *194* | Benzo[*b*]fluoranthene | 252 |
| *C3-Fluorenes* | *208* | Benzo[*j*]fluoranthene | 252 |
| Dibenzothiophene | 184 | Benzo[*k*]fluoranthene | 252 |
| C1-Dibenzothiophenes | 198 | Benzo[*e*]pyrene | 252 |
| *C2-Dibenzothiophenes* | 212 | Benzo[*a*]pyrene | 252 |
| *C3-Dibenzothiophenes* | 226 | Perylene | 252 |
| Phenanthrene | 178 | Indeno[1,2,3-*cd*]pyrene | 276 |
| Anthracene | 178 | Benzo[*ghi*]perylene | 276 |
| C1-Phenanthrenes/Anthracenes | 192 | Dibenz[*a,h*]anthracene | 278 |
| C2-Phenanthrenes/Anthracenes | 206 | Benzo[*k*]fluoranthene | 252 |
| C3-Phenanthrenes/Anthracenes | 220 | Cyclopenta[*cd*]pyrene | 226 |
| Fluoranthene | 202 | Naphtho[2,1-*a*]pyrene | 302 |
| Pyrene | 202 | Dibenz[*a,e*]pyrene | 302 |
| C1-Fluoranthenes/Pyrenes | 216 | Dibenz[*a,i*]pyrene | 302 |
| 2, 1d-benzonapthothiophene | 234 | Dibenz[*a,l*]pyrene | 302 |
| 1,2d-benzonapthothiophene | 234 | Dibenz[*a,h*]pyrene | 302 |

## 5.7 Instrumental determination of PAHs

The greatest sensitivity and selectivity in routine analysis for parent PAH is achieved by combining HPLC with fluorescence detection (HPLC-UVF) or capillary gas chromatography with mass spectrometry (GC-MS). However, for the analysis of parent and alkylated PAHs GC-MS is the method of choice. In terms of flexibility, GC-MS is the most capable technique, as in principle it does not limit the selection of determinands in any way, while HPLC is suited only to the analysis of parent PAHs. In the past, analyses have also been conducted using HPLC with UV-absorption detection and GC with flame-ionisation detection, but neither can be recommended for alkylated PAHs because of their relatively poor selectivity. Both in terms of the initial capital cost of the instrumentation, and the cost per sample analysed, HPLC-UVF is cheaper than GC-MS. With the advent of high-sensitivity benchtop GC-MS systems, however, this cost advantage is now not as marked as in the past, and the additional information regarding sources available makes GC-MS the method of choice.

Limits of determination within the range of 0.05 to 0.5 µg kg–1 wet weight for individual PAH compounds should be achievable by GC-MS. However this limit can be lowered in routine analysis.

### 5.7.1 GC-MS

The three injection modes commonly used are splitless, on-column and PTV (programmed temperature vaporiser). Automatic sample injection should be used wherever possible to improve the reproducibility of injection and the precision of the overall method. If splitless injection is used, the liner should be of sufficient capacity to contain the injected solvent volume after evaporation. For PAH analysis, the cleanliness of the liner is also very important if adsorption effects and discrimination are to be avoided, and the analytical column should not contain active sites to which PAHs can be adsorbed. Helium is the preferred carrier gas, and only capillary columns should be used. Because of the wide boiling range of the PAHs to be determined and the surface-active properties of the higher PAHs, the preferred column length is 25–50 m, with an internal diameter of 0.15 mm to 0.3 mm. Film thicknesses of 0.2 µm to 1 µm are generally used; this choice has little impact on critical resolution, but thicker films are often used when one-ring aromatic compounds are to be determined alongside PAHs, or where a high sample loading is needed. No stationary phase has been found on which all PAH isomers can be resolved; the most commonly used stationary phase for PAH analysis is 5% phenyl methylsilicone (DB-5 or equivalent). This will not, however, resolve critical isomers such as benzo[*b*], [*j*] and [*k*]fluoranthenes, or chrysene from triphenylene. Chrysene and triphenylene can be separated on other columns, if necessary such as a 60 m non polar column such a DB5MS. For PAHs there is no sensitivity gain from the use of chemical ionisation (either positive or negative ion), so analyses are usually conducted in electron-impact mode at 70eV. Quadrupole instruments are used in single ion monitoring to achieve greater sensitivity. The masses to be detected are programmed to change during the analysis as different PAHs elute from the capillary column. In SIM the molecular ion is used for quantification. Qualifier ions can be used to confirm identification but they are limited for PAHs. Triple quadropole mass spectrometry can also be used and will give greater sensitivity. Some instruments such as ion-trap and time of flight mass spectrometers exhibit the same sensitivity in both modes, so full scan spectra can be used for quantification.

An example of mass spectrometer operating conditions in SIM mode is given in Table A1.2. The ions are grouped and screened within GC time windows of the compounds. In general the number of ions should not be greater than 20. The dwell time is important parameter and should be close for each ion. For GC capillary column analysis a dwell time should not be shorter than 20 ms, while a sum of a dwell in each retention time windows should not be greater than 500 ms. An example of conditions that can be used along with dwell times are shown in Table A1.2.

**Table A.1.2**: Example of operational conditions for the GC-MS analysis of parent and alkylated PAHs.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Group  N° | Retention time  (min) | Dwell time  (ms) | Ions in group  (AMU) | | | | | |
| 1 | 8.00 | 100 | 128 | 136 | 142 |  |  |  |
| 2 | 21.00 | 100 | 152 | 156 | 160 |  |  |  |
| 3 | 23.70 | 100 | 154 | 164 | 168 | 170 |  |  |
| 4 | 26.80 | 80 | 166 | 176 | 180 | 182 | 184 |  |
| 5 | 31.60 | 80 | 178 | 184 | 188 | 194 | 196 | 198 |
| 6 | 35.30 | 100 | 192 | 198 |  |  |  |  |
| 7 | 36.60 | 100 | 206 | 212 |  |  |  |  |
| 8 | 39.40 | 80 | 202 | 206 | 212 | 216 | 220 | 226 |
| 9 | 44.65 | 100 | 216 | 220 |  |  |  |  |
| 10 | 45.30 | 100 | 226 | 228 | 230 | 234 | 240 |  |
| 11 | 48.58 | 90 | 242 | 248 |  |  |  |  |
| 12 | 52.00 | 100 | 252 | 256 | 264 | 266 |  |  |
| 13 | 59.00 | 100 | 266 | 276 | 278 | 288 |  |  |

Alkylated homologues of PAHs (C1–C4), mainly associated with petrogenic sources, contain a number of different isomers that can give very complex but distinct distribution profiles when analysed by GC-MS. Integration of each isomer separately is difficult for most alkylated PAHs. 1- and 2-Methyl naphthalene give well resolved peaks that can be quantified separately. C1-Phenanthrene/anthracene gives five distinct peaks corresponding to 3-methyl phenanthrene, 2-methyl phenanthrene, 2-methyl anthracene, 4- and 9-methyl phenanthrene and 1-methyl phenanthrene. These may be integrated as a group or as separate isomers. For all other alkylated PAHs the area for all isomers may be summed and quantified against a single representative isomer. This method will lead, however, to an overestimation of the concentration as may include non-alkylated PAHs. Examples of integrations of both parent and alkylated PAHs are shown in Appendix 1.

# 6. Calibration and quantification

## 6.1 Standards

The availability of pure PAH compounds are limited. Although most of the parent compounds can be purchased as pure compounds, the range of possible alkyl-substituted PAHs is vast and only a limited selection of them can be obtained. PAH standards are available for at least one isomer of most alkyl group listed in Table A1.1. A range of deuterated PAHs (normally 5 to 7) should be used as internal standards to cover the range of PAHs being analysed in samples. A range of fully-deuterated parent PAHs is available for use as standards in PAH analysis. Suitable standards could range from d8-naphthalene to d14-dibenz[*a,h*]anthracene. Crystalline PAHs of known purity should be used for the preparation of calibration standards. If the quality of the standard materials is not guaranteed by the producer or supplier (as for certified reference materials), then it should be checked by GC-MS analysis. Solid standards should be weighed to a precision of 10–5 grams. Calibration standards should be stored in the dark because some PAHs are photosensitive, and ideally solutions to be stored should be sealed in amber glass ampoules or sealed GC vials. Otherwise, they can be stored in a refrigerator in stoppered measuring cylinders or flasks that are gas tight to avoid evaporation of the solvent during storage.

## 6.2 Calibration

Multilevel calibration with at least five calibration levels is preferred to adequately define the calibration curve. In general, GC-MS calibration is linear over a considerable concentration range but may exhibit a change of slope at very low concentrations. Quantification should be conducted in the linear region of the calibration curve. A separate calibration curve may be used where sample concentrations are very low. An internal standard method should be employed, using a range of deuterated PAHs as internal standards.

## 6.3 Recovery

The recovery of analytes should be checked and reported. Given the wide boiling range of the PAHs to be determined, the recovery may vary with compound group, from the volatile PAHs of low molecular weight to the larger compounds. Deuterated standards can be added in two groups: those to be used for quantification are added at the start of the analytical procedure, whilst those from which the absolute recovery will be assessed are added prior to GC-MS injection. This allows the recovery to be calculated.

# 7. Analytical Quality Control

Planners of monitoring programmes must decide on the accuracy, precision, repeatability, and limits of detection and determination which they consider acceptable. Achievable limits of determination for each individual component are as follows:

* for GC-MS measurements: 0.05 μg kg−1 ww;
* Further information on analytical quality control procedures for PAHs can be found elsewhere (Law and de Boer, 1995). A procedural blank should be measured with each sample batch, and should be prepared simultaneously using the same chemical reagents and solvents as for the samples. Its purpose is to indicate sample contamination by interfering compounds, which will result in errors in quantification. The procedural blank is also very important in the calculation of limits of detection and limits of quantification for the analytical method. In addition, a laboratory reference material (LRM) should be analysed within each sample batch. The LRM must be homogeneous and well-characterised for the determinands of interest within the analytical laboratory. Ideally, stability tests should have been undertaken to show that the LRM yields consistent results over time. The LRM should be of the same matrix type (e.g. mussels) as the samples, and the determinand concentrations should be in the same range as those in the samples. Realistically, and given the wide range of PAH concentrations encountered, particularly in oil spill investigations, this is bound to involve some compromise. The data produced for the LRM in successive sample batches should be used to prepare control charts. It is also useful to analyse the LRM in duplicate from time to time to check within-batch analytical variability. The analysis of an LRM is primarily intended as a check that the analytical method is under control and yields acceptable precision, but a certified reference material (CRM) of a similar matrix should be analysed periodically in order to check the method bias. The availability of biota CRMs certified for PAHs is very limited, and in all cases the number of PAHs for which certified values are provided is small. At present, only NIST 1974a (a frozen wet mussel tissue) and NIST 2974 (a freeze-dried mussel tissue) are available. At regular intervals, the laboratory should participate in an intercomparison or proficiency exercise in which samples are circulated without knowledge of the determinand concentrations, in order to provide an independent check on performance.

# 8. Data reporting

The calculation of results and the reporting of data can represent major sources of error, as has been shown in intercomparison studies for PAHs. Control procedures should be established in order to ensure that data are correct and to obviate transcription errors. Data stored on databases should be checked and validated, and checks are also necessary when data are transferred between databases. Data should be reported in accordance with the latest ICES reporting formats.

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# Appendix 1

Examples of integration of parent and alkylated PAHs analysed by GC-MS. The standards used for the calibration of the alkylated PAHs are asterixed.



**indeno[*123-cd*]pyrene**

**benzo[*ghi*]perylene**

Technical Annex 4: PBDEs in biota



**benzofluoranthenes**

**benzo[*a*]pyrene**

**perylene**

**benzo[*e*]pyrene**

# 1 Introduction

This annex provides advice on polybrominated diphenyl ether (PBDE) analysis for biota. The analysis of PBDEs in biota generally involves extraction with organic solvents, clean-up (removal of lipid) and gas chromatographic separation with mass-spectrometric detection. All stages of the procedure are susceptible to insufficient recovery and/or contamination. Where possible, quality control procedures are recommended in order to check the method’s performance. These guidelines are intended to encourage and assist analytical chemists to reconsider their methods and to improve their procedures and/or the associated quality control measures where necessary.

Polybrominated diphenyl ethers (PBDEs) constitute a group of additive flame retardants that are predominately found in electrical equipment, textiles and furniture. PBDEs are used as additives to polymers and resins and are thought to be more easily released to the environment compared to reactive flame retardants. PBDEs consist of two phenyl rings, connected by an ether bridge, each ring containing up to 5 bromine atoms. There are a possible 209 PBDE congeners depending on the position and number of bromines, with molecular weights ranging from 249 to 960 daltons. Congeners are named according to the International Union of Pure and Applied Chemistry (IUAPAC) numbering format developed for chlorobiphenyl (CB) congeners. However, PBDE technical mixtures used as flame retardants contain only a limited number of these congeners (~20). Commercial PBDE mixtures are classified according to the degree of bromination. The penta mix contains mainly tetra- to hexa-BDEs, the octa mix mainly hexa- to octa-BDEs and the deca mix containing mainly deca-BDE. Penta-BDE is primarily used in furniture and upholstery, octa-BDE in plastics, and deca-PBDEs in textiles and polymers. In the EU, a restriction on the use of the penta and octa technical mixture was put in place on 15 August 2004, restricting the use of the penta and the octa technical mixtures to a limit of 0.1% by mass for all articles placed in the market according to the European Directive 2003/11/EC¹, 24 th amendment of 76/769/EEC.

PBDEs can be released to the environment during their production, while manufacturing other products, and during disposal of products containing these chemicals. In addition, PBDEs may continue to leak out of treated material and constitute a diffuse source of these compounds to the environment. Atmospheric transportation is a major pathway for PBDEs into the marine environment. Other possible pathways include direct discharge from point sources such as storm waters and waste water. PBDEs have been found to concentrate in the Arctic and bioaccumulate in native animals and humans.

Due to the similarity in structure between PBDEs and CBs, PBDEs are expected to persist in the marine environment and exhibit similar toxic properties. PBDEs have high (Log Kow >4) octanol water partition coefficients ranging from 4.3 for di-BDE to 10.33 for deca-BDE (Table 1). PBDEs are readily taken up by marine animals both across gill surfaces and from their diet, and may bioaccumulate.

# 2 Appropriate Species for Analysis of PBDEs

Guidance on the selection of appropriate species for contaminant monitoring is given in the OSPAR guidelines. Other species such as sole, hake and oysters may also be appropriate. Existing data indicates that PBDE concentrations for shellfish are very low and, therefore, detecting long term trends may be difficult using these species. High trophic level organisms and lipid rich tissue will accumulate higher levels of PBDEs and, therefore, may be more suitable for temporal trend monitoring.

# 3 Transportation

Fish samples should be kept cool or frozen (-20°C or lower) as soon as possible after collection. Live mussels should be transported in closed containers at temperatures between 5°C and 10°C, but preferably below 10°C. For live animals it is important that the transport time is short and controlled (e.g. maximum of 24 hours). Frozen fish samples should be transported in closed metal or glass (cleaned and pre-baked) containers at temperatures below -20°C.

# 4 Pretreatment and Storage

## 4.1 Contamination

Sample contamination may occur during sampling, sample handling, pre-treatment and analysis, due to the environment, the containers or packing materials used, the instruments used during sample preparation, and from the solvents and reagents used during the analytical procedures. Controlled conditions are therefore required for all procedures, including the dissection of fish organs on-board ship. It is important that the likely sources of contamination are identified and steps taken to preclude sample handling in areas where contamination can occur. A ship is a working vessel and there can always be procedures occurring as a result of the day-to-day operations (deck cleaning, automatic overboard bilge discharges, etc.) which could affect the sampling process. One way of minimising the risk is to conduct dissection in a clean area, such as within a laminar-flow hood away from the deck areas of the vessel.

## 4.2 Shellfish

### 4.2.1 Depuration

Depending upon the situation, it may be desirable to depurate shellfish so as to void the gut contents and any associated contaminants before freezing or sample preparation. This is usually applied close to point sources, where the gut contents may contain significant quantities of PBDEs associated with food and sediment particles which are not truly assimilated into the tissues of the mussels. Depuration should be undertaken in controlled conditions and in filtered water taken from the sampling site; depuration over a period of 24 hours is usually sufficient. The aquarium should be aerated.

### 4.2.2 Dissection and storage

Mussels should be shucked live and opened with minimal tissue damage by detaching the adductor muscles from the interior of at least one valve. The soft tissues should be removed and homogenised as soon as possible, and frozen in glass jars (pre-baked at 450oC) or aluminium tins at -20°C until analysis. When samples are processed, both at sea and onshore, the dissection must be undertaken by trained personnel on a clean bench wearing clean gloves and using clean stainless steel knives and scalpels. Stainless steel tweezers are recommended for holding tissues during dissection. After each sample has been prepared, all tools and equipment (such as homogenisers) should be cleaned by wiping down with tissue and solvent washed. Knives should only be sharpened using steel to prevent contamination of the blade from the oils used to lubricate sharpening blocks.

## 4.3 Fish

### 4.3.1 Dissection and storage

Ungutted fish should be wrapped separately in suitable material (e.g. solvent washed aluminium foil) and stored at < -20°C. If plastic bags or boxes are used, then they should be used as outer containers only, and should not come into contact with tissues. Organ samples (e.g. liver) should be stored in solvent washed containers made of glass, stainless steel or aluminium, or should be wrapped in solvent washed aluminium foil. In the latter case, care should be taken that the capacity of the freezer is not exceeded. Cold air should be able to circulate between the samples in order that the minimum freezing time can be attained (maximum 12 hours). The individual samples should be clearly and indelibly labelled and stored together in a suitable container at a temperature of -20°C ± 5ºC until analysis. If the samples are to be transported during this period (e.g. from the ship to the laboratory), then arrangements must be made which ensure that the samples do not thaw out during transport.

When samples are processed, both at sea and onshore, the dissection must be undertaken by trained personnel on a bench previously washed with detergent (e.g. Decon 90) wearing clean gloves and using solvent washed stainless steel knives and scalpels. Stainless steel tweezers are recommended for holding tissues during dissection. After each sample has been prepared, all tools and equipment (such as homogenisers) should be cleaned by wiping with tissue and rinsing with solvent.

### 4.3.2 Sub-sampling

When sampling fish muscle, care should be taken to avoid including any epidermis or subcutaneous fatty tissue in the sample. Samples should be taken underneath the red muscle layer. In order to ensure uniformity, the right side dorso-lateral muscle should be sampled. If possible, the entire right side dorsal lateral fillet should be homogenised and sub-samples taken for replicate PBDE determinations. If, however, the amount of material to be homogenised is too large, a specific portion of the dorsal musculature should be chosen. It is recommended that the portion of the muscle lying directly under the first dorsal fin is used in this case.

When dissecting the liver, care should be taken to avoid contamination from the other organs. If bile samples are to be taken then they should be collected first. If the whole liver is not to be homogenised, a specific portion should be chosen in order to ensure comparability.

When pooling of tissues (e.g. liver or muscle) is necessary, an equivalent quantity of tissue should be taken from each fish, e.g., 10% from each whole fillet.

# 5 Analysis

## 5.1 Precautionary Measures

Special precautions are required in the laboratory when analysing PBDEs due to their sensitivity to UV light. PBDEs are prone to photolytic degradation; if exposed to UV light debromination can occur, especially for BDE209 (Covaci *et al.,* 2003; de Boer and Wells, 2006). Therefore, incoming light to the laboratory should be minimised by placing UV filters on the windows and over fluorescent lightings, or by not using any artificial lighting within the laboratory. It is recommended that all calibration and spiking standards are prepared and stored in amber glassware.

The use of plastics, in the laboratory as well as during sampling, should be avoided as they can contain PBDEs. BDE209 can adsorb to dust particles and can be a source of contamination in the laboratory. Therefore, it is recommended that an ioniser be placed in the laboratory and the laboratory kept as dust free as possible. Heating of glass­ware in an oven (e.g. at 450°C overnight) can also be useful for removing PBDE contamination. In addition all glassware should be covered with aluminium foil to keep out any dust.

## 5.2 Solvent Purity and Blanks

BDE209 can stick to glassware (or any other chemically active sites). This can result in contamination of glassware. For work at low concentrations, the use of high-purity solvents is essential, particularly when large solvent volumes are being used for column clean-up. All batches of solvents should be checked for purity by concentration of an aliquot of solvent by at least the same volume factor as used in the overall analytical procedure. Batches which show significant contamination, so as to interfere with analysis, should be rejected. All glassware should be solvent-rinsed immediately prior to use as it will collect contamination from the laboratory atmosphere during storage. Pre-cleaning of all reagents (alumina, silica, sodium sulphate, hydromatrix etc.) is essential.

## 5.3 Preparation of materials

Solvents, reagents and adsorptive materials must be ‘free’ of PBDEs and other interfering compounds. If not, then they must be purified using appropriate methods. Reagents and absorptive materials should be purified by solvent extraction and/or by heating in a muffle oven as appropriate. Glass fibre materials (e.g. Soxhlet thimbles and filter papers used in pressurised liquid extraction (PLE)) should be cleaned by solvent extraction or pre-baked at 450oC overnight. It should be borne in mind that clean materials can be re-contaminated by exposure to laboratory air, particularly in urban locations, and so the method of storage after cleaning is of critical importance. Ideally, materials should be prepared immediately before use, but if they are to be stored, then the conditions should be considered critically. All containers which come into contact with the sample should be made of glass or aluminium, and should be pre-cleaned before use. Appropriate cleaning methods would include washing with detergents, rinsing with water of known quality, and finally solvent rinsing immediately before use.

## 5.4 Lipid determination

The determination of the lipid content of tissues can be of use in characterising the samples. This will enable reporting concentrations on a wet weight or lipid weight basis. The lipid content should be determined on a separate subsample of the tissue homogenate, as some of the extraction techniques used routinely for PBDE determination (e.g., PLE with fat retainers, alkaline saponification) destroy or remove lipid materials. The total lipid content of fish or shellfish should be determined using the method of Bligh and Dyer (1959) as modified by Hanson and Olley (1963) or an equivalent method such as Smedes (1999). Extractable lipid may be used, particularly if the sample size is small and lipid content is high. It has been shown that if the lipid content is high (>5%) then this will be comparable to the total lipid.

## 5.5 Dry weight Determination

The dry weight of samples should be determined gravimetrically so that concentrations can also be expressed on a dry weight basis.

## 5.6 Extraction and clean-up

The similarity in structure of the PBDEs to CBs means that techniques used for the analysis of CBs may also be applied to the analysis of PBDEs (de Boer *et al.*, 2001). PBDEs are lipophilic and so are concentrated in the lipids of an organism. A range of extraction methods have been used for the extraction of PBDEs from biota. These include the more traditional methods such as Soxhlet and the newer automated methods such as pressurised liquid extraction (PLE). Supercritical fluid extraction (SFE) has also been applied to PBDE extractions, although reproducibility was poor compared to Soxhlet(Covaci *et al.,* 2003). However, most laboratories are still using the traditional Soxhlet extraction. For soxhlets, hexane/acetone mixtures or toluene (particularly for BDE209) have been shown to give the best recoveries for the extraction of PBDEs combined with an extraction time of between 6 and 24 h. Hexane/acetone mixtures or toluene are also used with PLE (if no fat retainers used) with an extraction time of ~ 10 min per sample. PLE or soxhlet are therefore the preferred methods with PLE having the advantage of using less solvent, being fully automated and taking less time than Soxhlet.

Tissue extracts will always contain many compounds other than PBDEs, and a suitable clean-up is necessary to remove those compounds which may interfere with the subsequent analysis. Different techniques may be used, either singly or in combination, and the choice will be influenced by the selectivity and sensitivity of the final measurement technique and also by the extraction method employed. PBDEs are stable under acid conditions; therefore treatment with sulphuric acid or acid impregnated silica columns may be used in the clean-up. If Soxhlet extraction is used, then there is a much greater quantity of residual lipid to be removed before the analytical determination can be made than in the case of alkaline digestion. An additional clean-up stage may therefore be necessary. The most commonly used clean-up methods involve the use of alumina or silica adsorption chromatography, but gel permeation chromatography (GPC) is also employed. When using GPC the elution of PBDEs should be carefully checked particularly for BDE209. Destructive methods for lipid removal such as saponification have also been investigated; however this method can result in the degradation of the higher brominated PBDEs and, therefore is not recommended. When applying gel permeation chromatography (GPC), two serial columns are often used for improved lipid separation. Solvent mixtures such as dichloromethane/hexane or cyclohexane/ethyl acetate can be used as eluents for GPC. However, a second clean-up step is often required to separate the PBDEs from other orgnaohalogenated compounds. When silica columns are used, the PBDEs will elute in the second, more polar, fraction (along with the organochlorine pesticides). However, this will be dependent on the solvents used and the adsorbents and the degree of deactivation.

One advantage of using PLE extraction is that it is possible to combine the clean-up with the extraction, especially where mass spectrometry will be used as the detection method. Methods have been developed by Lund University for online clean-up and fractionation of dioxins, furans and PCBs with PLE for food, feed and environmental samples (Sporring et al., 2003). The first method utilises a fat retainer for the on-line clean-up of fat. Silica impregnated with sulphuric acid, alumina and florisil have all been used as fat retainers. A non-polar extraction solvent such as hexane should be used if fat retainers are used during PLE. This method can also be applied to the extraction of PBDEs. However, problems have been highlighted with BDE209 which can be lost during PLE extraction through adsorption on to the extraction system tubing. However, with careful optimisation it is possible to use PLE for BDE209. Losses of BDE209 may be accounted for by using labeled BDE209 as an internal standard.

## 5.7 Pre-concentration

Turbo-vap sample concentrators can be used to reduce solvent volume. This is a rapid technique, but needs to be carefully optimised and monitored to prevent both losses (both of volatiles and solvent aerosols) and cross-contamination. The use of rotary-film evaporators is more time consuming but more controllable However, evaporation of solvents using this technique should be performed at low temperature (water bath temperature of ≤ 30°C) and under controlled pressure conditions, in order to prevent losses of the more volatile PBDEs. For the same reasons, evaporation to dryness should be avoided at all costs. Syncore systems are also more controllable but as rapid as Turbo-vaps and have the advantage of automatically rinsing down the sides of the vial (if the flushback module fitted) while concentrating. Again water-bath temperatures should be minimised to prevent losses. When reducing the sample to the required final volume, solvents can be removed by a stream of clean nitrogen gas. Suitable solvents for injection into the gas chromatograph (GC) include hexane, heptane, toluene and *iso*-octane.

## 5.8 Selection of PBDEs to be determined

PBDE technical mixtures used as flame retardants contain only a limited number of the possible 209 congeners (~20). The penta mix contains mainly tetra- to hexa-BDEs, the octa mix mainly hexa- to octa-BDEs and the deca mix containing mainly deca-BDE. Nine BDE congeners have been detected in the penta mix, the major ones being BDE47 (37%) and BDE99 (35%). The octa mix contains hexa- to octa-brominated congeners, with the main congener being BDE183, a hepta-brominated congener. The deca mix contains 98% decaBDE (BDE209).

PBDE congeners currently analysed vary considerably, however the congeners found in environmental samples are relatively consistent. Most laboratories analyse for the penta-mix compounds, tetra- to hexa-BDEs. In addition, these congeners are thought to be the most toxic and likely to bioaccumulate. In biota the dominant congeners are normally BDE47, 99, 100, 153 and 154. BDE 209 is less frequently measured, due to the analytical difficulties. It is rarely found in biota, but can degrade to lower brominated BDEs. Law *et al*. (2006)proposed a minimum congener set for use when determining BDEs to cover all three technical mixtures and what is commonly found in biota and sediment. This list consisted of BDE28, BDE47, BDE99, BDE100, BDE153, BDE154, BDE183 and BDE209. This list is consistent with the congeners required by the QUASIMEME Scheme for biota and are routinely measured by the majority of laboratories. However, it is apparent that other congeners are found in marine samples (e.g. BDE 66 and 85) and so should also be analysed.

Standards are available for all these congeners. Table 1 lists the PBDEs most commonly monitored.

## 5.9 Instrumental determination of PBDEs

Splitless, pulsed-splitless, programmed temperature vaporiser (PTV) and on-column injectors have been used for the determination of PBDEs, all of which are capable of yielding good results. Automatic sample injection should be used wherever possible to improve the reproducibility of injection and the precision of the overall method. For PBDE analysis, the cleanliness of the liner is very important if adsorption effects and discrimination are to be avoided, and the analytical column should not contain active sites to which PBDEs, particularly BDE209, can be adsorbed. Helium is the preferred carrier gas, and only capillary columns should be used. Mainly non-polar columns are used eg. HT-8, DB1701 and STX-500 (DB1 is usually used for BDE209) Korytar *et al*. (2005) provide comprehensive information on various capillary columns used for PBDE analysis. Baseline separation should be achievable for all BDEs listed in Table 1. However, BDE31 may coelute with BDE28. Because of the wide boiling range of the PBDEs to be determined and the surface-active properties of the higher PBDEs, the preferred column length is 25–50 m, with an internal diameter of 0.1 mm to 0.3 mm. Film thicknesses around 0.2 µm are generally used.

BDE209 can be measured in the same run but will give a smaller and broader peak compared to other PBDEs. Detection limits will be approximately 10 fold higher for BDE209. Since the retention time is long, the determination of BDE209 is often done separately using thinner films (0.1 µm) and/or a shorter column, both of which have been found to improve the detection of BDE209.

**Table 1**:Congeners commonly monitored in environmental samples along with their degree of bromination, chemical name and the octanol water partition coefficient (Log KOW), where available (Braekevelt et al.).

|  |  |  |  |
| --- | --- | --- | --- |
| PBDE Congener | Number of Br | Name | Log KOW |
| BDE17 | 3 | 22’,4-tribromodiphenyl ether | 5.74 |
| BDE28\* | 3 | 2, 44’-tribromodiphenyl ether | 5.94 |
| BDE75 | 4 | 2, 44’, 6-tetrabromodiphenyl ether |  |
| BDE49 | 4 | 2, 34, 5’-tetrabromodiphenyl ether |  |
| BDE71 | 4 | 2, 3’, 4’, 6-tetrabromodiphenyl ether |  |
| BDE47\* | 4 | 2, 2’,4, 4’-tetrabromodiphenyl ether | 6.81 |
| BDE66 | 4 | 2, 3’,4, 4’-tetrabromodiphenyl ether |  |
| BDE77 | 4 | 3, 3’,4, 4’-tetrabromodiphenyl ether |  |
| BDE100\* | 5 | 2, 2’,4, 4’, 6-pentabromodiphenyl ether | 7.24 |
| BDE119 | 5 | 2, 3’,4, 4’, 6-pentabromodiphenyl ether |  |
| BDE99\* | 5 | 2, 2’,4, 4’, 5-pentabromodiphenyl ether | 7.32 |
| BDE85 | 5 | 2, 2’,3, 4, 4’-pentabromodiphenyl ether | 7.37 |
| BDE154\* | 6 | 2, 2’,4, 4’, 5, 6’-hexabromodiphenyl ether | 7.82 |
| BDE153\* | 6 | 2, 2’,4, 4’, 5, 5’-hexabromodiphenyl ether | 7.90 |
| BDE138 | 6 | 2, 2’,3, 4, 4’, 5’-hexabromodiphenyl ether |  |
| BDE190 | 7 | 23 3’,44’,56-heptabromodiphenyl ether |  |
| BDE183\* | 7 | 22',34 4',5',6-heptabromodipheny l ether | 8.27 |
| BDE209\* | 10 | Decabromodiphenyl ether | 10.33 |

\* Congeners proposed by Law *et al.* as a minimum congener set for use when determining BDEs; they are also included in the QUASIMEME scheme

### 5.9.1 Detection Methods

Either gas chromatography-mass spectrometry (GC-MS) or GC-MS-MS (ion trap or triple quadropole) should be used. GC-ECD is rarely used due to the limited linear range, and lack of selectivity. If GC-ECD is used then the clean-up will need to separate out all other organohalogenated compounds which may give co-elution problems. Both high and low resolution 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 PBDEs in environmental samples, the expense and limited availability means that most laboratories use low resolution GC-MS normally in ECNI mode. Lower brominated PBDEs (mono- and di-BDEs) show better sensitivity in EI mode. However, the higher brominated PBDEs (>3 bromines) give better sensitivity using the ECNI mode; limits of detection for these congeners are approximately 10 fold lower in ECNI compared to EI. ECNI shows improved sensitivity compared to positive impact chemical ionisation (PCI). Therefore, GC-ECNI-MS is used most frequently for the analysis of PBDEs in environmental samples. Either ammonia or methane may be used as the reagent gas when using chemical ionisation.

### 5.9.2 GC-MS

The base ions detected using NCI are the bromine ions (*m/z* = 79/81) for the tri- to hepta-BDEs. BDE congeners show the typical 79Br (50.5%) and 81Br (49.5%) isotope distribution pattern. One of the drawbacks of the CI mode is that isotopically labelled standards(13C)cannot be used as internal standards for quantification purposes when only the bromide ions are monitored. However, mono fluorinated BDEs may be used as internal standards. Alternatively using GC-ECNI-MS a recovery standard can be added prior to extraction. CB198 and other halogenated compounds not present in environmental samples can be used as recovery standards. Larger fragment ions, necessary for confirmation, are only found for BDE209. These are formed by the cleavage of the ether bond to give the pentabromo phenoxy ion (*m/z =* 484/486). In general an internal standard method should be used for the quantification of PBDEs.

One advantage of using EI is that 13C labelled internal standards may be used. The major ions formed in EI mode are the molecular ions which can be used for identification and quantification purposes. Other fragment ions are also formed in EI mode which can be used as confirmatory ions.

### 5.9.3 Possible pitfalls and solutions

Degradation of PBDEs, particularly BDE209, can occur on the GC. The presence of a hump or rising baseline before BDE209 is an indication of degradation during injection, whereas the presence of lower brominated BDE (nona-, octa- and eventually other lower brominated BDEs) indicates possible degradation during extraction and clean-up. To minimise this, the GC liners and injection syringe should be changed regularly. Silanising both the syringe and liner may help. When using on-column injection, the choice of retention gap can also have an effect on the degradation of BDE209 during analysis. Deactivated fused silica retention gaps are often used. The QUASIMEME (Quality Assurance of Information for Marine Environmental Monitoring) external quality assurance scheme has also highlighted the difficulties with the analysis of BDE209 with CV% for this congener ranging from 40 – 256%. As a result, many laboratories do not analyse for BDE209.

# 6 Calibration and Quantification

## 6.1 Standards

Standard solutions of known purity should be used for the preparation of calibration standards. If the quality of the standard materials is not guaranteed by the producer or supplier (as for certified reference materials), then it should be checked by GC-MS analysis. In addition, certified standard solutions are available from QUASIMEME and other suppliers for cross-checking. Calibration standards should be stored in the dark because some PBDEs are photosensitive, and ideally solutions to be stored should be sealed in amber, glass ampoules. Otherwise, they can be stored in a refrigerator in stoppered measuring cylinders or flasks that are gas tight to avoid evaporation of the solvent during storage.

Ideally, internal standards should fall within the range of the compounds to be determined, and should not include compounds which may be present in the samples. A range of 13C-labelled PBDEs are available for use as internal standards in PBDE analysis using GC-EIMS. However, when using GC-ECNI-MS these are of little value as, for the majority of congeners, only the bromine ions can be monitored. For BDE209 a high molecular weight fragment is formed during GC-ECNI-MS and, therefore, 13C labelled BDE209 should be used. When GC-ECNIMS is used mono fluorinated BDEs may be used as internal standards or a recovery standard added to each sample prior to extraction and the recovery calculated as a check on the method.

## 6.2 Calibration

Multilevel calibration with at least five calibration levels is preferred to adequately define the calibration curve. In general, GC-MS calibration is linear over a considerable concentration range but exhibits non-linear behaviour when the mass of a compound injected is low due to adsorption. The use of a syringe standard is recommended, for example BDE190. Quantification should be conducted in the linear region of the calibration curve, or the non-linear region must be well characterised during the calibration procedure. Internal standardisation should be used for the quantification of PBDEs.

# 7 Analytical Quality Control

Planners of monitoring programmes must decide on the accuracy, precision, repeatability, and limits of detection and determination which they consider acceptable. Achievable limits of determination for each individual component are as follows:

* for GC-ECNI-MS measurements: 0.05 μg kg−1 wet weight for tri- to hepta-BDEs and 0.50 μg kg−1 wet weight for BDE209;
* for GC-EIMS: 0.5 μg kg−1 wet weight.
* for high resolution GC-MS: 0.02 ng kg−1 wet weight for tri- to hepta-BDEs and 0.5 ng kg−1 wet weight for BDE209.

A procedural blank should be measured with each batch of samples, and should be prepared simultaneously using the same chemical reagents and solvents as for the samples. Its purpose is to indicate sample contamination by interfering compounds, which will result in errors in quantification. Recoveries should be checked for all samples. Recoveries should be between 70 and 120% if not samples should be repeated. The procedural blank is also very important in the calculation of limits of detection and limits of quantification for the analytical method. In addition, a laboratory reference material (LRM) or certified reference material (CRM) should be analysed within each sample batch. The LRM must be homogeneous and well-characterised for the determinands of interest within the analytical laboratory. Ideally the LRM or CRM should be of the same matrix type (e.g., liver, muscle, mussel tissue) as the samples, and the determinand concentrations should be in the same range as those in the samples. The data produced for the LRM or CRM in successive sample batches should be used to prepare control charts. It is also useful to analyse the LRM or CRM in duplicate from time to time to check within-batch analytical variability. The analysis of an LRM is primarily intended as a check that the analytical method is under control and yields acceptable precision. A CRM may be analysed periodically in order to check the method bias. CRMs certified for PBDEs are available (Wise et al.). At regular intervals, the laboratory should participate in an intercomparison or proficiency exercise in which samples are circulated without knowledge of the determinand concentrations, in order to provide an independent check on performance.

# 8 Data Reporting

The calculation of results and the reporting of data can represent major sources of error. Control procedures should be established in order to ensure that data are correct and to obviate transcription errors. Data stored on databases should be checked and validated, and checks are also necessary when data are transferred between databases. If possible data should be reported in accordance with the latest ICES reporting formats.

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Technical Annex 5: Hexabromocyclododecane (HBCD) in biota

# 1 Introduction

This annex provides advice on hexabromocyclododecane (HBCD)analysis for biota. The analysis of HBCD in biota generally involves extraction with organic solvents, clean-up (removal of lipid) and either gas chromatographic separation with mass-spectrometric (MS) detection or liquid chromatography with MS detection. All stages of the procedure are susceptible to insufficient recovery and/or contamination. Where possible, quality control procedures are recommended in order to check the method’s performance. These guidelines are intended to encourage and assist analytical chemists to reconsider their methods and to improve their procedures and/or the associated quality control measures where necessary.

HBCD is produced by the bromination of cycldodec-15 9-triene and has been used since the late 1970s. HBCD is an additive flame retardant that is predominately used in foams and expanded polystyrene and in textile back coatings. HBCD can be released to the environment during its production and while manufacturing other products, and during disposal of products containing this chemical. In addition, HBCD may continue to leak out of treated material and constitute a diffuse source of this compound to the environment. Atmospheric transportation is thought to be a major pathway for HBCD into the marine environment; in addition, point sources may exist. HBCD has been found in remote areas of Sweden and Finland and in the Arctic.

Theoretically, there are sixteen possible stereoisomers of HBCD; 6 enantiomeric pairs and 4 meso forms. However, in technical HBCD mixtures mainly three of the 6 enatiomeric pairs are found, namely α-, β- and γ-HBCD, with the dominant isomer being γ-HBCD (Law *et al.*, 2005). In sediment the γ- isomer also dominates but in biota the major isomer is α-HBCD. β-HBCD is always a minor component. HBCD has a high octanol water partition coefficient (Log Kow = 5.8) and, the potential to bioaccumulate.

# 2 Appropriate Species for Analysis of HBCD

Guidance on the selection of appropriate species for contaminant monitoring is given in the CEMP guidelines. Other species such as sole, hake and oysters may also be appropriate. Existing data indicates that HBCD concentrations for shellfish are very low and, therefore, detecting long term trends may be difficult using these species. High trophic level organisms and lipid rich tissue will accumulate higher levels of HBCD and, therefore, may be more suitable for temporal trend monitoring.

# 3 Transportation

Fish samples should be kept cool or frozen (at a temperature of -20°C or lower) as soon as possible after collection. Live mussels should be transported in closed containers at temperatures between 5°C and 10°C. For live animals it is important that the transport time is short and controlled (e.g. maximum of 24 hours). Frozen fish samples should be transported in closed metal or glass (cleaned and pre-baked) containers at temperatures below -20°C.

# 4 Pre-treatment and Storage

## 4.1 Contamination

Sample contamination may occur during sampling, sample handling, pre-treatment and analysis, due to the environment, the containers or packing materials used, the instruments used during sample preparation, and from the equipment, solvents and reagents used during the analytical procedures. Controlled conditions are therefore required for all procedures, including the dissection of fish on-board ship. It is important that the likely sources of contamination are identified and steps taken to preclude sample handling in areas where contamination can occur. A ship is a working vessel and there can always be procedures occurring as a result of the day-to-day operations (deck cleaning, automatic overboard bilge discharges, etc.) which could affect the sampling process. One way of minimising the risk is to conduct dissection in a clean area, such as within a laminar-flow hood, away from the deck areas of the vessel.

## 4.2 Shellfish

### 4.2.1 Depuration

Depending upon the situation, it may be desirable to depurate shellfish so as to void the gut contents and any associated contaminants before freezing or sample preparation. This is usually applied close to point sources, where the gut contents may contain significant quantities of HBCD associated with food and sediment particles which are not truly assimilated into the tissues of the mussels. Depuration should be undertaken in controlled conditions and in filtered water taken from the sampling site; depuration over a period of 24 hours is usually sufficient. The aquarium should be aerated and temperature controlled?

### 4.2.2 Dissection and storage

Mussels should be shucked live and opened with minimal tissue damage by detaching the adductor muscles from the interior of at least one valve. The soft tissues should be removed and homogenised as soon as possible, and frozen in solvent washed glass jars (pre-baked at 450oC) or aluminium tins at -20°C until analysis.

When samples are processed, both at sea and onshore, the dissection must be undertaken by trained personnel on a clean bench wearing clean gloves and using clean stainless steel knives and scalpels. Stainless steel tweezers are recommended for holding tissues during dissection. After each sample has been prepared, all tools and equipment (such as homogenisers) should be cleaned by wiping down with tissue and solvent washed. Knives should only be sharpened using steel to prevent contamination of the blade from the oils used to lubricate sharpening blocks.

## 4.3 Fish

### 4.3.1 Dissection and storage

Ungutted fish should be wrapped separately in suitable material (e.g. aluminium foil) and frozen. If plastic bags or boxes are used, then they should be used as outer containers only, and should not come into contact with tissues. Organ samples (e.g. livers) should be stored in solvent washed containers, made of glass, stainless steel or aluminium, or should be wrapped in pre-cleaned aluminium foil. Cold air should be able to circulate between the samples in order that the minimum freezing time can be attained (maximum 12 hours). The individual samples should be clearly and indelibly labelled and stored together in a suitable container at a temperature of -20°C until analysis. If the samples are to be transported during this period (e.g. from the ship to the laboratory), then arrangements must be made which ensure that the samples do not thaw out during transport.

When samples are processed, both at sea and onshore, the dissection must be undertaken by trained personnel on a bench previously washed with detergent (e.g. Decon 90) wearing clean gloves and using solvent washed stainless steel knives and scalpels. Stainless steel tweezers are recommended for holding tissues during dissection. After each sample has been prepared, all tools and equipment (such as homogenisers) should be cleaned by wiping with tissue and rinsing with solvent.

### 4.3.2 Subsampling

When sampling fish muscle, care should be taken to avoid including any epidermis or subcutaneous fatty tissue in the sample. Samples should be taken underneath the red muscle layer. In order to ensure uniformity, the right side dorso-lateral muscle should be sampled. If possible, the entire right side dorsal lateral fillet should be homogenised and sub-samples taken for replicate HBCD determinations. If, however, the amount of material to be homogenised is too large, a specific portion of the dorsal musculature should be chosen. It is recommended that the portion of the muscle lying directly under the first dorsal fin is used in this case.

When dissecting the liver, care should be taken to avoid contamination from the other organs. If bile samples are to be taken then they should be collected first. If the whole liver is not to be homogenised, a specific portion should be chosen in order to ensure comparability. When pooling of tissues is necessary, an equivalent quantity of tissue should be taken from each fish, e.g. 10 % from each whole fillet.

# 5 Analysis

## 5.1 Solvent Purity and Blanks

For work at low concentrations, the use of high-purity solvents is essential and particularly when large solvent volumes are being used for extraction and column clean-up. All batches of solvents should be checked for purity by concentration of an aliquot of solvent by at least the same volume factor as used in the overall analytical procedure. Batches which show significant contamination, which will interfere with analysis, should be rejected. All glassware should be solvent-rinsed immediately prior to use as it will collect contamination from the laboratory atmosphere during storage. Heating of glass­ware in an oven (e.g. at 450°C for 24 hours) can also be useful in removing contamination. Pre-cleaning of all reagents (alumina, silica, sodium sulphate, hydromatrix etc) is essential.

## 5.2 Preparation of materials

Solvents, reagents and adsorptive materials must be free of HBCD and other interfering compounds. If not, then they must be purified using appropriate methods. Reagents and absorptive materials should be purified by solvent extraction and/or by heating in a muffle oven as appropriate. Glass fibre materials (e.g. Soxhlet thimbles and filter papers used in pressurised extraction (PLE)) should be cleaned by solvent extraction and/or pre-baked at 450oC overnight. It should be borne in mind that clean materials can be re-contaminated by exposure to laboratory air, particularly in urban locations, and so storage after cleaning is of critical importance. Ideally, materials should be prepared immediately before use, but if they are to be stored, then the conditions should be considered critically. All containers which come into contact with the sample should be made of glass or aluminium, and should be pre-cleaned before use. Appropriate cleaning methods would include washing with detergents, rinsing with water and finally solvent rinsing immediately before use.

## 5.3 Lipid determination

The determination of the lipid content of tissues can be of use in characterising the samples. This will enable reporting concentrations on a wet weight or lipid weight basis. The lipid content should be determined on a separate subsample of the tissue homogenate, as some of the extraction techniques used routinely for HBCD determination (e.g. PLE with fat retainers) destroy or remove lipid materials. The total lipid should be determined using the method of Bligh and Dyer (1959) as modified by Hanson and Olley (1963) or an equivalent method such as Smedes (1999). Extractable lipid may be used, particularly if the sample size is small and lipid content is high. It has been shown that if the lipid content is high (>5%) then this will be comparable to the total lipid. Gravimetric determination of the dry matter content of the sample is recommended.

## 5.4 Extraction and clean-up

HBCD is lipophilic and, therefore, can concentrate in the lipids of an organism. HBCD can be extracted using extraction techniques used for other lipophilic, non-polar compounds such as CBs and PBDEs (Morris *et al.*, 2006). A range of extraction methods have been used for the extraction of HBCD from biota. These include the more traditional methods such as Soxhlet or Ultra Turrax homogenisation and newer automated methods such as pressurised liquid extraction (PLE). However, most laboratories are still using the traditional Soxhlet extraction. For Soxhlets, hexane/acetone mixtures are commonly used combined with an extraction time of between 6 and 24 hrs. Hexane/acetone mixtures are also used with PLE (if no fat retainers are used) with an extraction time of ~ 10 min per sample. PLE or Soxhlet are therefore the preferred methods with PLE having the advantage of using less solvent, being fully automated and taking less time than Soxhlet.

Tissue extracts will always contain many compounds other than HBCD, and a suitable clean-up is necessary to remove those compounds which may interfere with the subsequent analysis. Different techniques may be used, either singly or in combination, and the choice will be influenced by the selectivity and sensitivity of the final measurement technique and also by the extraction method employed. If Soxhlet extraction is used, then there is a much greater quantity of residual lipid to be removed before the analytical determination can be made. The most commonly used clean-up methods involve the use of alumina or silica adsorption chromatography, but gel permeation chromatography (GPC) can also be employed. For GPC, two serial columns are often used for improved lipid separation. Solvent mixtures such as dichloromethane/hexane or cyclohexane/ethyl acetate can be used as eluents for GPC. Depending on the detection method being used and the lipid content of the sample it may be necessary to use a second clean-up step to separate HBCD from other interfering compounds. HBCD is stable under acid conditions; therefore treatment with sulphuric acid or acid impregnated silica columns may be used in the clean-up.

One advantage of using PLE extraction is that it is possible to combine the clean-up with the extraction, especially where mass spectrometry is being used as the detection method. Methods have been developed by Lund University for online clean-up and fractionation of dioxins, furans and PCBs with PLE for food, feed and environmental samples (Sporring *et al.* 2003). The first method utilises a fat retainer for the on-line clean-up of fat. Silica impregnated with sulphuric acid, alumina and florisil have all been used as fat retainers. A non-polar extraction solvent such as hexane should be used if fat retainers are used during PLE. This method can also be applied to the extraction of HBCD. However, if tetrabromobisphenol A (TBBP-A) is also to be extracted, this method is not possible due to retention on the fat retainer.

## 5.5 Pre-concentration

Turbo-vap sample concentrators can be used to reduce solvent volume. The use of rotary-film evaporators is more time consuming but more controllable. Buchi Syncore systems are also more controllable and are as rapid as Turbo-vaps and have the advantage of automatically rinsing down the sides of the vial (if flushback module fitted) while concentrating. In contrast to PBDEs and CBs where the evaporation steps have to be carefully optimised to avoid losses of the lower brominated/chlorinated compounds, loss of HBCD during concentrations is not an issue. When reducing the sample to a final volume, solvents can be removed by a stream of clean nitrogen gas. Suitable solvents for injection into the gas chromatograph (GC) include pentane, hexane, heptane and *iso*-octane. For analysis by LC-MS samples are normally taken to dryness and reconstituted in methanol.

## 5.6 Instrumental determination of HBCD

Analysis of HBCD is less straightforward than the analyses of PBDEs and a different approach is normally required. HBCD can be determined by gas chromatography-mass spectrometry (GC-MS), but the analysis can be problematic. The uncertainty is greater than for PBDEs analysed using the same method (Covaci *et al.*, 2003). In addition, the three main HBCD diastereoisomers found in technical mixtures cannot be separated by GC and a total concentration only can be determined. A liquid chromatography (LC) method is required to separate the three diastereoisomers, with separation of enantiomers being possible with a chiral HPLC column.

### 5.6.1 GC-MS

Few publications analyse HBCD along with the PBDEs by GC-MS, although it has been done using both GC- electron capture negative ionisation (ECNI) and high resolution GC-MS. GC-electron capture detection (ECD) is rarely used due to the limited linear range, and lack of selectivity. If GC-ECD is used then the clean-up will need to separate out all other organohalgenated compounds which may give co-elution problems. Splitless, pulsed-splitless, programmed temperature vaporiser (PTV) and on-column injectors have been used for the determination of HBCD. Automatic sample injection should be used wherever possible to improve the reproducibility of injection and the precision of the overall method. Mainly non-polar columns are used, for example HT-8, DB-5, STX-500. Both high and low resolution GC-MS can be used in conjunction with either electron ionisation (EI) or ECNI. Most laboratories using GC for HBCD use low resolution GC-MS normally in ECNI mode. ECNI shows improved sensitivity compared to EI or positive impact chemical ionisation (PCI). When GC-ECNI-MS is used, the bromine ion is monitored. One of the drawbacks of the CI mode is that isotopically labelled standards(13C)cannot be used as internal standards for quantification purposes when only the bromine ions are monitored. Larger fragment ions, required for structural confirmation are not formed in ECNI mode. Either ammonia or methane may be used as the reagent gas when using chemical ionisation.

HBCD isomers interconvert at temperatures >160oC, therefore the three HBCD diastereoisomers cannot be separated and a broad hump is obtained in the GC chromatogram. In addition, the three diastereoisomers will have different response factors and, therefore, the concentration of HBCD cannot be determined accurately by GC-MS (Wells and de Boer, 2006). Furthermore HBCD degrades at 240oC, therefore, there may be significant losses of HBCD during GC analysis. Cold on-column injection, short GC columns and thin stationary films can minimise the degradation of HBCD. When analysing for HBCD by GC-MS the liner should be changed after each batch of samples to keep it as clean as possible. Co-elution of HBCD with certain PBDEs can also be a problem.

### 5.6.2 LC-MS

A reverse phase column should be used for analysis of HBCD by LC-MS. The three diastereoisomers found in the technical mixture should separate easily using a column such as a C18 and either methanol/water or acetonitrile/water, normally with ammonium acetate (10 mM), as the mobile phase. Typically the flow rate will be around 250 µl min-1 and a gradient programme will be required. HPLC with chiral columns such as permethylated β-cyclodextrin columns can also be used to separate the enantiomers of the α, β, γ-HBCD diastereoisomers. Either electrospray or atmospheric pressure chemical ionisation (APCI) can be used. However, electrospray is more sensitive and is therefore recommended. Clean-up of the samples before analysis is important to avoid matrix effects and ion suppression. The deprotonated molecular ion (*m/z* = 640.7) should be the major ion, fragment ions may also be identified to be used as qualifier ions. LC-MS has been reported to have poorer detection limits compared to GC-MS, with the sensitivity being approximately 10 times less than that of the GC-NCIMS method. Using LC-MS and with an injection volume of ~15 μl, it should be possible to detect around 0.5 ng on column (Morris *et al.*, 2004). LC-MS-MS can usually overcome the problem of higher detection limits.

# 6 Calibration and Quantification

## 6.1 Standards

HBCD standard solutions for each of the three major stereoisomers (α-, β- and γ-HBCD) of known purity should be used for the preparation of calibration standards. If the quality of the standard materials is not guaranteed by the producer or supplier (as for certified reference materials), then it should be checked by GC-MS analysis. In addition, certified standard solutions are available from QUASIMEME and other suppliers for cross-checking. Calibration standards should be stored in sealed amber glass ampoules. Otherwise, they can be stored in a refrigerator in stoppered measuring cylinders or flasks that are gas tight to avoid evaporation of the solvent during storage.

Ideally, internal standards should fall within the range of the compounds to be determined, and should not include compounds which may be present in the samples. Deuterated and 13C-labelled HBCD standards are available for the three major diastereoisomers for use as internal standards in HBCD analysis using GC-EIMS or LC-MS. However, deuterated standards are less expensive and are therefore the preferred option. As HBCD is prone to ion suppression it is recommended that a labelled standard should be used for each isomer being analysed by LC-MS. When using GC-ECNI-MS these are of little value as only the bromine ions can be monitored. When GC-ECNI-MS is used for the analysis a recovery standard should be added to each sample prior to extraction and the recovery calculated as a check on the method.

## 6.2 Calibration

Multilevel calibration with at least five calibration levels is preferred to adequately define the calibration curve. In general, GC-MS calibration is linear over a considerable concentration range but exhibits non-linear behaviour when the mass of a compound injected is low due to adsorption. Quantification should be conducted in the linear region of the calibration curve, or the non-linear region must be well characterised during the calibration procedure. External standardisation is used for HBCD with GC-ECNI-MS as the bromine ions only are monitored. An internal standard method may be used when GC-EIMS or LC-MS is used.

# 7 Analytical Quality Control

Planners of monitoring programmes must decide on the accuracy, precision, repeatability, and limits of detection and determination which they consider acceptable. Achievable limits of determination for each individual component are as follows:

* for GC-ECNI-MS: 0.05 μg kg−1 wet weight
* for LC-MS: 0.5 μg kg−1 wet weight.
* for LC-MS/MS: 0.05μg kg−1 wet weight

A procedural blank should be measured with each batch of samples, and should be prepared simultaneously using the same chemical reagents and solvents as for the samples. Its purpose is to indicate sample contamination by interfering compounds, which will result in errors in quantification. The procedural blank is also very important in the calculation of limits of detection and limits of quantification for the analytical method. For GC-EIMS or LC-MS analysis, labelled standards can be added after or prior to extraction, whilst those from which the absolute recovery will be assessed are added prior to GC-MS injection. This ensures that the calculated HBCD concentrations are corrected for the recovery obtained in each case. For GC-ECNI-MS, recovery of HBCD should be checked and reported. In the case of GC-ECNI-MS a recovery standard such as CB198 should be added prior to extraction and the recovery calculated for each sample, by reference to an external standard.

In addition, a laboratory reference material (LRM) or certified reference material (CRM) should be analysed within each sample batch if available. The LRM must be homogeneous and well-characterised for the determinands of interest within the analytical laboratory. Ideally the LRM or CRM should be of the same matrix type (e.g., liver, muscle, mussel tissue) as the samples, and the determinand concentrations should be in the same range as those in the samples. The data produced for the LRM or CRM in successive sample batches should be used to prepare control charts. It is also useful to analyse the LRM or CRM in duplicate from time to time to check within-batch analytical variability. The analysis of an LRM is primarily intended as a check that the analytical method is under control and yields acceptable precision. A CRM may be analysed periodically in order to check the method bias. The availability of biota CRMs certified for HBCD is very limited. At regular intervals, the laboratory should participate in an intercomparison or proficiency exercise in which samples are circulated without knowledge of the determinand concentrations, in order to provide an independent check on performance.

# 8 Data Reporting

The calculation of results and the reporting of data can represent major sources of error. Control procedures should be established in order to ensure that data are correct and to obviate transcription errors. Data stored on databases should be checked and validated, and checks are also necessary when data are transferred between databases. If possible data should be reported in accordance with the latest ICES reporting formats.

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Technical Annex 6: Perfluorinated compounds in biota

# 1. Introduction

Per- and polyfluorinated compounds (PFCs) are man-made chemicals and are ubiquitous in the environment (1). PFCs are widely used as processing additives during fluoropolymer production and as surfactants in consumer applications, including surface coatings for carpets, furniture and paper products. They are also components in breathable, waterproof fabrics, fire-fighting foams and insulators for electric wires (2). From the production and use of these products, PFCs can be released into the environment.

In this document, the name PFCs refers to compounds with a hydrophilic functional group and a hydrophobic fully fluorinated chain which can vary in length. The polyfluorinated acids have high water solubilities, low pKa values and are therefore dissociated at environmentally relevant pH values (2). Perfluorinated sulfonamides and fluorotelomer alcohols (FTOHs) are neutral compounds currently discussed as precursors to perfluorooctane sulfonate (PFOS) and perfluorinated carboxylic acids (PFCAs) (3).

The objective of this technical annex is to provide advice on the analysis of PFCs in biota. The detection of PFCs at ppb levels is complex because of a risk of contamination during sample handling, storage, preparation and instrumental analysis. Several methods to determine PFCs in biota tissue are applied in various laboratories, but they generally apply extraction with medium polar organic solvents, clean-up steps and liquid-chromatography (LC) with mass spectrometric detection (MS). International Standard Organisation (ISO) has already promoted a standard for the determination of PFOS and perfluorooctanoic acid (PFOA) in water (4), but at present no standard is available for the analysis of biota samples.

# 2 Analyses

Table 1.5.5.4.1 gives an overview of PFCs relevant for analysis in biota. They are chosen from the following groups: Perfluorinated sulfonates (PFSAs), perfluorinated sulfinates (PFSiAs), PFCAs and perfluorinated sulfonamides. For monitoring purposes, the high-volume chemicals PFOS and PFOA are considered the most important PFCs and should be included in biota monitoring. Additionally, it is suggested that long-chained PFCs (≥ C8) should be included in analysis due to their bioaccumulative potential. The substitute perfluorobutane sulfonate is likely to bioaccumulate to a lesser extent and might be more relevant for water monitoring. However, until more information is available, perfluorobutane sulfonate might be included in biota monitoring as well. Table 1.5.5.4.1 also includes suggestions for appropriate internal standards (IS). It is strongly recommended to use mass-labelled compounds as IS for PFC-analysis.

|  |  |  |  |
| --- | --- | --- | --- |
| **Table 1.5.5.4.1**:Full names, acronyms, formulas, and Chemical Abstract System (CAS) numbers of PFCs relevant for biota analysis. | | | |
| **Analyte** | **Acronym** | **Formula** | **CASa-Number** |
| Perfluorobutanoic acid | ***PFBA*** | C3F7COOH | 375-22-4 |
| Perfluoropentanoic acid | ***PFPA*** | C4F9COOH | 2706-90-3 |
| Perfluorohexanoic acid | ***PFHxA*** | C5F11COOH | 307-24-4 |
| Perfluoroheptanoic acid | ***PFHpA*** | C6F13COOH | 375-85-9 |
| Perfluorooctanoic acid | ***PFOA*** | C7F15COOH | 335-67-1 |
| Perfluorononanoic acid | ***PFNA*** | C8F17COOH | 375-95-1 |
| Perfluorodecanoic acid | ***PFDA*** | C9F19COOH | 335-76-2 |
| Perfluoroundecanoic acid | ***PFUnDA*** | C10F21COOH | 4234-23-5 |
| Perfluorododecanoic acid | ***PFDoDA*** | C11F23COOH | 307-55-1 |
| Perfluorotridecanoic acid | ***PFTriDA*** | C15F25COOH | 72629-94-8 |
| Perfluorotetradecanoic acid | ***PFTeDA*** | C13F27COOH | 376-06-7 |
| Perfluorohexadecanoic acid | ***PFHxDA*** | C15F31COOH | 67905-19-5 |
| Perfluorobutane sulfonate | ***PFBS*** | C4F9SO2O- | 29420-49-3 (potassium salt) |
| Perfluorohexane sulfonate | ***PFHxS*** | C6F13SO2O- | 3871-99-6  (potassium salt) |
| Perfluoroheptane sulfonate | ***PFHpS*** | C7F15SO2O- | n.a. |
| Perfluorooctane sulfonate | ***PFOS*** | C8F17SO2O- | 1763-23-1  (sodium salt) |
| Perfluoro-1-decanesulfonate | PFDS | C10F21SO2O- | 13419-61-9  (sodium salt) |
| 1H,1H,2H,2H-perfluorooctane sulfonate | THPFOS (6:2 FTS) | C6F13C2H4SO3- | 27619-97-2 |
| Perfluorooctane sulfinate | ***PFOSi*** | C8F17SO2- | n.a. |
| Perfluorooctane sulfonamide | PFOSA | C8F17SO2NH2 | 754-91-6 |
| **Internal Standards** |  |  |  |
| Perfluoro-n-(1,2,3,4-13C4)butanoic acid | [13C4]-PFBA | [2,3,4-13C3]F713COOH | n.a. |
| Perfluoro-n-(1,2-13C2)hexanoic acid | ***[13C2]-PFHxA*** | C4F9[2-13C]F213COOH | n.a. |
| Perfluoro-n-[1,2,3,4-13C4]octanoic acid | [13C4]-PFOA | C4F9[2,3,4-13C3]F613COOH | n.a. |
| Perfluoro-n-[1,2,3,4,5-13C5]nonanoic acid | ***[13C5]-PFNA*** | C4F9[2,3,4,5-13C4]F813COOH | n.a. |
| Perfluoro-n-[1,2-13C2]decanoic acid | ***[13C2]-PFDA*** | C8F1713CF213COOH | n.a. |
| Perfluoro-n-[1,2-13C2]undecanoic acid | ***[13C2]-PFUnDA*** | C9F1913CF213COOH | n.a. |
| Perfluoro-n-[1,2-13C2]dodecanoic acid | ***[13C2]-PFDoDA*** | C10F2113CF213COOH | n.a. |
| Perfluoro-1-hexane[18O2]sulfonate | ***[18O2]-PFHxS*** | C6F13S[18O2]O- | n.a. |
| Perfluoro-1-[1,2,3,4-13C4]octanesulfonate | ***[13C4]-PFOS*** | C4F9[1,2,3,4-13C4]F8SO2O- | n.a. |
| Perfluoro-1-[1,2,3,4-13C4]octanesulfinate | [13C4]-PFOSi | ***C4F9[1,2,3,4-13C4]F8SO2-*** | n.a. |
| a, Chemical Abstract System; n.a., not available | | | |

# 3. Species and tissue for analysis

The same species as those commonly used in the monitoring of other bioaccumulative and persistent contaminants, such as organochlorine compounds, are recommended. The most suitable monitoring species are described in the main text of these guidelines.

However, the accumulation behaviour of PFCs is different from that of other halogenated contaminants. PFCs primarily bind to the plasma protein albumin and accumulate in blood, especially serum, and organs, for example in liver and kidney (5). Therefore, the highest concentrations are often found in blood and liver.

Regarding mussel analysis, the soft tissue is recommended, while liver should be favoured in environmental PFC monitoring of fish. At present, experience with PFC monitoring of seabird eggs is limited, but the compounds have been detected e.g. in guillemot and herring gull eggs (6, 7).

# 4. Sampling, transportation and storage

The dissection and the collection of eggs and blood has to be carried out by trained staff/personnel as incorrect handling of biota samples can contaminate the sample for PFC analysis. Materials and clothes which contain or can adsorb fluorinated compounds must be avoided. In particular the containers or bags which come in direct contact with the sample should not contain fluorinated polymers like Teflon™. Instead, polypropylene materials are recommended. After collection, samples should be stored in closed containers at a temperature lower than −20°C until sample preparation. The handling time at room temperature should be short because of the possible degradation of precursors to PFCAs and PFSAs.

# 5. Sample preparation

The sample preparation for analysis of PFCs requires clean conditions if possible on a clean bench. The laboratory has to be kept as free as possible from any material which can contain fluorinated compounds (e.g. Teflon™). Every material which can come in contact with the sample must be free of fluorinated compounds. Materials used in the PFC analysis should be cleaned with solvents such as methanol and acetone and covered with solvent rinsed aluminium foil to keep out any dust. The septa of vials should be Teflon™-free such as Barrier™ septa made of silicone polymer and aluminium. Solvents including ultrapure water should be of high purity and must be tested for residues of PFCs prior to use.

Within every sample batch, a method blank should be analysed. If measurable blanks occur, the analytical instrumentation and every sample preparation step have to be checked for contamination.

## 5.1 Homogenisation

An Ultra-turrax® disperser with plastic dispersing (e.g. polycarbonate and polysulfone) is one option to obtain a homogeneous extract. Depending on matrix and expected concentrations, an appropriate sample amount is weighed in polypropylene tubes for the extraction. After homogenisation the extract should be spiked with an IS mixture at concentrations close to the environmental level. Before extraction the sample should be incubated with the IS for about 12 h at 4°C so that the IS can be incorporated into the matrix.

## 5.2 Extraction

Three methods are commonly used for the extraction of PFCs in biota. One is published by *Hansen et al.* (6) and uses an ion pair extraction with tetrabutylammonium (TBA) and the extraction solvent methyl *tert*-butyl ether (MTBE). The second method is described by Powley *et al.* (7) and uses ultrasonic extraction (UE) with a subsequent clean-up. The third method is described by So *et al.* (8) and includes alkaline digestion followed by solid phase extraction (SPE) on WAX™ cartridges. In the following section, the UE method with acetonitrile or methanol is described because of its easy handling and good recoveries, but the other two methods will be acceptable alternatives. The UE method includes a minimum of three extractions, each with 10-fold solvent of the sample volume and 30-min extraction time. After the extraction, the three extracts are combined for clean-up.

## 5.3 Clean-up

Because of matrix effects on ionisation enhancement/suppression in electrospray tandem mass spectrometry (ESI-MS-MS), a clean-up of the extracts is necessary. 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)). Preliminary results indicate that retention on silica can be used for lipid removal, but is likely to lead to losses of PFOSA. Lipids can also be removed from methanol or acetonitrile extracts by precipitation at −20°C (9). The extract is then centrifuged for 1 minute and the supernatant is decanted into a clean vial.

After lipid removal, additional clean-up steps will be required. An appropriate method is described by Powley *et al.* (7). Briefly, 25 mg of graphitised carbon adsorbent (e.g. ENVI-Carb™, 100 m2 g-1, 120/400 mesh) and 50µL acetic acid are added into a small tube. The extract is concentrated to 1 mL and transferred into the tube. The extract is mixed, centrifuged and finally, 0.5 mL of the supernatant is transferred to another flask.

Alternatively, commercially available SPE cartridges can be used (e.g. ENVI-Carb™, 100 mg, 1 mL, 100–400 mesh). If necessary, a second freezing out step can be used if the matrix still interferes with the measurement. Additional clean up might be required, depending on sample type and concentration levels.

Sample extracts should be concentrated according to the required sensitivity. Concentration techniques at low temperature (< 40°C) and controlled pressure conditions are preferred in order to avoid losses of the more volatile PFCs. Evaporation to dryness should be avoided.

An injection standard (InjS) can be added to the final extract for correction of the injection volumes and calculation of the recoveries of the mass-labelled IS. The InjS should not occur in environmental samples; this can be avoided if a mass-labelled InjS is used.

The solvent composition of the final extract should correspond to the mobile phase of the LC method in order to obtain a satisfactory peak shape of the compounds, in particular of the short-chain PFCs. Unless the samples are analysed immediately, the vials should be kept at 4°C or lower.

# 6. Instrumental analysis

LC coupled with a tandem mass spectrometer and interfaced with an electrospray ionisation source in a negative-ion mode (LC-(-)ESI-MS/MS) (6, 10) or LC coupled with an (‑)ESI time-of-flight mass spectrometer (LC-ESI-QTOF-MS) (11) is the best choice for PFC analysis. Tandem MS and QTOF-MS have the advantage of low instrumental noise with a high selectivity.

## 6.1 Liquid chromatography

For the liquid-chromatography C8 or C18 reserved phase columns can be used. A guard column may improve the peak performance and extend the lifetime of the chromatography column. As the mobile phase, water with methanol or acetonitrile can be used, both with 2–10 mM ammonium acetate as an ionisation aid. Gradients from 10% to 100% methanol or acetonitrile will be necessary for the separation of the compounds listed in Table 1.5.5.4.1.

Modifications of the instrument might be necessary to minimise contact with fluorine-containing materials (12). For example, Teflon™-containing tubing, filters for the mobile phase solvents and degassers can be sources for contaminations. A scavenger cartridge can be installed between the pump and injector to trap contaminants from the mobile phase.

To ensure stability of retention times, the use of a temperature controlled column oven is strongly recommended.

## 6.2 Detection methods

The most widely used instrument for detection of PFCs is by tandem MS. Typical precursor and product ions are given in Table 1.5.5.4.2. MS-parameters for the individual compounds, such as collision energy, declustering potential and cone voltage, have to be optimised for each instrument. The sensitivity of tandem MS is usually about one order of magnitude higher than that of QTOF-MS (13).

**Table 1.5.5.4.2** Precursor and product ions for PFCs analysed using LC-(-)ESI-MS/MS.

| Analyte | Precursorion (m/z) | Product ion (m/z) |
| --- | --- | --- |
| *PFBA* | 112.9 | 168.7 |
| *PFPA* | 262.8 | 218.9 |
| *PFHxA* | 312.9 | 268.8 |
| *PFHpA* | 362.9 | 318.9 |
| *PFOA* | 413.0 | 368.9 |
| *PFNA* | 462.9 | 418.9 |
| *PFDA* | 512.9 | 469.0 |
| *PFUnDA* | 562.9 | 519.0 |
| *PFDoDA* | 613.0 | 568.9 |
| *PFTriDA* | 663.1 | 618.9 |
| *PFTeDA* | 713.0 | 669.0 |
| *PFHxDA* | 812.8 | 769.1 |
| *PFBS* | 298.9 | 79.8 |
| *PFHxS* | 398.9 | 79.8 |
| *PFHpS* | 449.0 | 79.3 |
| *PFOS* | 499.0 | 79.7 |
| PFDS | 598.9 | 79.5 |
| THPFOS (6:2 FTS) | 426.9 | 406.7 |
| *PFOSi* | 482.8 | 418.9 |
| PFOSA | 497.9 | 77.9 |
| [13C4]-PFBA | 216.8 | 171.8 |
| *[13C2]-PFHxA* | 314.9 | 269.9 |
| [13C4]-PFOA | 417.0 | 371.8 |
| *[13C5]-PFNA* | 467.9 | 423.0 |
| *[13C2]-PFDA* | 514.9 | 469.8 |
| *[13C2]-PFUnDA* | 565.0 | 519.8 |
| *[13C2]-PFDoDA* | 614.9 | 569.9 |
| *[18O2]-PFHxS* | 403.0 | 83.9 |
| *[13C4]-PFOS* | 502.9 | 79.5 |
| [13C4]-PFOSi | 486.9 | 422.9 |

# 7. Calibration and quantification

## 7.1 Standards

The use of commercially available standards with a purity of >99% is recommended. The purity of standards should be verified, as impurities from the same homologue group and isomers can occur.

Suggestions for mass-labelled IS are given in Table 1.5.5.4.1. Mass-labelled IS and an InjS are strongly recommended for the quantification of PFCs in biota. The IS and InjS have to be added before the extraction and the measurement, respectively. If possible the corresponding mass-labelled IS should be used for each target analyte. In case a mass-labelled standard is not available, an IS with similar physicochemical characteristics and recovery rates to the target compound can be used but matrix suppression/ enhancement effects must first be checked in LC-ESI-MS/MS.

## 7.2 Calibration

The calibration curves must include the IS and InjS in the same range as the spike level. Linearity has to be checked for the calibration range and the correlation coefficient (R) should be better than 0.99. The lower linear range is defined by the quantification limits and blank level of the method. The blank response should be lower than 20% of the lowest calibration standard. A multilevel calibration should have at least five calibration levels.

In case of matrix effects, standard addition may be an alternative calibration option.

## 7.3 Quantification

Every detection and quantification must satisfy defined criteria for quality assurance (14). If possible, two mass transitions should be recorded for each target analyte, one for quantification (quantifier) and one for qualitative identification (qualifier). The abundance ratio of these two masses in the sample is compared with that of the calibration standards. Usually, a positive detection is obtained if the ratio in the sample deviates less than 30% from the average ratio of the calibration standards. Relative retention times should also be used for identification.

For quantification the peak height must have a signal to noise ratio of over 10, exceed a measured blank by a factor of 5 and be above the lowest calibration point.

Some PFSAs and sulfonamides show more than one peak in the chromatogram, which is due to the presence of branched isomers. The ratio of the isomers can be different between the calibration standards and environmental samples. These isomers cannot be quantified precisely because of the lack of calibration standards. If the peak area of the branched isomer exceeds 10% of that of the linear isomer, it is recommended to estimate its concentration based on the response factor of the linear standard.

The PFCAs and PFSAs are almost dissociated completely in environmental matrices. If salts are used for the preparation of calibration standards, quantification should be calculated for the corresponding acids.

# 8. Quality Assurance and Quality Control

Prior to the analysis of environmental samples, the method should be subject to a full method validation according to the requirements of the monitoring programme. This should include the determination of limits of detection, limits of quantification, trueness, precision, linearity of calibration, measurement uncertainty, and robustness.

Every sample batch should include a procedural blank which is prepared in the same way as the samples. The number of samples per batch may differ between laboratories and depend on how many samples can be processed under comparable conditions.

If mass labelled standards for the analytes are used, absolute recoveries between 50% and 150% are acceptable. In other cases, recoveries should be between 80% and 120%.

Within each batch, at least one sample should be extracted in duplicate. Laboratory control samples should be included in each batch of samples. The results should be documented and monitored in control charts.

At present, no certified reference material is available for PFCs in biota. Possible bias in the analytical method should be checked by the analysis of spiked laboratory control samples.

The laboratories should demonstrate their competence by participation in proficiency testing or intercalibration exercises relevant for the monitoring programme.

# 9. Data reporting

For routine analysis, the data report should be in accordance with the relevant monitoring program; it should include information about, for example, sampling, sample processing, storage, and analysis. Results should be reported along with the associated measurement uncertainty.

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Technical Annex 7: Organotin compounds in biota

This annex is intended as a supplement to the general guidelines. It is not a complete description or a substitute for detailed analytical instructions. The annex provides guidelines for the measurement of organtins, in marine biota in monitoring programmes. Target compounds include tributyltin (TBT), dibutyltin (DBT) and monobutyltin (MBT) and also triphenyltin (TPhT), diphenyltin (DPhT), and monophenyltin (MPhT).

# 1. Species

Target species for the monitoring of organotin compounds are shellfish, in particular bivalves like *Mytilus edulis* or *Mytilus galloprovincialis*. *Mytilus edulis* occurs in shallow waters along almost all coasts of the Contracting Parties. It is therefore suitable for monitoring in nearshore waters. No distinction is made between *M. edulis* and *M.galloprovincialis* because the latter, which may occur along the coast from Spain and Portugal to the sorthern coasts of UK, cannot easily be discerned from *M. edulis*. A sampling size range of 3‑6 cm is specified to ensure availability throughout the whole maritime area. The Pacific oyster *(Crassostrea gigas)* should be sampled in areas where *Mytilus sp.* is not available. The sampling size should be within the length range 9‑14 cm to ensure individuals of the 2 year age class.

Gastropods can also be used for TBT indicators, for instance in relation to biological effect monitoring. However, gastropods do not feed as continuously as bivalves and have a higher capacity of TBT metabolism, possibly resulting in a higher variability of TBT body burdens in gastropods compared with bivalves. In addition, correlation between imposex and TBT body burdens in the environment can be low, because of a time-lag between current TBT levels and imposex induced irreversibly in the early life stages and also because of non-continous feeding strategies. In some sensitive gastropod species, imposex can also be induced by TBT at lower levels than analytical detection limits generally achieved.

# 2. Sampling

Two alternative sampling strategies are described: sampling to minimise natural variability and length-stratified sampling. References of relevance to sampling and statistics include Gilbert (1987); Bignert *et al.* (1993 and 1994); Nicholson and Fryer (1996); and Nicholson *et al.* (1997). Advice on sampling strategies for temporal trend and spatial monitoring in shellfish are provided in OSPAR’s general CEMP Guidelines for Monitoring Contaminants in Biota and in Techncial Annex 1: Organic Contaminants.

# 3. Transportation

Samples should be kept cool and frozen at <-20°C as soon as possible after collection. Length and weight should be determined before freezing. Live mussels should be transported in closed containers at temperatures between 5‑15°C, preferably <10°C. Frozen samples should be transported in closed containers at temperatures <-20°C. More rigorous conditions will be necessary for samples for biological effects monitoring, *e.g.* storage in liquid nitrogen.

# 4. Pre-treatment and storage

## 4.1 Contamination

Sample contamination may occur during sampling, sample handling, pre-treatment, and analysis (Oehlenschläger, 1994), due to the environment, the containers or packing material used, the instruments used during sample preparation or from the chemical reagents used during the analytical procedures. Controlled conditions are therefore required for all procedures, including the dissection of organisms on board ship.

## 4.2 Depuration

Mussels should be placed on a polyethylene tray elevated above the bottom of a glass aquarium. The aquarium should be filled with sub-surface sea water collected from the same site as the samples and which has not been subject to contamination from point sources if possible. The aquarium should be aerated and the mussels left for 20‑24 hours at water temperatures and salinity close to those from which the samples were removed.

## 4.3 Opening of the shells

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

## 4.4 Dissection and storage

The soft tissues should be removed and deep frozen (-20°C) as soon as possible in containers appropriate to the intended analysis. TBT is stable in cockles and oysters stored at -20°C in the dark over a 7 month period. Longer storage can cause significant loss of TBT due to degradation (Gomez-Ariza *et al.*, 1999). The dissection of the soft tissue must be done under clean conditions on a clean bench by scientific personnel, wearing clean gloves and using clean stainless steel knives. After each sample has been prepared, the tools should be cleaned regularly. Washing in acetone or alcohol and high purity water is recommended. When the analysis is eventually undertaken, all fluids that may initially separate on thawing should be included with the materials homogenised. Homogenisation should be performed immediately prior to any sub-dividing of the sample.

# 5. Analysis

## 5.1 Preparation of materials

Solvents, chemicals and adsorption materials must be free of organotin compounds or other interfering compounds. If not they should be purified using appropriate methods. Solvents should be checked by concentrating the volume normally used in the procedure to 10% of the final volume and then analysing for the presence of organotin compounds and other interfering compounds using a GC. If necessary, the solvents can be purified by redistillation. Chemicals and adsorption materials should be purified by extraction and/or heating. Glass fibre materials (*e.g.* thimbles for Soxhlet extraction) should be pre-extracted. Alternatively, full glass thimbles with a G1 glass filter at the bottom can be used. Generally, paper filters should be avoided in filtration and substituted for by appropriate glass filters. As all super cleaned materials are prone to contamination (*e.g.* by the adsorption of organotin compounds and other compounds from laboratory air), materials ready for use should not be stored for long periods. All containers, skills, glassware *etc.* which come into contact with the sample must be made of appropriate material and must have been thoroughly pre-cleaned. Glassware should be extensively washed with detergents, heated at >250°C and rinsed immediately before use with organic solvents or mixtures such as hexane/acetone. Alternatively, all glassware can be washed in 10% HCl (or even in concentrated HCl) and then rinsed with distilled water.

### 5.2 Lipid determination

Organotin data are not usually expressed on a lipid basis. Lipid content is not a good normalisator because of poor correlations to organotin content. However, the determination of the lipid content of tissues can be of use in characterising the samples. If required, the lipid content should be determined on a separate subsample of the tissue homogenate, as some of the extraction techniques used routinely for organotin determination may destroy lipid materials. The total fat weight should be determined using the method of Bligh and Dyer (1959) or Smedes (1999).

## 5.3 Dry weight determination

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

## 5.4 Determination of organotins by gas chromatography

### 5.4.1 Calibration and preparation of calibrand solutions

#### 5.4.1.1 External calibration

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

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

ii) by using commercially readily available derivatised standards (e.g. Quasimeme <http://www.quasimeme.org/>).

Standard solutions can be prepared in (m)ethanol or another solvent depending on the instrumental method used. Addition of an internal standard (tripropyltin chloride TPrTCl or 13C labelled or deuterated TBT if using GC analysis with mass selective detection) to all standard and samples solutions is recommended. When using tripropyltin chloride, which is an underivatised standard, the recovery efficiency of the whole procedure can be determined.

A new calibration solution should always be cross-checked to the old standard solution.

Calibrand solutions should be stored in a refrigerator in gas tight containers to avoid evaporation of solvent during storage. It is important to determine the expiry date of standard dilutions in order to avoid a concentration shift due to deterioration of analytes or evaporation of solvents.

#### 5.4.1.2 Isotope Dilution-Mass Spectrometry

When using Isotope Dilution-Mass Spectrometry technique (IDMS), external calibration is not required.

### 5.4.2 Homogenisation and drying

Homogenisation should be carried out on fresh tissue. Care should be taken that the sample integrity is maintained during the actual homogenisation and during drying. When the analysis is undertaken, all fluids that may initially separate on thawing should be included with the materials homogenised. Homogenisation should be performed immediately prior to succeeding procedures. When grinding samples after drying, classical techniques using a ball mill can be used. Cryogenic homogenisation of dried or fresh materials at liquid nitrogen temperatures using a PTFE device (*cf.* Iyengar and Kasperek, 1977) or similar techniques can be applied (*cf.* Iyengar, 1976; Klussmann *et al*., 1985).

### 5.4.3 Extraction

Release of organotin compounds from the biological matrix is a critical step, due to the strong matrix binding of the analytes and possible species degradation. Recovery standards should be added prior to extraction, however correction procedures should be used with care as equilibration between the spiked and the target compounds is not always guaranteed. Different extraction techniques are commonly used, such as microwave assisted extraction, mechanical shaking and digestion. Microwave assisted extraction (MAE) as well as mechanical shaking provide quantitative recoveries with negligible degradation of the TBT compounds (Centineo *et al.*, 2004). However, it must be taken into account that considerable loss of DBT, due to degradation was reported for MAE. Digestion techniques can be used to extract butyltins, though species degradation is not always under control using this technique. Mechanical shaking seems to be a suitable technique. Alternatively, pressurised liquid extraction (accelerated solvent extraction) can be used to extract organotin compounds. Extraction usually takes place in an aqueous methanolic acidic environment, with subsequent extraction to an organic phase, such as pentane or hexane. Acidic conditions enhance the extraction efficiency, acetic acid is usually preferred to other acids to ensure stability of butyltins compounds. Complexing agents such as tropolone are often employed. Extraction can be performed on wet as well as on freeze-dried samples. Wet tissue must be dried by mixing with anhydrous sodium sulphate or other anhydrous materials.

### 5.4.4 Derivatisation

#### 5.4.4.1 Alkylation

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

Sodium Tetraethylborate (NaBEt4): Derivatisation with this complexing agent has been developed to minimise the analysis time. The NaBEt4 procedure allows a simultaneous extraction-derivatisation in a buffered medium (optimum pH 4-5). NaBEt4 derivatisation produces more thermally stable derivatives. However, NaBEt4 is extremely air sensitive, since it is considered as pyrophoric, care must be taken to keep its chemical integrity. Although solutions in water have been shown to be stable for about 1 month at 4 ³C, it is recommended to prepare them freshly for use. Solutions of the reagent in an organic solvent (e.g.tetrahydrofuran, methanol or ethanol) seem to be more stable (Smedes *et al.*, 2000). The determination of organotin compound in complex matrices, such as biological matrices with high lipid content, has led to several problems, including low recovery and low derivatisation efficiency. A clean-up step might be subsequently required.

Sodium Diethyldithiocarbamate (NaDDTC): NaDDTC is preferable to Grignard reagents as it does not require anhydrous conditions but it does not simultaneously derivatise and extract like NaBEth4. Yet this step can be combined with Grignard reagent to provide better derivatisation for a wider spectrum of organotins.

#### 5.4.4.2 Hydride generation

The butyltin species are converted to an hydride form by sodium tetrahydroborate (NaBH4). Hydride generation produces a large volume of hydrogen as a by-product, which facilitates the purging of butyltin hydrides from a large volume of sample.

### 5.4.5 Clean-up

The most commonly used clean-up methods involve the use of alumina or silica adsorption chromatography. For the latter, phenyltin compounds like triphenyltin may not co-elute with butyltins. Gel permeation chromatography and similar high performance liquid chromatography (HPLC) based methods are also employed. The major advantages of using HPLC-based clean-up methods are their ease of automation and reproducibility.

### 5.4.6 Pre-concentration

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

## 5.5 Instrumental determination

Most of the analytical techniques developed for the speciation of organotin compounds are based on GC. GC remains the preferred separation technique owing to its high resolution and the availability of sensitive detectors such as (pulsed) flame photometry ((P)FPD), mass spectrometry (MS) or inductively coupled plasma-mass spectrometry (ICP-MS)

As an alternative approach, high performance liquid chromatography has become a popular technique. It mainly uses fluorescence, ultraviolet, and more recently inductively coupled plasma optical emission spectrometry (ICP-OES), inductively coupled plasma mass spectrometry (ICP-MS), and mass spectrometry detectors such as atmospheric pressure chemical ionisation mass spectrometry (APCI-MS-MS) and electrospray ionisation mass spectrometry (ESI-MS). ICP-MS and (P)FPD detectors have been applied widely because of their inherent selectivity and sensitivity.(P)FPD has been shown to have greater selectivity and lower detection limits (by a factor of 25 to 50 times) for organotin compounds than those obtained with conventional FPD (Bravo *et al.*, 2004).

### 5.5.1 Gas chromatography

The two injection modes commonly used are splitless and on-column injection. Automatic sample injection should be used wherever possible to improve the reproducibility of injection and the precision of the overall method. If splitless injection is used, the liner should be of sufficient capacity to contain the injected solvent volume after evaporation. Helium must be used for GC-MS, GC-FPD and GC-ICP-MS. The preferred column length is 25–30 m, with an internal diameter of 0.15 mm to 0.3 mm. Film thicknesses of 0.3 μm to 1 µm are generally used. The most commonly used stationary phase for organotin analysis is 5% phenyl methyl siloxane. Mass spectrometric analyses are usually conducted in electron-impact mode at 70eV.

### 5.5.2 High Performance Liquid Chromatography

All stainless steel parts of the HPLC system that come into contact with the sample should be replaced by polyether ketone (PEEK) components. Reverse phase columns (e.g. octadecylsilane C18) are commonly used (Wahlen and Catterick, 2003) and the mobile phase can consist, for example, of a mixture of acetonitrile, water and acetic acid with 0.05% triethylamine, pH 3.1–3.4 (65:25:10 variable depending on columns used).

### 5.5.3 Detection

Flame photometry (FPD), equipped with a 610 nm band-pass filter, selective for tin compounds), mass spectrometry (MS) or inductively coupled plasma-mass spectrometry (ICP-MS) are mainly used as detectors for gas chromatography and high performance liquid chromatography.

# 6. Quality assurance

References of relevance to QA procedures include HELCOM (1988); HELCOM COMBINE manual, QUASIMEME (1992); Oehlenschläger (1994); ICES (1996); and Morabito *et al.* (1999).

## 6.1 System performance

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

## 6.2 Recovery

The recovery should be checked and reported. One method is to add an internal (recovery) standard to each sample immediately before extraction (e.g. tripropyltin) and a second (quantification) standard immediately prior to injection (e.g. tetrapropyltin). The recovery of MBT may be lower than for other organotin compounds, pobrably because of a lower derivatisation efficiency.

When using Isotope Dilution-Mass Spectrometry technique, the loss of target analytes is compensated. However, the recovery should still be calculated and should be between 50% and 150%.

## 6.3 Calibrand solutions and calibration

See Section 5.4.1.

## 6.4 Blanks

A procedural blank should be measured for each sample series and should be prepared simultaneously using the same chemicals and solvents as for the samples. Its purpose is to indicate sample contamination by interfering compounds, which will lead to errors in quantification. Even if an internal standard has been added to the blank at the beginning of the procedure, a quantification of peaks in the blank and subtraction from the values obtained for the determinands must not be performed, as the added internal standard cannot be adsorbed by a matrix.

## 6.5 Accuracy and precision

A Laboratory Reference Material (LRM) should be included, at least one sample for each series of identically prepared samples. The LRM must be homogeneous, well characterised for the determinands in question and stability tests must have shown that it produces consistent results over time. The LRM should be of the same type of matrix (*e.g.* liver, muscle tissue, fat or lean fish) as the samples, and the determinand concentrations should occur in a comparable range to those of the samples. If the range of determinand concentrations in the samples is large (> factor of 5) two reference materials should be included in each batch of analyses to cover the lower and upper concentrations. The data produced for the LRM in successive sample batches should be used to prepare control charts. It is also useful to analyse the LRM in duplicate from time to time to check within-batch analytical variability. The analysis of an LRM is primarily intended as a check that the analytical method is under control and yields acceptable precision, but a certified reference material (CRM such as ERM-CE 477 (mussel, certified for TBT, DBT, MBT) or NIES No. 11 (fish tissue certified for TBT and non-certified reference value for TPhT)) of a similar matrix should be analysed periodically in order to check the method bias. Additionally a duplicate of at least one sample should be run with every batch of samples. Each laboratory should participate in interlaboratory comparison studies and proficiency testing schemes on a regular basis, preferably at an international level.

## 6.6 Data collection and transfer

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

# 7. Data recording and reporting parameters

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

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

The following parameters should be recorded:

## 7.1 Sampling and biological parameters

|  |
| --- |
| Shellfish   1. location of sampling site (name, latitude, and longitude); 2. date and time of sampling (GMT); 3. sampling depth with respect to low tide (for sub-tidal sites only); 4. irregularities and unusual conditions; 5. name and institution of sampling personnel; 6. number of pooled samples; 7. number of individuals in pool; 8. mean, minimum and maximum length and standard deviation; 9. mean dry shell weight; 10. mean soft tissue weight (wet weight); 11. condition index. |

## 7.2 Analytical and quality assurance parameters

|  |
| --- |
| 1. LRM and CRM results for a set of organotin compounds, reported on a wet weight basis; 2. descriptions of the extraction, cleaning and instrumental determination methods; 3. mean tissue lipid weight and method of extraction; 4. the mean soft dry weight and method of determining water content if this differs from air drying to constant weight at 105°C (if sufficient material is available); 5. the detection limit for each organotin compound. Specific performance criteria, including detection limits and precision, are usually set by the programme. A typical detection limit for single contaminants is 1 µg/kg wet weight, although this might be difficult to achieve for phenyltins compounds.  * QA information according to the requirements specified in the programme. |

## 7.3 Lipids

|  |
| --- |
| * total lipids (*e.g.* Bligh and Dyer, 1959; or Smedes, 1999) (expressed as % or g/kg wet weight). |

## 7.4 Parameters

|  |
| --- |
| 1. organic contaminants of interest to monitoring programmes for which these guidelines apply: organotin compounds suite required for analysis 2. Butyltin compounds: Tributyltin (TBT), dibutyltin (DBT) and monobutyltin (MBT) 3. Phenyltin compounds: Triphenyltin (TPhT), diphenyltin (DPhT) and monophenyltin (MPhT) |

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Technical Annex 8: Determination of chlorobiphenyls in biota

# Analytical Method

# 1. Introduction

This technical annex provides advice on chlorobiphenyl (CB) analysis for all biota samples. The guideline is an update of an earlier version (OSPAR, 1999) taking into account evolutions in the field of analytical chemistry and also covering the determination of the planar CBs, i.e. the mono-*ortho* (CB105, CB114, CB118, CB123, CB156, CB157, CB167 and CB189) and non-*ortho* substituted CBs (CB81, CB77, CB126 and CB169). When reviewing the literature, it should be noted that planar, coplanar and dioxin-like CBs / PCBs are all equivalent terms.

The analysis of CBs in biota generally involves extraction with organic solvents, clean-up (removal of lipids and fractionation), and gas chromatographic separation with electron capture or mass-spectrometric detection. All stages of the procedure are susceptible to insufficient recovery and/or contamination. Where possible, quality control procedures are required in order to check the method’s performance. These guidelines are intended to encourage and assist analytical chemists to reconsider their methods and to improve their procedures and/or the associated quality control measures where necessary. Due to the low concentrations of, particularly, non-*ortho* substituted CBs in biota compared to those of other CBs, their determination requires an additional separation and concentration step. Therefore, in the relevant sections a distinction will be made between the non-*ortho* CBs and the others.

These guidelines can also be used for several other groups of organochlorine compounds, e.g. DDTs and their metabolites, chlorobenzenes and hexachlorocyclohexanes. Recoveries in the clean-up procedures must be checked carefully. In particular, treatment with H2SO4 results in a loss of some compounds (e.g. dieldrin and endosulfan (de Boer and Wells, 1996)).

These guidelines are not intended as a complete laboratory manual. If necessary, further guidance should be sought from specialised laboratories. Whichever analytical procedure is adopted, the laboratory must demonstrate the validity of the procedure. Analyses must be carried out by trained staff.

# 2. Analysis

## 2.1. Precautionary measures

Solvents, chemicals and adsorption materials must be free of CBs or other interfering compounds. If not they should be purified using appropriate methods. Solvents should be checked by concentrating the volume normally used in the procedure to 10% of the final volume if practical and then analysing for the presence of CBs and other interfering compounds. If necessary, the solvents can be purified by redistillation but this practice is not favoured by most analytical laboratories as they generally opt to purchase high quality solvents directly. Chemicals and adsorption materials should be purified by extraction and/or heating. Glass fibre materials (*e.g.* thimbles for Soxhlet extraction) should be pre-extracted. Alternatively, glass thimbles with a G1 glass filter at the bottom can be used. Generally, paper filters should be avoided in filtration and substituted for by appropriate glass filters. As all pre-cleaned materials are prone to contamination (*e.g.* by the adsorption of CBs and other compounds from laboratory air), materials ready for use should not be stored for long periods. All containers, tools, glassware *etc.* which come into contact with the sample must be made of appropriate material and must have been thoroughly pre-cleaned. Glassware should be extensively washed with detergents, heated at >250°C and rinsed immediately before use with organic solvents or mixtures such as hexane/acetone. In addition all glassware should preferably be covered with aluminium foil and stored in cupboards to keep out any dust. Old and scratched glassware is more likely to cause blank problems because of the larger surface and therefore greater chance of adsorption. Furthermore, scratched glassware can be more difficult to clean effectively, as shown during analysis of brominated flame retardants (QUASIMEME, 2007).

## 2.2 Lipid determination

The determination of the lipid content of tissues can be of use in characterising the samples and reporting concentrations in biota on a wet weight or lipid weight basis. The total lipid content of fish or shellfish should be determined using the method of Bligh and Dyer (1959) as modified by Hanson and Olley (1963) or an equivalent method such as Smedes (1999). Extractable lipid methods may be used, particularly if the sample size is small and lipid content is high. It has been shown that if the lipid content is high (> 5%) then the extractable lipid content will be comparable to that of the total lipid. If extraction techniques are applied which destroy or remove lipid materials (e.g., PLE with fat retainers), the lipid content should be determined on a separate subsample of the tissue homogenate. Other relevant information concerning lipid determination are provided by QUASIMEME, 1994 and Roose *et al*., 1996.

## 2.3. Dry weight determination

Dry weight determinations should be carried out by drying homogenised sub-samples of the material to be analysed to constant weight at 105°C.

## 2.4. Homogenisation and drying

Prior to analysis, the samples should be sufficiently homogenised. Homogenisation is generally carried out on fresh tissue. Care should be taken that the sample integrity is maintained during the actual homogenisation. When the analysis is undertaken, all fluids that may initially separate on thawing should be included with the materials homogenised. Homogenisation should be performed prior to extraction and clean-up procedures. When homogenising samples after drying, classical techniques using a ball mill can be used. Cryogenic homogenisation of dried or fresh materials at liquid nitrogen temperatures using a PTFE device (*cf.* Iyengar and Kasperek, 1977) or a similar technique is also possible (*cf.* Iyengar, 1976; Klussmann *et al*., 1985).

CBs can be extracted from either wet or dried samples, although storage, homogenisation and extraction are easier when the samples are dry. Drying the samples may, however, alter the CB concentrations e.g. by the loss of compounds through evaporation or by contamination. Potential losses and contamination should be checked as part of the method validation.

Chemical drying can be performed by grinding with e.g. Na2SO4 or MgSO4 until the sample reaches a free-flowing consistency. It is essential that at least several hours elapse between grinding and extraction to allow for complete dehydration of the sample, as the presence of residual water will decrease the extraction efficiency.

Freeze-drying is also a popular technique, although its application should be carefully considered. Possible losses or contamination must be checked. Losses through evaporation are diminished by keeping the temperature in the evaporation chamber below 0°C. Contamination during freeze-drying can be reduced by putting a lid, with a hole of about 3 mm in diameter, on the sample container.

## 2.5. Extraction

Recovery standards should be added prior to extraction. When using Soxhlet extraction, a combination of polar and apolar solvents is recommended. Alternatively, saponification may be used. This technique is highly effective, but conditions should be controlled as saponification could result in the decomposition of some pesticides and, under certain conditions, of some CB congeners.

Although the use of binary non-polar/polar solvent mixtures and Soxhlet extraction is still the benchmark for CB extraction, there have been numerous attempts to find alternative procedures, which are less time-consuming, use less solvent and/or enable miniaturisation. Amongst these novel approaches are pressurised liquid extraction (PLE) and related subcritical water extraction (SWE), microwave-assisted extraction (MAE), matrix solid-phase dispersion (MSPD), ultrasound extraction (US) and supercritical fluid extraction (SFE).

From among the techniques mentioned, PLE or Accelerated Solvent Extraction (ASE) has – so far – been most successful. Soxhlet methods are easily translated into PLE as the same solvent compositions can be used. The method further allows interesting modifications that include in-cell clean-up of samples by adding fat retainers, such as acid-impregnated silica, florisil or alumina, to the cell. New promising techniques have been described, e.g. the use of a small carbon column in the extraction cell, which selectively adsorbs dioxin-like compounds (subsequently isolated by back-flushing with toluene), but these are not established for routine analysis (Sporring *et al*., 2003). PLE and MAE have the shared advantage over SFE that they are matrix-independent, which facilitates method development and changing-over from the classical Soxhlet extraction. Recent years have also seen an increased use of ultrasound-based techniques for the isolation of analytes from solid samples. With most applications, extraction efficiency is satisfactory, and sonication time often is 30 min or less (Roose and Brinkman, 2005).

All the methods described above are in principle suitable for extracting CBs from biota. However, Soxhlet extraction is still the reference for alternative approaches.

## 2.6 Clean-up

The extraction procedures above will result in the co-extraction of lipids, which will need to be removed from the extract. Furthermore, tissue extracts will always contain many compounds other than CBs, and a suitable clean-up is necessary to remove those compounds which may interfere with the subsequent analysis. Different techniques may be used, either singly or in combination, and the choice will be influenced by the selectivity and sensitivity of the final measurement technique and also by the extraction method employed. Most CBs are stable under acid conditions; therefore treatment with sulphuric acid or acid impregnated silica columns may be used in the clean-up.

The most commonly used clean-up methods involve the use of alumina or silica adsorption chromatography, but gel permeation chromatography (GPC) is also employed. Any water residues in the extract should be removed prior to clean-up, e.g. by adding Na2SO4.

As CBs are apolar, clean-up using normal-phase chromatography is the most appropriate technique for their separation from other compounds. Using an apolar solvent (*e.g.* hexane or *iso*-octane) as an eluent, CBs normally elute very rapidly. All polar solvents used in the extraction should be removed before further clean-up. The last concentration step is usually performed by evaporation with a gentle stream of nitrogen. Evaporation to dryness should always be avoided but, for the analysis of planar CBs, very small final sample extract volumes might be necessary to achieve detectable concentrations.

Deactivated Al2O3 (5-10% water) is often used as a primary clean-up technique. Al2O3 can yield a sufficiently clean extract for a GC-ECD analysis of the sample. Al2O3 effectively removes lipid compounds from the extracts (although samples with a very high lipid content and low CB concentrations may require additional clean-up).

Deactivated silica (1-5% water) does not retain CBs (including non-*ortho* CBs) and only retains polycyclic aromatic hydrocarbons (PAHs) slightly when eluted with hexane or *iso*-octane. When organochlorine pesticides are also to be determined in the same extract, deactivation of the silica with a few percent of water is necessary.

For high activity silica (heated overnight at 180°C) the retention of CBs is negligible, while PAHs are more strongly retained. The CBs and a few other organochlorine compounds can be eluted with apolar solvents. More polar solvents (*e.g.* hexane/acetone) should be avoided as some interfering organochlorine pesticides would be eluted as well.

For the separation of CBs from lipids or oil components, reversed‑phase HPLC can be used. In reversed-phase chromatography, CBs elute during a solvent gradient of 80 to 90% methanol together with numerous other compounds of the same polarity. Most of the above mentioned extraction methods and clean-up procedures yield an extract containing an apolar solvent. These cannot be injected directly for reversed-phase chromatography, and so compounds must be transferred between solvents several times *e.g.* before injection and after elution. When using polar solvents for extraction, reversed-phase columns could be used directly for clean-up. When eluting an acetonitrile extract from a C18 solid phase extraction (SPE) column with acetonitrile, high molecular hydrocarbons are strongly retained while CBs elute in the first few column volumes.

The above mentioned normal-phase chromatographic procedures on silica and Al2O3 can be transferred to HPLC having the advantages of higher resolution and better reproducibility.

When using GPC, the elution of CBs should be carefully checked. Two serial columns are often used for improved lipid separation. Solvent mixtures such as dichloromethane/hexane or cyclohexane/ethyl acetate can be used as eluents for GPC. However, a second clean-up step is often required to separate the CBs from other organohalogenated compounds and/or to remove residual lipids.

One advantage of using PLE extraction is that it is possible to combine the clean-up with the extraction, especially where mass spectrometry will be used as the detection method. If Soxhlet extraction is used for biota, then there is a much greater quantity of residual lipid to be removed than in the case of PLE with fat retainers. An additional clean-up stage may therefore be necessary. Methods have been developed for online clean-up and fractionation of dioxins, furans and CBs with PLE for food, feed and environmental samples (Sporring *et al*., 2003), utilising a fat retainer for the on-line clean-up of fat. Silica impregnated with sulphuric acid, alumina and florisil have all been used as fat retainers. A non-polar extraction solvent such as hexane should be used if fat retainers are used during PLE.

Non-*ortho* CBs require a more specialised clean-up that is generally associated with the analysis of dioxins. Although initial clean-up may very well proceed along the lines described above, the larger sample intake results in the presence of even more co-extractive compounds and care has to be taken that the capacity of the adsorption columns is not exceeded and/or that lipids are adequately removed. Often, more rigorous procedures are applied to remove the excess material by e.g. shaking the sample with concentrated sulphuric acid. A more efficient alternative is to elute the sample over a silica column impregnated with sulphuric acid (40 % w/w).

Non-*ortho* CBs are nearly always separated from the other CBs using advanced separation techniques. One very efficient method is to inject the extracts (after concentrating them) into a HPLC system coupled to a PYE (2-(1-pyrenyl) ethyldimethylsilylated silica) column. Column dimensions are typically 4.6 x 150 mm, but combinations of several columns in-line are sometimes used. The use of PYE columns not only allow the separation of ortho, mono-ortho and non-ortho CBs from one another on the basis of structural polarity, but also from dibenzo-p-dioxins and dibenzofurans. The eluting solvent is an apolar solvent such as iso-hexane. Coupled to a fraction collector, the use of a HPLC system allows the automatic clean-up of a considerable number of samples at a time. Alternatively, HPLC systems equipped with porous graphitised carbon can be used. Column sizes are in the order of 50 x 4.7 mm and care has to be taken that the column is not overloaded. Similarly to PYE columns, they will separate non-*ortho* CBs from the other CBs and from dioxins. Fully automated systems, such as Powerprep™, that combine several steps are routinely used.

## 2.7 Pre-concentration

Evaporation of solvents using a rotary-film evaporator was, until recently, the most common method. However, evaporation of solvents using this technique should be performed at low temperature (water bath temperature of ≤ 40°C) and under controlled pressure conditions, in order to prevent losses of the more volatile CBs. To reduce the sample to the final volume, solvents can be removed by blowing-down with a gentle stream of nitrogen. Only nitrogen of a controlled high quality should be used.

Turbovap sample concentrators can also be used to reduce solvent volume. This is a rapid technique, but needs to be carefully optimised and monitored to prevent both losses (both of volatiles and solvent aerosols) and cross-contamination. The use of rotary-film evaporators is more time consuming but more controllable. Here also, evaporation to dryness should be avoided at all costs. Syncore™ parallel evaporators (Buchi, Switzerland) can be used with careful optimisation of the evaporation parameters. The Buchi Syncore™ Analyst also uses glass tubes but the system is sealed, avoiding contamination from the laboratory air during evaporation. It does not use a nitrogen stream, thus reducing the loss of volatiles and if the flushback module is fitted the sides of the tubes are rinsed automatically thus reducing the loss of the heavier components. Again water-bath temperatures should be minimised to prevent losses. When reducing the sample to the required final volume, solvents can be removed by a stream of clean nitrogen gas. Suitable solvents for injection into the gas chromatograph (GC) include hexane, heptane, toluene and *iso*-octane.

## 2.8 Calibration and preparation of calibrant solutions

Internal standards (recovery and quantification standards) should be added in a fixed volume or weight to all standards and samples. The ideal internal is a CB which is not found in the samples. All CBs with a 2,4,6‑substitution (e.g. CB112, CB155, CB198) are, in principle, suitable for this purpose. Alternatively, 1,2,3,4‑tetrachloronaphthalene or homologues of dichloroalkylbenzylether can be used. For GC analysis with mass selective detection (GC-MS), 13C labelled CBs should be used at each degree of chlorination. This is especially critical for the determination of the non-ortho CBs. If possible, the labelled calibrant solutions should correspond to the unlabelled determinants. For the non-ortho CBs, a labelled standard is available for each congener and use of all of them is recommended. When preparing a calibration solution for a new determinant for the first time, two independent stock solutions of different concentrations should always be prepared simultaneously to allow cross checking. A new calibration solution should also be cross-checked to the old standard solution. Crystalline CBs of known purity can be used for preparing calibration solutions but, for health and safety reasons, the purchase of solutions is recommended for planar CBs. In recent years, a lot of certified commercial custom made standards have become available and laboratories have been switching to these. If the quality of the standard materials is not guaranteed by the producer or supplier, it should be checked by GC preferably with mass spectrometric detection. Solid standards should be weighed to a precision of 10-5 grams. Calibration solutions should preferably be stored in ampoules in a cool and dark place. Commercially available screw-cap vials with a capillary opening (CertanTM) combine of advantages of ampoules and vials, and, have proven to be reliable. When stored in containers the weight loss during storage should be recorded.

## 2.9 Instrumental determination

### 2.9.1 Injection techniques

The two modes commonly used are splitless and on-column injection as, in split injection, strong discrimination effects may occur. The liner should possess sufficient capacity with respect to the injected volume after evaporation, but should not be oversized so as to avoid poor transfer to the column and losses by adsorption. Liners with a light packing of (silylated) glass wool may improve the performance for CBs, but may cause degradation of some organochlorine compounds like DDT, which are often included in national monitoring programmes.

Recently, other techniques such as temperature‑programmed or pressure‑programmed injection have become more prominent. They offer additional advantages such as an increased injection volume without the negative effects previously associated with that technique, but should be thoroughly optimised before use. Increasing the injection volume will allow either the elimination of an extra evaporation step or the lowering of the analytical detection limits, or both.

### 2.9.2 Carrier gas

Hydrogen is the preferred carrier gas and is indispensable for columns with very small inner diameters. Helium is also acceptable and is the standard carrier gas for use with GC-MS techniques.

### 2.9.3 Columns

Only capillary columns should be used. The following parameters are recommended:

|  |  |
| --- | --- |
| Minimum Length | 50 m (for microcolumns of internal diameter <0.1 mm, shorter columns can be suitable). |
| Maximum internal diameter | 0.25 mm. Note that for diameters <0.15 mm the elevated pressure of the carrier gas needs special instrumental equipment as most of the instruments are limited to 400 kPa. |
| Film thickness | 0.2 - 0.4 µm. |

Columns which do not fulfil these requirements generally do not offer sufficient resolution to separate CB28, CB105 and CB156 from closely eluting CBs. A wide range of stationary phases can be used for CB separation. The chemical composition is different for columns from different producers and this influences the maximum temperature at which the column can be operated. Further advice may be found in the producers’ catalogues, where compositions, applications and tables from which to compare products from different manufacturers are included.

In recent years, new chromatographic phases have become available that result in an improved separation of critical CB pairs. A good example is the HT-8 phase (1,7-dicarba-closo-dodecarborane phenylmethyl siloxane) (Larsen *et al*., 1995) that shows a remarkable selectivity for CBs (Table 1). This column is currently recommended for CB analysis.

### 2.9.4 Detection

The electron capture detector (ECD) is still frequently used for CB analysis. Injection of chlorinated solvents or oxygen-containing solvents should be avoided when ECD is used due to the generation of large interfering signals. When using mass selective detectors (MSD), the electron-capture negative-ion chemical ionisation mode (ECNICI) is extremely sensitive for pentachlorinated to decachlorinated CBs, and is approximately ten fold more sensitive than ECD. However, the sensitivity of MS systems has improved considerably, allowing analysis also to be undertaken using electron impact ionisation (EI). Previously, the use of ECNICI was often necessary in order to detect the low concentrations of, in particular, the non-*ortho* CBs. Suggested target and qualifier ions for *ortho* CBs (including mono-*ortho* CBs) are shown in Table 1 and in Table 2 for non-*ortho* CBs.

Next to conventional GC-MS, the use of ion-trap with its tandem MS² option – i.e., yielding improved selectivity – is receiving increased attention. The use of GC-ITMS provides a less expensive alternative to high-resolution mass spectrometry (HRMS), which is commonly used to determine PCDD/Fs and, as such, is also ideally suited for the detection of all CB groups.

**Table 1**:Example of retention times for selected CB congeners using a 50 m HT8 column (0.25 mm i.d. and 0.25 µm film), along with possible target and qualifier ions. Temperature programme: 80oC, hold for 1 minute, ramp 20oC/minute, to 170 oC, hold 7.5 minutes, ramp 3 oC/minute to 300 oC, hold for 10 minutes.

| **CB congener** | **MW** | **RT** | **Target Ion** | **Qualifier Ion** | **Number of chlorines** |
| --- | --- | --- | --- | --- | --- |
| ***13C-CB28*** | **270** | 28.371 | 268 | 270 | 3 |
| CB31 | **258** | 28.071 | 256 | 258 | 3 |
| CB28 | **258** | 28.388 | 256 | 258 | 3 |
| ***13C-CB52*** | **304** | 30.317 | 304 | 302 | 4 |
| CB52 | **292** | 30.336 | 292 | 290 | 4 |
| CB49 | **292** | 30.698 | 292 | 290 | 4 |
| CB44 | **292** | 32.024 | 292 | 290 | 4 |
| CB74 | **292** | 34.881 | 292 | 290 | 4 |
| CB70 | **292** | 35.199 | 292 | 290 | 4 |
| ***13C-CB101*** | **340** | 36.612 | 338 | 340 | 5 |
| CB101 | **326** | 36.630 | 326 | 328 | 5 |
| CB99 | **326** | 37.062 | 326 | 328 | 5 |
| CB97 | **326** | 38.267 | 326 | 328 | 5 |
| CB110 | **326** | 39.277 | 326 | 328 | 5 |
| CB123\* | **326** | 41.2 | 326 | 328 | 5 |
| CB118\* | **326** | 41.563 | 326 | 328 | 5 |
| CB105\* | **326** | 43.443 | 326 | 328 | 5 |
| CB114\* | **326** | 42.2 | 326 | 328 | 5 |
| ***13C-CB153*** | **374** | 42.567 | 372 | 374 | 6 |
| CB149 | **362** | 40.328 | 360 | 362 | 6 |
| CB153 | **362** | 42.584 | 360 | 362 | 6 |
| CB132 | **362** | 42.236 | 360 | 362 | 6 |
| CB137 | **362** | 43.744 | 360 | 362 | 6 |
| ***13C-CB138*** | **374** | 44.437 | 372 | 374 | 6 |
| CB138 | **362** | 44.487 | 360 | 362 | 6 |
| CB158 | **362** | 44.663 | 360 | 362 | 6 |
| CB128 | **362** | 46.307 | 360 | 362 | 6 |
| ***13C-CB156*** | **374** | 48.406 | 372 | 374 | 6 |
| CB156\* | **362** | 48.366 | 360 | 362 | 6 |
| CB167\* | **362** | 46.2 | 360 | 362 | 6 |
| CB157\* | **362** | 48.698 | 360 | 362 | 6 |
| ***13C-CB180*** | **408** | 48.829 | 406 | 408 | 7 |
| CB187 | **396** | 44.787 | 394 | 396 | 7 |
| CB183 | **396** | 45.264 | 394 | 396 | 7 |
| CB180 | **396** | 48.846 | 394 | 396 | 7 |
| CB170 | **396** | 50.684 | 394 | 396 | 7 |
| ***13C-CB189*** | **406** | 53.182 | 406 | 408 | 7 |
| CB189\* | **396** | 53.196 | 394 | 396 | 7 |
| ***13C - CB194*** | **442** | 57.504 | 442 | 440 | 8 |
| CB198 | **430** | 50.347 | 430 | 428 | 8 |
| CB194 | **430** | 57.514 | 430 | 428 | 8 |
| \*mono-*ortho* CBs | | | | | |

**Table 2**: Possible target and qualifier ions for non*-ortho* CBs, including labelled internal standards

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **CB** | **Target ion (*m/z)*** | **Qualifier (*m/z*)** | **Qualifier (*m/z*)** | **Qualifier (*m/z*)** |
| 13CB81 | 304 | 302 | NA | NA |
| CB81 | 292 | 290 | 220 | 222 |
| 13CB77 | 304 | 302 | NA | NA |
| CB77 | 292 | 290 | 220 | 222 |
| 13CB126 | 338 | 340 | NA | NA |
| CB126 | 326 | 328 | 254 | 256 |
| 13CB169 | 372 | 374 | NA | NA |
| CB169 | 360 | 362 | 218 | 220 |

### 2.9.5 Separation, identification and quantification

When using GC-ECD and, to a certain extent, GC-MS, two columns with stationary phases of different polarity should be used, as column-specific co-elution of the target CBs with other CBs or other organochlorine compounds can occur on a single column. Using columns of differing selectivity’s can resolve these co-elution problems. The temperature programme used must be optimised for each column to achieve sufficient separation of the CB congeners to be determined. An isothermal period in the programme around 200-220°C of approximately 30 minutes is recommended. Care should be taken that CBs of interest do not coelute with other CB congeners (for example CB28 and CB31). When using GC-ECD, compounds are identified by their retention time in relation to the standard solutions under the same conditions. Therefore GC conditions should be constant. Shifts in retention times should be checked for different areas of the chromatogram by identifying characteristic, unmistakable peaks (*e.g.* originating from the internal standard or higher concentrated CBs such as CB153 and CB138. Using a GC-MS system, the molecular mass or characteristic mass fragments or the ratio of two ion masses can be used to confirm the identity of resolved CBs. Since calibration curves for most CBs are usually non-linear when using GC-ECD, but should be linear when using GC-MS, a multilevel calibration of at least five concentrations is recommended. The calibration curve must be controlled and the best fit must be applied over the relevant concentration range. One should strive to work within the linear range of the detector. Analysis of the calibration solutions should be carried out in a mode encompassing the concentrations of the sample solutions (or alternatively by injecting matrix-containing sample solutions and matrix-free standard solutions distributed regularly over the series). When the chromatogram is processed with the help of automated integrators, the baseline may not always be set unambiguously and always needs to be inspected visually. When using GC-ECD, peak height is preferable to peak area for quantification purposes. From the two columns of different polarity the more reliable result (in terms of absence of co-elutions) should be reported.

Recent years have witnessed the emergence of so-called comprehensive two-dimensional gas chromatography (GC x GC) – a technique that can be used to considerably improve analyte/matrix as well as analyte/analyte separation. Briefly, a non-polar x (semi-)polar column combination is used, with a conventional 25–30 m long first-dimension, and a short, 0.5-1 m long, second-dimension column. The columns are connected via an interface called a modulator. The latter device serves to trap, and focus, each subsequent small effluent fraction from the first-dimension column and, then, to launch it into the second column. The main advantages of the comprehensive approach are that the entire sample (and not one or a few heart-cuts, as in conventional multidimensional GC (Dallüge *et al*., 2003) is subjected to a completely different separation, that the two-dimensional separation does not take any more time than the first-dimension run, and that the re-focusing in the modulator helps to increase analyte detectability. The most interesting additional benefit for CBs is, that structurally related as CB congeners show up as so-called ordered structures in the two-dimensional GC x GC plane. The very rapid second-dimension separation requires the use of detectors with sufficiently high data acquisition rates. Initially, only flame ionisation detectors could meet this requirement. However, today there is also a micro-ECD on the market that is widely used for GC x GC-µECD of halogenated compound classes. Even more importantly, analyte identification can be performed by using a time-of-flight mass spectrometer (Dallüge *et al*., 2002) or – with a modest loss of performance, but at a much lower price – one of the very recently introduced rapid-scanning quadrupole mass spectrometers (Korytar *et al*., 2005; Adahchour *et al*., 2005). So far, the use of GC x GC has been limited to qualitative applications and still seems inappropriate for routine quantification of analytes.

# 3 Quality assurance

Planners of monitoring programmes must decide on the accuracy, precision, repeatability, and limits of detection and determination which they consider acceptable. References of relevance to QA procedures include HELCOM, 1988; QUASIMEME 1992; Wells *et al*., 1992; Oehlenschläger, 1994; Smedes *et al.*, 1994 and ICES, 1996.

## 3.1 System performance

The performance of the GC system should be monitored by regularly checking the resolution of two closely eluting CBs. A decrease in resolution points to deteriorating GC conditions. The signal-to-noise ratio yields information on the condition of the detector. A dirty ECD detector or MS ion source can be recognised by the presence of an elevated background signal together with a reduced signal-to-noise ratio. Chromatograms should be inspected visually by a trained operator.

## 3.2 Recovery

The recovery should be checked and reported. One method is to add an internal (recovery) standard to each sample immediately before extraction and a second (quantification) standard immediately prior to injection. If smaller losses occur in extraction or clean-up, or solutions are concentrated by uncontrolled evaporation of solvents (*e.g.* because vials are not perfectly capped), such losses can be compensated for by normalisation. If major losses are recognised and the reasons are unknown, the results should not be reported, as recoveries are likely to be irreproducible. A control for the recovery standard is recommended by adding the calibration solution to a real sample. Recoveries should be between 70 and 120%, if not, analysis of samples should be repeated.

## 3.3 Blanks

A procedural blank should be measured for each sample series and should be prepared simultaneously using the same chemicals and solvents as for the samples. Its purpose is to indicate sample contamination by interfering compounds, which will lead to errors in quantification. Even if an internal standard has been added to the blank at the beginning of the procedure, a quantification of peaks in the blank and subtraction from the values obtained for the determinands must not be performed, as the added internal standard cannot be adsorbed by a matrix. An alternative may be using a CB-free oil as a matrix blank.

## 3.4 Accuracy and precision

Analysis of a Laboratory Reference Material (LRM) should be included, at least one sample within each batch of samples. The LRM must be homogeneous, well characterised for the determinands in question and stability tests must have shown that it produces consistent results over time. The LRM should be of the same type of matrix (*e.g.* liver, muscle tissue, fat or lean fish) as the samples, and the determinant concentrations should occur in a comparable range to those of the samples. If the range of determinant concentrations in the sample is large (greater than a factor of 5) it is preferable to include two reference materials within each batch of analyses to cover the lower and upper concentrations. It is good practice to run duplicate analyses of a reference material to check within-batch analytical variability. A quality control chart should be recorded for a selected set of CBs. When introducing a new LRM or when it is suspected from the control chart that there is a systematic error possibly due to an alteration of the material, a relevant Certified Reference Material (CRM) of a similar matrix to the material analysed should be used to check the LRM. Additionally a duplicate of at least one sample should be run with every batch of samples. Each laboratory should participate in interlaboratory comparison studies and proficiency testing schemes on a regular basis, preferably at an international level.

## 3.5 Data collection and reporting

The calculation of results and the reporting of data can represent major sources of error. Control procedures should be established in order to ensure that data are correct and to obviate transcription errors. Data stored on databases should be checked and validated, and checks are also necessary when data are transferred between databases. If possible data should be reported in accordance with the latest ICES reporting formats.

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Technical Annex 9: Analysis of dioxins, furans and dioxin-like PCBs in biota (revised in 2010/2011)

# 1. Introduction

Polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans (dioxins/furans – PCDD/Fs) are ubiquitous in the environment, primarily as unintentional by-products of combustion and industrial processes. They enter the aquatic environment via several routes, including atmospheric deposition. As they are strongly hydrophobic compounds, sediments are the eventual sink in the aquatic environment, providing a source of potential exposure to aquatic organisms (Hurst et al., 2004). Generally highly resistant to metabolism, PCDD/Fs bioaccumulate and biomagnify and have reached high concentrations in e.g. fish from the Baltic Sea, which resulted in recommendations to restrict the use of those fish for human consumption (Verta et al., 2007). This guideline only addresses the 17 tetra- through octa-chlorinated 2,3,7,8-substituted PCDD/F congeners, and the non- and mono-ortho substituted polychlorinated biphenyls (PCBs), which are able to exhibit similar effects as the 2,3,7,8-substituted dioxins and furans. The general chemical structures of PCDD/Fs and PCBs are given in Figure 1.

In this guideline, the term “dioxin-like PCBs” (dl-PCBs) is used for the non-ortho and mono-ortho PCB congeners listed in Table 1. The coplanar structure of non-ortho substituted PCB congeners allows a configuration similar to that of PCDD/Fs. Mono-ortho substituted PCBs may take a steric position close to coplanarity and are consequently less toxic than non-ortho PCBs. Nevertheless, they have been considered due to their relatively high concentrations compared to those of non-ortho PCBs or PCDD/Fs (Daelemans et al., 1992). The exposure to dl-PCBs is mainly via the food-chain, as the compounds are highly lipophilic and bioaccumulate and biomagnify in lipid-rich tissue (e.g. Dyke et al., 2003).

PCDD/Fs and dl-PCBs have been shown to produce various toxic responses, including immunotoxicity, developmental and reproductive effects and carcinogenesis (OSPAR, 2005). The initial mechanism of toxicity is via the aryl hydrocarbon receptor (AhR), leading to changes in gene expression, cell growth and cell differentiation (Nebert et al., 1993; Hurst et al., 2004). Due to their persistence, high toxicity, bioaccumulation potential and ability for long-range transport, they are controlled under the Stockholm Convention for Persistent Organic Pollutants (POPs). Their spatial and temporal monitoring in the aquatic environment is important to evaluate the risk to wildlife and human health (Hurst et al., 2004). Due to the low concentrations at which adverse effects can be observed, the analytical methodology for the analysis of PCDD/Fs and dl-PCBs differs from those for other organochlorine compounds, as described in this guideline.



Figure 1: General formula of PCDDs, PCDFs and PCBs. The possible number of chlorine atoms results in 75 PCDD congeners, 135 PCDF congeners (x=1–4, y=0–4), and 209 PCB congeners (x=1–5, y=0–5).

# 2. Analytes and Toxicity Equivalent Factors (TEFs)

Environmental monitoring should include the 17 tetra- through octa-chlorinated 2,3, 7,8-substituted PCDD/Fs and the dl-PCBs listed in Table 1.

In the context of food and feed analysis and compliance checks with maximum residue limits, the concept of TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) Toxicity Equivalency Factors (TEFs) is commonly used, to account for mixtures of several PCDD/Fs and other compounds with dioxin-like activity usually present in these samples. Each congener has been assigned a TEF relative to that of the most toxic dioxin congener, 2,3,7,8-TCDD, that was given a TEF of 1.0. The concentrations of the individual congeners are multiplied with their respective TEFs, and the sum of this gives the total concentration of dioxin-like compounds, expressed in TCDD Equivalents (TEQs). Thus, concentrations of mixtures can be expressed in terms of their dioxin-like activity in TEQs, relative to the most potent 2,3,7,8-TCDD. Two parallel TEF systems are currently in use: TEFs established by the World Health Organization (WHO-TEF/TEQ) and TEFs developed by NATO/CCMS (International TEFs or I-TEFs/TEQ). The use of I-TEFs, however, is decreasing. The WHO-TEF-system is reviewed every five years, and Table 1 presents the most recent values, as of 2006 (Van den Berg et al., 2006).

According to OSPAR (2005), the scientific relevance of using TEQs to express results is greater for human exposure than for evaluation of pollution sources and emissions, for which information on congener patterns can be of more importance. Furthermore, the system assumes additive effects of the individual congeners, while both synergistic and antagonistic effects have also been reported (OSPAR, 2005). It is therefore recommended for environmental monitoring to report concentrations of individual PCDD/Fs and dl-PCB congeners in biota as absolute concentrations, i.e. pg/g wet weight (ww), with additional information on dry matter and lipid content (see chapter “Data reporting”). The TEF concept can be applied in a subsequent risk assessment, if appropriate.

As part of the TEQ approach, there are different ways of handling results below limits of quantification (LoQ):

The concept of upper bound requires using the limit of quantification for the contribution of each non-quantified congener to the TEQ.

The concept of lower bound requires using zero for the contribution of each non-quantified congener to the TEQ.

The concept of medium bound requires using half of the limit of quantification calculating the contribution of each non-quantified congener to the TEQ.

As mentioned above, results of environmental monitoring should preferably be reported for individual congeners, in absolute concentrations. However, OSPAR (2005) mentioned the ongoing food analysis programmes which might be complementary to environmental monitoring. In this context, information on the handling of concentrations below LoQ will be important. Thus, results expressed as TEQ values should be reported as both upper bound and lower bound values (at least, indication of which calculation mode was used should be given). COMMISSION DIRECTIVE 2002/69/EC specifies that, for samples containing 1 pg WHO TEQ/g fat, the difference between upper bound and lower bound level should not exceed 20%. For lower contamination levels, this difference may be in the range of 25 to 40%.

**Table 1**:Chlorinated dibenzo-p-dioxins (CDDs), chlorinated dibenzofurans (CDFs) and dl-PCBs with their Toxicity Equivalent Factors (TEFs) according to the systems developed by the World Health Organization (WHO2005-TEF, Van den Berg et al., 2006) and NATO/CCMS (I-TEF).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Homologue group | Congener | I-TEF | WHO2005-TEF | IUPAC no. |
| PCDDs | | | | |
| TCDD | 2,3,7,8 | 1 | 1 |  |
| PeCDD | 1,2,3,7,8 | 0.5 | 1 |  |
| HxCDD | 1,2,3,4,7,8 | 0.1 | 0.1 |  |
|  | 1,2,3,6,7,8 | 0.1 | 0.1 |  |
|  | 1,2,3,7,8,9 | 0.1 | 0.1 |  |
| HpCDD | 1,2,3,4,6,7,8 | 0.01 | 0.01 |  |
| OCDD | 1,2,3,4,6,7,8,9 | 0.001 | 0.0003 |  |
| PCDFs | | | | |
| TCDF | 2,3,7,8 | 0.1 | 0.1 |  |
| PeCDF | 1,2,3,7,8 | 0.05 | 0.03 |  |
|  | 2,3,4,7,8 | 0.5 | 0.3 |  |
| HxCDF | 1,2,3,4,7,8 | 0.1 | 0.1 |  |
|  | 1,2,3,6,7,8 | 0.1 | 0.1 |  |
|  | 1,2,3,7,8,9 | 0.1 | 0.1 |  |
|  | 2,3,4,6,7,8 | 0.1 | 0.1 |  |
| HpCDF | 1,2,3,4,6,7,8 | 0.01 | 0.01 |  |
|  | 1,2,3,4,7,8,9 | 0.01 | 0.01 |  |
| OCDF | 1,2,3,4,6,7,8,9 | 0.001 | 0.0003 |  |
| Non-*ortho* PCBs | | | | |
| TeCB | 3,3',4,4' |  | 0.0001 | 77 |
| TeCB | 3,4,4’,5 |  | 0.0003 | 81 |
| PeCB | 3,3',4,4',5 |  | 0.1 | 126 |
| HxCB | 3,3',4,4',5,5' |  | 0.03 | 169 |
| Mono-*ortho* PCBs | | | | |
| PeCB | 2,3,3',4,4' |  | 0.00003 | 105 |
|  | 2,3,4,4',5 |  | 0.00003 | 114 |
|  | 2,3',4,4',5 |  | 0.00003 | 118 |
|  | 2',3,4,4',5 |  | 0.00003 | 123 |
| HxCB | 2,3,3’,4,4’,5 |  | 0.00003 | 156 |
|  | 2,3,3',4,4',5 |  | 0.00003 | 157 |
|  | 2,3',4,4',5,5' |  | 0.00003 | 167 |
| HpCB | 2,3,3',4,4',5,5' |  | 0.00003 | 189 |

# 3. Biota samples

OSPAR (2005) presented a monitoring strategy for PCDD/Fs, which identified biota as one of the important matrices for environmental monitoring (the other one being marine sediments). Aquatic organisms can accumulate hydrophobic compounds like PCDD/Fs and dl-PCBs and reach concentrations considerably above those of the surrounding waters. The ratio between the concentration in biota and in the water is the bioconcentration factor (BCF), which is between 2000 and 9000 for PCDD/Fs (OSPAR, 2005). As the BCF varies with species and compound, it is important to design a sampling programme which minimises confounding factors, i.e. to choose the same species, sampling area and sampling period.

The species selected for monitoring should fulfil certain requirements:

* Reflect concentration changes in the sampling area, i.e. ensure a link between exposure and concentration in the organisms.
* Accumulate compounds without showing adverse effects.
* Representative of and abundant in the area (to ensure sufficient sample material for analysis).
* Relatively easy to handle.

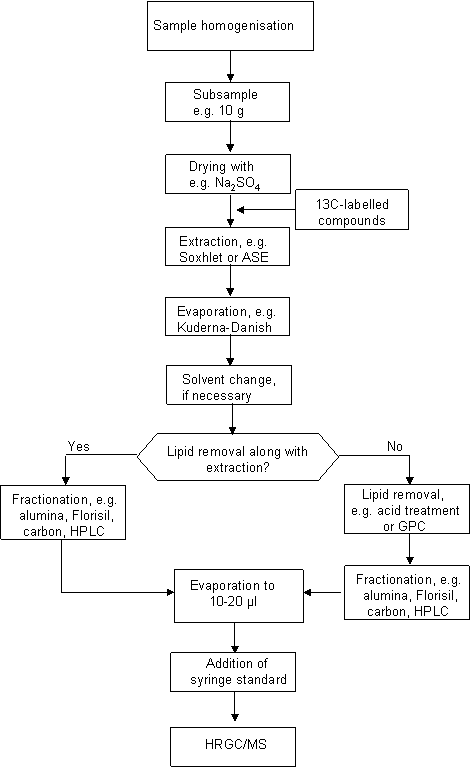
Analogous to the monitoring of other organohalogen compounds, mussels and fish are suitable and commonly used for monitoring of PCDD/Fs and dl-PCBs (OSPAR, 2005). Highest PCDD/F concentrations are found in fish liver and muscle tissue of fatty fish such as herring and salmon. National food agencies often analyse PCDD/Fs and dl-PCBs in commercial fish and fish products, in order to monitor compliance with EU limit values. While different approaches will be necessary in environmental analyses, OSPAR (2005) recommends the monitoring of fish and shellfish as part of the monitoring strategy for dioxins.

In general, the same recommendations are valid as described for other organochlorine compounds, i.e. in the OSPAR guideline on organic contaminants (OCs) in biota, which also contains details on sample dissection and homogenization. It should be pointed out, however, that the risk of sample contamination is considerably higher, given the extremely low concentrations of PCDD/Fs and dl-PCBs in most biota samples. The staff collecting and handling the samples should be well-trained and properly instructed in how to avoid contamination.

For mussel samples, it is important to remove any sediment particles from their intestinal system, by depuration in a glass aquarium with filtered water from the sampling location for approximately 24 hours. Mussel samples must not be frozen prior to dissection, but should be transported at temperatures between 5 and 15°C, suitable for the area of origin, in a clean container. After dissection, all samples should be stored in the dark at < ‑20°C prior to analysis. Under these conditions, long-term storage of tissue samples is possible (De Boer and Smedes, 1997). More details on the practical aspects of sample handling and preparation are given in the OSPAR guideline on OCs in biota.

# 4. Analytical methods

An example of a suitable method for the analysis of biota samples is given in Figure 2.



**Figure 2**: Analytical method suitable for analysis of biota samples within environmental monitoring.

## 4.1 Preparatory steps

It is essential to avoid contamination during all analytical steps. Reagents should be of high purity or cleaned by extraction or solvent rinse. All solvents used must be checked for presence of residues of target or interfering compounds (e.g. polychlorinated diphenyl ethers). The purity of standards should be checked. Reusable glassware should be rinsed with solvent, disassembled, washed with a detergent solution and further rinsed with ultrapure water and solvent. Baking glassware is common practice as part of the cleaning process, but the formation of active sites on the glass surface that may adsorb the target compounds has been reported (USEPA, 1994).

The preparation of stock solutions and standards can follow the guidelines developed for OCs in biota. However, care has to be taken to monitor and to avoid contamination. Furthermore, the high toxicity of the compounds might require a particularly careful handling; see comments under “Safety”. Commercially available diluted stock solutions can be used to reduce safety issues. As valid for the entire analytical method, only trained personnel should perform these steps.

PCDD/Fs and dl-PCBs are normally determined by isotope dilution, using high resolution gas chromatography and high resolution mass spectrometry (HRGC/HRMS). 13C-labelled standards of all congeners to be analysed are added prior to extraction of the samples. These internal standards are used to quantify the native PCDD/Fs and PCBs and to check the method performance in each sample (recovery surrogates). Table 2 provides a list of all 13C12 labelled congeners available for use as internal standards while Table 3 provides the minimum number of internal standards to be used for the quantification of PCDD/Fs congeners.

**Table 2**: 13C12 labelled congeners that can be used as the internal standards

|  |  |
| --- | --- |
| **PCDD/F congeners** | **PCB congeners** |
| 2,3,7,8-13C12-TCDD | 13C12-CB-77 |
| 1,2,3,7,8-13C12-PeCDD | 13C12-CB-81 |
| 1,2,3,4,7,8-13C12-HxCDD | 13C12-CB-126 |
| 1,2,3,6,7,8-13C12-HxCDD | 13C12-CB-169 |
| 1,2,3,7,8,9-13C12-HxCDD |  |
| 1,2,3,4,6,7,8-13C12-HpCDD | 13C12-CB-105 |
| 13C12-OCDD | 13C12-CB-114 |
|  | 13C12-CB-118 |
| 2,3,7,8-13C12-TCDF | 13C12-CB-123 |
| 1,2,3,7,8-13C12-PeCDF | 13C12-CB-156 |
| 2,3,4,7,8-13C12-PeCDF | 13C12-CB-157 |
| 1,2,3,4,7,8-13C12-HxCDF | 13C12-CB-167 |
| 1,2,3,6,7,8-13C12-HxCDF | 13C12-CB-189 |
| 2,3,4,6,7,8-13C12-HxCDF |  |
| 1,2,3,7,8,9-13C12-HxCDF |  |
| 1,2,3,4,6,7,8-13C12-HpCDF |  |
| 1,2,3,4,7,8,9-13C12-HpCDF |  |
| 13C12-OCDF |  |

**Table 3**: Minimum number of internal standards to be used for calibration of PCDD and PCDF homologue groups

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Substance** | **PCDD-Homologues** | | **PCDF-Homologues** | |
|  | **Native** | **13C12-labelled** | **Native** | **13C12-labelled** |
| Tetrachloro homologues | 2,3,7,8 | 2,3,7,8 | 2,3,7,8 | 2,3,7,8 |
| Pentachloro homologues | 1,2,3,7,8 | 1,2,3,7,8 | 1,2,3,7,8 | 2,3,4,7,8  1,2,3,7,8 |
| Hexachloro homologues | 1,2,3,4,7,8  1,2,3,6,7,8  1,2,3,7,8,9 | 1,2,3,7,8,9 | 1,2,3,4,7,8  1,2,3,6,7,8  1,2,3,7,8,9  2,3,4,6,7,8 | 2,3,4,6,7,8 |
| Heptachloro homologues | 1,2,3,4,6,7,8 | 1,2,3,4,6,7,8 | 1,2,3,4,6,7,8  1,2,3,4,7,8,9 | 1,2,3,4,6,7,8 |

## 4.2 Lipid determination

The total lipid content should be determined in all biota samples, using the method of Bligh and Dyer (1959) as modified by Hanson and Olley (1963) or an equivalent method such as Smedes (1999). For normalisation purposes, the total lipid content is preferred to the extractable lipid content (De Boer, 1988).

## 4.3 Extraction

Soxhlet extraction is commonly used for biota samples. Immediately prior to use, the Soxhlet apparatus should be pre-extracted with e.g. dichloromethane:hexane (1:1) for approximately 3 hours (USEPA, 1994). According to the USEPA method, an adequate amount of tissue (e.g. 10 g of wet tissue) is spiked with the labelled compounds and mixed with sodium sulphate. The sample is allowed to dry for 12–24 hours and should be remixed prior to transfer to a glass Soxhlet thimble. Soxhlet extraction proceeds for 18–24 hours using e.g. fresh dichloromethane:hexane (1:1) (USEPA, 1994).

More recently, pressurised liquid extraction (PLE) has become a common and faster alternative to Soxhlet extraction (Focant et al., 2004). PLE uses organic solvents at temperatures above their boiling point maintained in the liquid phase under high pressure. The extraction cell which contains the sample is heated (e.g. 100°C) and filled up with an appropriate solvent (e.g. toluene, dichloromethane) up to a pressure of 140 bars. The minimum extraction time should be 10 minutes in static mode, and several extraction cycles are recommended (n = 2-3). To further reduce analysis time, PLE can be combined with in-line clean-up procedures using preferably sulphuric acid impregnated silica as fat retainer (Björklund et al., 2006). Proper fat-fat retainer ratios are important to avoid fat remaining in the sample after extraction. Mixed (polar/non-polar) solvent combinations cannot be used with this technique. It is understood that the combination of increased pressure and temperature is sufficient to extract all PCDD/Fs and dl-PCBs from the matrix.

## 4.4 Clean-up

The extracts are concentrated using suitable evaporation devices, e.g. rotary evaporation, Turbovap, Syncore or Kuderna-Danish. The risk of cross-contamination is fairly high for rotary evaporation, so the evaporator should be pre-cleaned, e.g. by 100 ml of clean solvent. If the extracts are to be cleaned up by adsorption chromatography on e.g. silica gel, a solvent change to hexane is necessary. The purification procedures have two objectives: i) removal or destruction of lipids and ii) removal of interfering compounds. Due to the very low levels of PCDD/Fs in biota samples, the elimination of interferences is essential.

For the first part, addition of concentrated sulphuric acid is commonly applied, either in combination with a column chromatography clean-up or by direct addition of silica impregnated with sulphuric acid to the extracts. The column chromatography clean-up suggested by USEPA (1994) for lipid removal in biota extracts includes 2 g of silica gel, 2 g of potassium silicate, 2 g of anhydrous Na2SO4, 10 g of silica gel (impregnated with sulphuric acid) and another 2 g of anhydrous sodium sulphate, to be packed bottom-to-top into a column of 25 mm ID. The column is pre-eluted with 100 ml of hexane and after loading of the sample, eluted with 200 ml of hexane. Ready to use, multilayer clean-up columns are also available commercially. Alternatively, approximately 30 -100 g of sulphuric acid impregnated silica gel can be added to the extract, while stirring for 2-3 hours. The treatment with sulphuric acid impregnated silica requires strict safety procedures as the small particles can cause serious health damage after inhalation. Gel permeation chromatography (GPC) has also been applied for lipid removal, but often a series of GPC columns is needed to ensure a 100% fat removal. Alternatively, an additional clean-up step using concentrated sulphuric acid might be applied after GPC to remove residual lipids from samples with a higher lipid content.

For removal of interferences, HPLC, GPC and column chromatography using alumina, silica gel, Florisil and activated carbon are possible alternatives. USEPA (1994) suggests adsorption chromatography on alumina or Florisil and carbon as minimum additional clean-up steps after lipid removal. Depending on whether acid or basic alumina is chosen, the eluents should be dichloromethane:hexane (1:4) or (1:1), respectively. The material for the carbon column can be e.g. Carbopack™-C. Interferences are removed in a washing step with e.g. hexane, dichloromethane:cyclohexane and dichloromethane:toluene. Then, the column is inverted and the analytes are eluted with toluene.

HPLC can also be used for purification and fractionation of the extracts. 2-(1- pyrenyl)ethyldimethylsilylated (PYE) silica columns and porous graphitised carbon are suitable columns for this purpose (Echols et al., 1998). When coupled in series, nitrophenylpropylsilica column (Nucleosil, 5 µm particles, 250 x 4.6 mm) and PYE (Cosmosil, 5 µm particles, 150 x 4.6 mm) enable the separation of PCDD/Fs from dl-PCBs (Bandh et al. 1996). Fully automated clean-up systems are also available commercially (e.g. PowerPrep™ system). The European research project DIFFERENCE recommended at least three clean up or fractionation steps to ensure sufficiently clean extracts (Van Loco et al., 2004; Van Leeuwen et al., 2007).

## 4.5 Concentration and syringe standards

After clean-up, a keeper is added (e.g. iso-octane or nonane) and the extracts are concentrated to near dryness, i.e. 10-20 µl. A syringe standard mix should also be added to evaluate the recovery of labelled internal standards. For example 13C12 -1,2,3,4-TCDD can be used for recovery determinations of TCDD/Fs and PeCDD/Fs internal standards while 13C12 -1,2,3,7,8,9-HxCDD can be used for recovery determinations of HxCDD/Fs, HpCDD/Fs and OCDD/F internal standards.

# 5. Instrumental analysis

The PCDD/F content in environmental samples is commonly monitored using high resolution gas chromatography (HRGC) and high resolution mass spectrometry (HRMS), but low resolution mass spectrometry (LRMS) may be a suitable alternative if the required minimum performance criteria are met (see “HRGC/LRMS”).

## 5.1 GC-analysis

The GC analysis should be optimised with regard to separation and sensitivity. Fishman et al. (2007) provided a comprehensive review of GC columns available for dioxin analysis. Generally 50-60 m, 5% diphenyl 95% dimethylpolysiloxane columns are a common choice. However, these columns could exhibit multiple co-elutions for both PCBs and PCDD/Fs (Reiner et al., 2006), depending on the matrix to be analysed. The use of RTx-Dioxin 2 column has been reported in the literature as a suitable alternative to DB-5 columns. Combining this phase with reduced inner diameter and phase thickness (for example a 40m x 0.18mm x 0.18µm) enables the analysis of the 17 PCDD/F congeners in 40 minutes, with data fulfilling QA/QC requirements and providing better selectivity, especially for 2,3,7,8-TCDD and 2,3,7,8-TCDF (Reiner et al., 2006; Cochran et al., 2007).

Potential interferences for dl-PCBs on common GC-columns are summarised in Table 4 (Reiner et al., 2006). Complete separation can be achieved by multi-analysis on columns of different polarity. Recent developments indicate possibilities of full separation of relevant PCB congeners on one column, e.g. on an SGE HT8-PCB capillary column. A full separation of all PCB congeners is also possible by using comprehensive multi-dimensional GC (GCxGC) (e.g. Haglund et al., 2008; Skoczynska et al., 2008).

**Table 4**: Possible interferences for selected dl-PCBs using a 5% phenyl column (Reiner et al., 2006)

|  |  |
| --- | --- |
| **PCB congener** | **Potential interference** |
| CB-81 | CB-87 |
| CB-77 | CB-110 |
| CB-123 | CB-149 |
| CB-126 | CB-178 and CB-129 |
| CB-156 | CB-171 |
| CB-157 | CB-201 |

Various injection techniques are possible, e.g. on-column injection, splitless injection, pressure-pulsed splitless injection and programmed temperature vaporizing (PTV) injection. The most suitable injection volume depends on the dioxin concentrations in the sample and the sensitivity of the instrumental analysis. In HRGC/HRMS analysis, 1-2 µl are common injection volumes.

## 5.2 Compound identification

The HRMS system should be operated at a minimum of 10,000 resolving power throughout all the runs, and resolution should be checked regularly during the sequence of runs. The individual PCDD/Fs, dl-PCBs or labelled compounds are identified by comparing the GC retention time and ion abundance ratio of two exact masses monitored (Tables 5 and 6) with the corresponding retention time of an authentic labelled internal standard and the theoretical or acquired ion abundance ratio of the two exact masses. The congeners for which there are no labelled analogues are identified when relative retention time and ion abundance ratios agree within predefined limits. The following criteria should be met for identification of an individual dl-PCB, PCDD/F or labelled compound in a standard, blank or sample:

* The signal for the two exact masses specified in Tables 5 and 6 should be present and within ± 2 s.
* The signal-to-noise ratio (S/N) for the GC peak at each exact mass has to be at least 3 for each congener detected in a sample extract, and at least 10 for all congeners in the calibration standard.
* The ratio of the integrated areas of the two exact masses specified in Tables 5 and 6 has to be within 15% of the theoretical shown in Table 7.
* The relative retention time of a native PCDD/F and dl-PCB has to be within a time window of ± 0.003 s based on the retention time of the corresponding 13C12-labelled standard. The relative retention time of congeners for which there are no labelled analogues has to be within ± 0.002.

If interferences preclude identification, extract a new, further cleaned up aliquot and analyse again. If interferences cannot be removed flag the data to indicate results are maximum concentrations.

## 5.3 Compound quantification

Quantitative analysis is performed using selected ion monitoring (SIM), in one of the two following ways:

* For the PCDD/Fs and dl-PCBs for which labelled analogues have been added to the sample (Table 2), the GC/MS system is calibrated, and the concentration of each compound is determined using the isotope dilution technique.
* For the PCDD/Fs and dl-PCBs for which labelled analogues are not added to the sample (see Table 3 for PCDD/Fs), the GC/MS system is calibrated for each compound using a labelled isomer with the most similar structure and the concentration of each compound is determined using the internal standard technique.

Calibration curves should be based on a minimum of 5 calibration points. Mass drift correction is mandatory, usually based on a lock-mass m/z of perfluorokerosene (PFK) or perfluorotributylamine (PFTBA, FC43).

**Table 5**: Masses for the detection and quantification of PCDD/Fs

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Substance** | **Dibenzofurans** | | **Dibenzo-p-dioxins** | |
| **Native** | **13C12-labelled** | **Native** | **13C12-labelled** |
| Tetra-CDD/F | 303.9016 | 315.9419 | 319.8965 | 331.9368 |
| 305.8987 | 317.9389 | 321.8937 | 333.9339 |
| Penta-CDD/F | 339.8598 | 351.9000 | 355.8547 | 367.8949 |
| 341.8569 | 353.8970 | 357.8518 | 369.8919 |
| Hexa-CDD/F | 373.8208 | 385.8610 | 389.8157 | 401.8559 |
| 375.8179 | 387.8580 | 391.8128 | 403.8529 |
| Hepta-CDD/F | 407.7818 | 419.8220 | 423.7767 | 435.8169 |
| 409.7789 | 421.8190 | 425.7738 | 437.8140 |
| Octa-CDD/F | 441.7428 | 453.7830 | 457.7377 | 469.7779 |
| 443.7399 | 455.7801 | 459.7348 | 471.7750 |

**Table 6**: Masses for the detection and quantification of PCBs

|  |  |  |
| --- | --- | --- |
| **Homologue groups** | **Native CBs** | **13C12-labeled CBs** |
| Tetrachlorobiphenyls | 289.9223 | 301.9626 |
| 291.9194 | 303.9597 |
| Pentachlorobiphenyls | 325.8804 | 337.9207 |
| 327.8775 | 339.9177 |
| Hexachlorobiphenyls | 359.8415 | 371.8817 |
| 361.8385 | 373.8788 |
| Heptachlorobiphenyls | 393.8025 | 405.8427 |
| 395.7995 | 407.8398 |

The isotope ratio between the two ions of the molecular isotope cluster, which are recorded, has to match the theoretical value within ± 15 % (see Table 7).

**Table 7**: Tolerance limits of isotope ratios for PCDD/Fs and dl-PCBs

|  |  |  |  |
| --- | --- | --- | --- |
| **Chlorine atoms** | **Isotope Ratio Lower Limit** | **Isotope Ratio**  **Theoretical Value** | **Isotope Ratio Upper Limit** |
| 4 | 0.65 | 0.77 (M/M+2) | 0.89 |
| 5 | 0.55 | 0.64 (M+4/M+2) | 0.75 |
| 6 | 0.69 | 0.81 (M+4/M+2) | 0.94 |
| 7 | 0.83 | 0.96 (M+4/M+2) | 1.10 |
| 8 | 0.76 | 0.89 (M+2/M+4) | 1.02 |

## 5.4 HRGC/LRMS

Low resolution mass spectrometry (LRMS) has also been applied to the analysis of PCDD/Fs and/or dl-PCBs. Limits of detections are higher than those obtained with HRMS detectors, but can be compensated by e.g. larger injection volumes. A very efficient extract clean-up is of the utmost importance to exclude any interferences. A technique commonly applied is GC-LRMS using ion trap mass analyzers working in tandem mode (Focant et al., 2005; Malavia et al., 2008, Eppe et al., 2004). Table 8 provides information on precursor and product ions obtained by GC-ion trap MS. GC-LRMS (quadrupole) can be an option for dl-PCBs in particular.

Both HRMS and LRMS techniques have to demonstrate that they meet the requirements regarding separation and sensitivity described in the monitoring programme, see also comments under “Quality Assurance and Quality Control”. When using LRMS the maintenance of the instrument is crucial and could be time consuming (e.g. frequent cleaning of the ion source). The sensitivity for 2,3,7,8-TCDD may be critical.

**Table 8**: Precursor ions and product ions for the determination of PCDD/Fs and dl-PCBs by HRGC-ion trap tandem MS

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| 1. **Target Compounds** | 1. **Native** | | 1. **13C12-labelled** | |
|  | 1. **Precursor Ion (m/z)** | 1. **Product** 2. **Ions** 3. **(m/z)** | 1. **Precursor Ion (m/z)** | 1. **Product** 2. **Ions** 3. **(m/z)** |
| 1. TCDD | 1. 322 (M+2) | 1. 257 + 259 | 1. 334 (M+2) | 1. 268 + 270 |
| 1. PeCDD | 1. 356 (M+2) | 1. 291 + 293 | 1. 368 (M+2) | 1. 302 + 304 |
| 1. HxCDD | 1. 390 (M+2) | 1. 325 + 327 | 1. 402 (M+2) | 1. 336 + 338 |
| 1. HpCDD | 1. 424 (M+2) | 1. 359 + 361 | 1. 436 (M+2) | 1. 370 + 372 |
| 1. OCDD | 1. 460 (M+4) | 1. 395 + 397 | 1. 472 (M+4) | 1. 406 + 408 |
| 1. TCDF | 1. 306 (M+2) | 1. 241 + 243 | 1. 318 (M+2) | 1. 252 + 254 |
| 1. PeCDF | 1. 340 (M+2) | 1. 275 + 277 | 1. 352 (M+2) | 1. 286 + 288 |
| 1. HxCDF | 1. 374 (M+2) | 1. 309 + 311 | 1. 386 (M+2) | 1. 320 + 322 |
| 1. HpCDF | 1. 408 (M+2) | 1. 343 + 345 | 1. 420 (M+2) | 1. 354 + 356 |
| 1. OCDF | 1. 444 (M+4) | 1. 379 + 381 |  |  |
| 1. CB-81, 77 | 1. 292 (M+2) | 1. 220 + 222 | 1. 304 (M+2) | 1. 232 + 234 |
| 1. CB-123, 118, 114, 105, 126 | 1. 326 (M+2) | 1. 254 + 256 | 1. 338 (M+2) | 1. 266 + 268 |
| 1. CB-167, 156, 157, 169 | 1. 360 (M+2) | 1. 288 + 290 | 1. 372 (M+2) | 1. 300 + 302 |
| 1. CB-189 | 1. 394 (M+2) | 1. 322 + 324 | 1. 406 (M+2) | 1. 334 + 336 |

# 6 Quality Assurance and Quality Control

The laboratory is required to operate a formal quality assurance programme. Indicative values for accuracy and precision are given under “Verification method” in Table 9. An example of a comprehensive QA/QC approach is described in method 1613B by USEPA (1994).

The analytical method requires high sensitivity and low detection limits, usually in the pg-range, for both PCDD/Fs and dl-PCB congeners (OSPAR, 2005), and should meet the requirements for LoQ specified in the monitoring programme. For individual PCDD/Fs, LoQ of 0.3 pg/g wet weight should be achievable, with the exception of OCDD (1 pg/g wet weight). For non-*ortho* PCBs, LoQ should be as low as 5 pg/g wet weight, while for mono-*ortho* PCBs requirements on LoQ are less strict as their concentrations in biota samples are usually higher, in particular concentrations of congeners CB-105, CB-118 and CB-156. The selectivity of the method should be sufficient to avoid interfering compounds, i.e. the individual congeners should be separated from each other and any interferences present. The recovery of the individual internal standards added prior to extraction should be between 60-120%.

All sample series should include procedural blanks and measurements of certified/laboratory reference materials. Blanks should be as low as possible, at least below 20% of the lowest concentration of interest. Certified reference materials should be analysed regularly, although only few are available for the determination of PCDD/Fs and dl-PCBs in biota, for example from NIST (cod liver oil), Cambridge Isotope Laboratories (fish) and the National Research Council Canada (fish) (De Boer and McGovern, 2001). The laboratory should further prove its competence by regular participation in relevant laboratory proficiency tests. It is essential that the matrix and concentration range of the proficiency testing samples are comparable with the samples routinely analysed within the monitoring programme (De Boer, 2001; Wells and de Boer, 2006).

# 7 Screening methods based on bioassays

Bioassays are not currently applied in the monitoring under the OSPAR Co-ordinated Environmental Monitoring Programme (CEMP), but have been suggested as screening tools for monitoring PCDD/Fs and dl-PCBs in foodstuffs (COMMISSION DIRECTIVE 2002/69/EC), with the requirement to meet the criteria given in Table 9. Screening tools might be useful in, for instance, choice of sampling sites, and will therefore be briefly discussed in this guideline. Hurst et al. (2004) also emphasised that monitoring programmes were moving towards effect-based monitoring, with biological relevance becoming more important. The tool must be capable of rapid, inexpensive and high-throughput screening producing interpretable and meaningful results (Hurst et al., 2004).

**Table 9**:Quality criteria for screening and verification methods (COMMISSION DIRECTIVE 2002/69/EC)

|  |  |  |
| --- | --- | --- |
|  | Screening method | Verification method |
| False negatives | < 1% |  |
| Accuracy |  | ± 20% |
| Precision (expressed as the coefficient of variation between repeated measurements) | < 30% | < 15% |

The dioxin responsive chemically activated luciferase expression (DR-CALUX) assay is mechanism specific and utilises the interaction of compounds with the AhR. However, it is not compound specific and produces a response with all compounds capable of interactions with the AhR. COMMISSION DIRECTIVE 2002/69/EC demands that the TEQ-values determined by bioassays should be the sum of PCDD/Fs and dl-PCBs in the sample, however, Hurst et al. (2004) and Van Leeuwen et al. (2007) demonstrated some disagreement between the results of the bioassay and the conventional targeted HRGC/HRMS analysis. The differences may be caused by unknown compounds producing a dioxin-like response in the CALUX assay (e.g. brominated or mixed halogenated dioxin analogues, polychlorinated naphthalenes, PAHs etc.) – or compounds antagonising the AhR (e.g. di-ortho-substituted PCBs).

These deviations from results of chemical analysis were also considered as an advantage by Hurst et al. (2004), as the assay allows a more accurate assessment of the true potency of dioxin-like compounds present in the samples. In order to obtain specific responses to PCDD/Fs and dl-PCBs in the sample, the extracts require specific clean-up methods to exclude interferences from other dioxin-like compounds. As mentioned above, environmental monitoring aims at presenting concentrations of individaul compounds in the respective samples, rather than toxicity assessments.

# 8 Safety

The chemical compounds dealt with in this guideline are hazardous and must only be handled by trained personnel familiar with handling of PCDD/F and dl-PCBs, and associated risks as well as precautionary measures. USEPA (1994) recommends that laboratories purchase diluted standard solutions instead of preparing primary solutions.

# 9 Data reporting

Results are reported in pg/g ww. The lipid content and water content of the samples should be reported as well. For normalizing purposes the total lipid content should be determined, rather than the extractable lipid content (De Boer, 1988). Concentrations are reported to two significant figures. Minimum performance criteria such as LoQ and measurement uncertainty along with information on blanks and reference materials should be included in the report.

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1. Revised in 2018

   Addition of Technical Annexes 4 and 5 agreed in 2008.

   Replacement of Technical Annex 3 (PAHs in biological materials) in 2009

   Addition of Technical Annexes 6 (perfluorinated compounds in biota) and 7 (organotin compounds in biota) agreed in 2009

   Revised version of Technical Annex 1 agreed in 2010

   Addition of Technical Annexes 8 (chlorobiphenyls) and 9 (dioxins/furans and planar CBs) agreed in 2010

   Technical Annex 9 revised in 2010/2011

   Guidelines revised in 2012 (see HASEC 12/13/1, Annex 7)

   Revised technical annex 2 on Metals 2017, extending the methods description and newer references [↑](#footnote-ref-1)
2. “Narrow length range” means that the length of the individuals collected should be constant from year to year at each site or should at least fall within a very narrow range. The length range could however vary between sites and hence is not specified in the table. [↑](#footnote-ref-2)
3. The same sex should be sampled each year. [↑](#footnote-ref-3)
4. One egg taken randomly from each of 10 clutches. [↑](#footnote-ref-4)
5. The eggs should be collected as early as possible to avoid collecting replacement eggs. [↑](#footnote-ref-5)
6. Please use information on the actual timing of spawning in your sampling area. It is recommended to use Fishbase [www.fishbase.org](http://www.fishbase.org):

   <http://www.fishbase.org/Reproduction/SpawningList.php?ID=69&GenusName=Gadus&SpeciesName=morhua&fc=183&StockCode=79> [↑](#footnote-ref-6)