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Monitoring Guidelines/Protocols for Sampling and Sample Preservation of Marine Biota for IMAP Common Indicator 17: Heavy and Trace Elements and Organic Contaminants

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UNEP/MAP
Athens, 2020

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Annex II: UNEP/FAO/IOC/IAEA (1988). Reference methods No 7 (Rev. 2): Sampling of selected marine organisms and sample preparation for trace metal analysis (2.1.2);

Annex III: HELCOM (2012). Annex B-12, Appendix 1. Technical note on biological material sampling and sample handling for the analysis of persistent organic pollutants (PAHs, PCBs and OCPs) and metallic trace elements (2.1.3);

Annex IV: IAEA (2011). Sample work-up for the analysis of selected chlorinated hydrocarbons in the marine environment. Reference Methods for Marine Pollution Studies No 71 (4.2.1);

Annex V: HELCOM (2012). COMBINE - Guideline for the determination of heavy metals in biota. Annex B-12, Appendix 4: Technical note on the determination of trace metallic elements in biota (5.2.1);

Annex VI: IAEA (2013). Recommended methods for the determination of petroleum hydrocarbons in biological samples (5.2.2);

Annex VII: OSPAR (2008). JAMP Guidelines for monitoring contaminants in biota and sediments (5.2.4);

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Note by the Secretariat

In line with the Programme of Work 2020-2021 adopted by COP21 the MED POL Programme has prepared the Monitoring Guidelines related to IMAP Common Indicators 13, 14, 17 and 20 for consideration of the Integrated Meeting of the Ecosystem Approach Correspondence Groups on Monitoring (December 2020), whilst the Monitoring Guidelines for Common Indicator 18, along with the Monitoring Guidelines related to data quality assurance and reporting are under finalization for consideration of the Meeting on CorMon on Pollution Monitoring planned to be held in April 2021.

These Monitoring Guidelines present coherent manuals to guide technical personnel of IMAP competent laboratories of the Contracting Parties for the implementation of the standardized and harmonized monitoring practices related to a specific IMAP Common Indicator (i.e. sampling, sample preservation and transportation, sample preparation and analysis, along with quality assurance and reporting of monitoring data). For the first time, these guidelines present a summary of the best available known practices employed in marine monitoring by bringing integrated comprehensive analytical practices that can be applied in order to ensure the representativeness and accuracy of the analytical results needed for generation of quality assured monitoring data.

The Monitoring Guidelines/Protocols build upon the knowledge and practices obtained over 40 years of MED POL monitoring implementation and recent publications, highlighting the current practices of the Contracting Parties' marine laboratories, as well as other Regional Seas Conventions and the EU. A thorough analysis of presently available practices of UNEP/MAP, UNEP and IAEA, as well the HELCOM, OSPAR and European Commission Joint Research Centre was undertaken to assist an innovative approach for preparation of the IMAP Monitoring Guidelines/Protocols.

The Monitoring Guidelines/Protocols also address the problems identified during realization of the Proficiency testing being organized by UNEP/MAP-MEDPOL and IAEA for two decades now, given that many unsatisfactory results within inter-laboratory testing may be connected to inadequate laboratory practices of the IMAP/MEDPOL competent laboratories.

In order to support national efforts, this Monitoring Guidelines provides two Technical Note for sampling and sample preservation of marine biota for the analysis of IMAP Common Indicator 17: a) Technical Note for the sampling of marine biota for the analysis of heavy metals and organic contaminants which includes the following four IMAP Protocols: i) Protocol for the collection of fish for heavy metal and organic contaminants analysis; ii) Protocol for the collection of bivalves for heavy metal and organic contaminants analysis; iii) Protocol for the dissection of fish to collect muscle and liver; and iv) Protocol for the dissection of bivalves; and b) Technical Note for the sample preservation of marine biota for the analysis of heavy metals and organic contaminants which includes the following two IMAP Protocols: i) Protocol for the treatment of biota samples prior to analysis of heavy metals; and ii) Protocol for the treatment of biota samples prior to analysis of organic contaminants, for consideration of Integrated Meeting of the Ecosystem Approach Correspondence Groups on Monitoring (CORMON) Biodiversity and Fisheries, Pollution and Marine Litter, and Coast and Hydrography.

The Monitoring Guidelines/Protocols, including this one related to sampling and sample preservation of marine biota for the analysis of IMAP Common Indicator 17 establish a sound ground for further regular update of monitoring practice for the purpose of successful IMAP implementation.

List of Abbreviations / Acronyms

CI	Common Indicator
COP	Conference of the Parties
CORMON	Correspondence Group on Monitoring
EcAp	Ecosystem Approach
EEA	European Environmental Agency
EC	European Commission
EU	European Union
FAO	Food and Agriculture Organization of the United Nation
HELCOM	Baltic Marine Environment Protection Commission - Helsinki Commission
IAEA	International Atomic Energy Agency
IOC	International Oceanographic Commission
IMAP	Integrated Monitoring and Assessment Programme of the Mediterranean Sea and Coast and Related Assessment Criteria
MAP	Mediterranean Action Plan
MED POL	Programme for the Assessment and Control of Marine Pollution in the Mediterranean Sea
MED QSR	Mediterranean Quality Status Report
OSPAR	Convention for the Protection of the Marine Environment for the North-East Atlantic
PoW	Programme of Work
QA/QC	Quality Assurance/Quality Control
QSR	Quality Status Report
US EPA	United States Environmental Protection Agency

1 Introduction

1. Heavy metals and organic contaminants are entering the Mediterranean marine environment discharged from land-based and sea-based pollution sources, as well as from atmospheric deposition. The UNEP/MAP Integrated Monitoring and Assessment Programme (IMAP) (UNEP/MAP, 2019[1]; UNEP (2019a[2]) includes the analysis of specific sedentary marine sentinel organisms (bivalves and benthic feeding fish) in order to assess pollution impact on the marine organisms. The suggested species for monitoring contaminants are a benthic feeding fish (e.g. *Mullus barbatus*) and bivalves (e.g. *Mytilus galloprovincialis*, *Donax trunculus*). However, in case different species of fish and bivalves are used by the Contracting Parties to the Barcelona Convention for assessing marine pollution, explanation has to be provided to UNEP/MAP Secretariat on the reason behind the selection of a different sentinel species for CI17 monitoring.

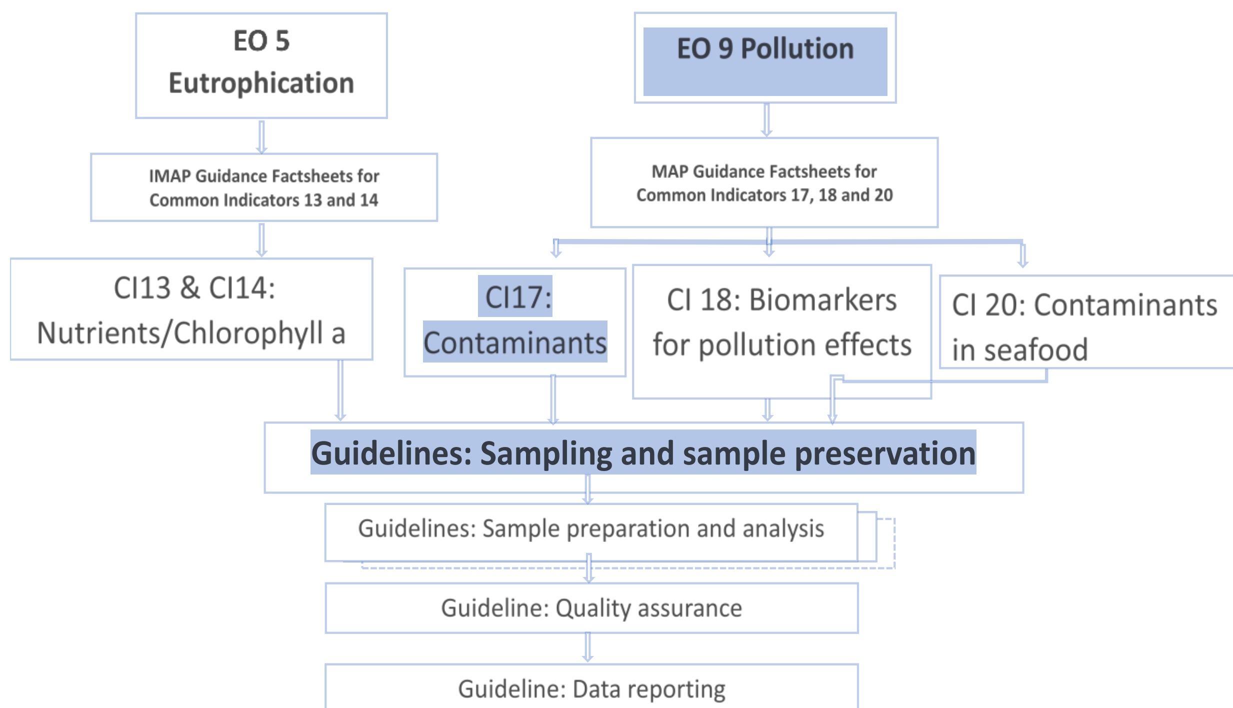
2. Standardize protocols for sampling and processing of marine biota samples is important in view of assuring comparable quality assurance of the data, as well as comparability between sampling areas and different national monitoring programmes. Also, sampling protocols provide guidance on the suitability of selected sampling sites, the number of required samples, the biometric indices to be recorded, the appropriate handling to avoid cross-contamination, and the storage conditions in view of maintaining the sample's integrity during the transfer from the sampling site to the analytical laboratory. Furthermore, protocols are providing guidance on the procedures to dissect the organisms (fish and bivalves) in order to collect the appropriate tissue for analysis (muscle and liver of fish and whole body of bivalves), taking care to avoid cross-contamination by metals or organic contaminants, depending on the foreseen analysis.

3. The Protocols on of this Guidelines, as provided here-below aim at streamlining sampling and processing of marine biota samples in view of assuring comparable quality assurance of the data, as well as comparability between sampling areas and different national monitoring programmes. They also provide the guidance on the suitability of selected sampling sites, the number of required samples, the biometric indices to be recorded, the appropriate handling to avoid cross-contamination, and the storage conditions in view of maintaining the sample's integrity during the transfer from the sampling site to the analytical laboratory to ensure the representativeness and the integrity of the samples. Furthermore, they guide on the procedures to dissect the organisms (fish and bivalves) in order to collect the appropriate tissue for analysis (muscle and liver of fish and whole body of bivalves), taking care on a need to avoid cross-contamination by metals or organic contaminants, depending on the foreseen analysis. They are not intended to be analytical training manuals, but guidelines for Mediterranean laboratories, which should be tested and modified in order to validate their final results.

4. In order to avoid unnecessary repetitions, reference is also made to the protocols already published and publicly accessible, which can also be used by the Contracting Parties' competent laboratories participating in IMAP implementation. Namely, the six here-below elaborated IMAP Protocols build on previous UNEP/MAP - IAEA Recommended Methods, such as Reference Methods No 6 on sampling of selected marine organisms and sample preparation for trace metal analysis (UNEP/FAO/IOC/IAEA, 1987, Annex I) and Reference Methods No 7 (Rev. 2) on sampling and dissecting marine organisms (UNEP/FAO/IOC/IAEA, 1988, Annex II), which were prepared in the framework of the MED POL monitoring programme. IMAP Protocols are also streamlined with similar Guidelines/Protocols for marine biota sampling, sample processing and preservation, which were developed by other Regional Seas Organisations, such as OSPAR (Annex VII) and HELCOM (Annex III), given suitability of any of these Guidelines for application in the context of IMAP. Given the suitability of any of these Guidelines in the context of IMAP, they could be further used by interested IMAP competent Mediterranean laboratories for developing their laboratory specific sampling and sample processing methodologies.

[1] UNEP/MAP (2019). UNEP/MED WG.467/5. IMAP Guidance Factsheets: Update for Common Indicators 13, 14, 17, 18, 20 and 21: New proposal for candidate indicators 26 and 27.
UNEP (2019a). UNEP/MED WG.463/6. Monitoring Protocols for IMAP Common Indicators related to pollution;

5. The below flow diagram informs on the category of this Monitoring Guideline related to sample preparation and analysis of marine biota for IMAP Common Indicator 17 within the structure of all Monitoring Guidelines prepared for IMAP Common Indicators 13, 14, 17, 18 and 20.



Flow Diagram: Monitoring Guidelines for IMAP Ecological Objectives 5 and 9

2 Technical note for the sampling of marine biota for the analysis of heavy metals and organic contaminants

6. Sampling is a very important step in the analysis of marine biota, since it affects the representatives of the sample, which is the basis of every Quality Assurance scheme. The fish and bivalves collected should reflect the condition of other organisms of the same species in the marine area under consideration. The sampling location and conditions (including seafloor nature, sampling depth, location of pollution sources) have to be chosen carefully, taking into consideration other oceanographic data (such as temperature, turbidity, trophic level) in the sampling area. The handling of biota after collection is also of primary importance, in order to follow appropriate procedures to avoid cross contamination of the samples from the ship's environment and the storage of samples. Also, the appropriate preservation of samples during transportation from the sampling site to the laboratory for further analysis is crucial, in order to avoid the deterioration of the biota tissues that may result in loss of determinant or contamination from the packaging materials. Finally, once the biota samples arrive at the laboratory, additional processing is required to dry and homogenize the samples and to store the dried samples in appropriate conditions in order to avoid any alteration of the contaminants' concentrations in the samples.

7. Under this Technical Note, this Guidelines for Sampling and Sample Preservation of Marine Biota for IMAP Common Indicator 17 provides the following Protocols:

- Protocol for the collection of fish for heavy metal and organic contaminants analysis;
- Protocol for the collection of bivalves for heavy metal and organic contaminants analysis;
- Protocol for the dissection of fish to collect muscle and liver;
- Protocol for the dissection of bivalves.

2.1 Protocol for the collection of fish for heavy metal and organic contaminants analysis

8. The most common fish species used for marine pollution monitoring in the Mediterranean region is the mullet (*Mullus barbatus*) (UNEP, 2019a). However, in different areas, according to local conditions, other benthic fish may be used for monitoring contaminants. A list of available reference species (Code list) for Data Dictionaries and Data Standards of the IMAP (Pilot) Info System for E09 (CI17 and CI20) is presented in the document UNEP/MED WG.467/8 (UNEP, 2019b[3]).

9. For fish sampling, in line with the IMAP Monitoring Protocols for CI17 (UNEP, 2019a), 3-5 parallel composite samples (5-6 specimen for each fish sample) are taken yearly from the same size class at each trend monitoring. Fish having a length of 12-16 cm should be included if possible in the selected size classes, to be in line with the Protocol for fish collection for the CI18. Fish can be collected by gill net fishing or trawling using a square-meshed net of 40 mm or, if justified, by a diamond meshed net of 50 mm as required by the EU legislation (EC 1967/2006[4]). Guidelines for collection of fish are presented in UNEP/FAO/IOC/IAEA (1987) (Annex I) and UNEP/FAO/IOC/IAEA (1988) (Annex II). Fish could be sampled from a research vessel or from a small fishing boat. Guidelines on sampling and processing of fish samples are also provided by HELCOM (2012a) (Annex III.) and OSPAR (2008) (Annex VII).

10. It has to be underlined that concentrations of chemical pollutants in marine biota tissues can be influenced by many environmental factors (such as seasonal fluctuations of temperature, organic matter, nutrients) and biological factors (such as the phase of reproductive cycle, weight fluctuations, changes in relative tissue composition, the massive development of gonadic tissues during gametogenesis and the loss of weight during spawning). In order to avoid such variations, it is recommended that sampling take place in the off-spawning period (EC. 2010). Also, in order to evaluate the influence of common biological and environmental factors it is suggested to record the date, seawater temperature, salinity, phytoplankton development, at sampling time.

11. IMAP Monitoring Protocols for CI17 (UNEP, 2019a), requires a fish sampling frequency at least once per year. The same sampling frequency is required in the EU Directive 2008/105/EC.

12. Fish samples should be protected from contamination, which may occur during sampling, sample handling, storage and transfer to the laboratory for further analysis. In case fish are dissected on board, the work must be carried out by personnel capable of identifying and removing the desired organs according to the requirements of the investigation. Fish samples have to be handled with care to avoid any contact with metals (for heavy metal analysis) or possible sources of organic contaminants (for chlorinated hydrocarbons and PAHs analysis). Detailed procedures for fish dissection and the measures to be taken in order to avoid sample contamination during handling, are presented in Protocol for the fish dissection to collect muscle and liver. Upon fish collection additional information on length, wet weight and sex should be recorded. In case of pooling, number of specimens and length range should also be recorded.

13. In case fish samples have to be transported to the laboratory for dissection, they have to be handled and stored in such a way, as to avoid sample deterioration or contamination. A ship has several potential metal contamination sources (metallic hull and superstructures, paint). To prevent metal contamination fish samples intended for heavy metal analysis should be handled in metal-free areas (working surfaces with plastic coatings or cover) and stored in plastic bags for transport to the laboratory. Regarding PAHs and chlorinated hydrocarbons, possible contamination sources in a ship include fuel and lubrication, as well as exhaust from the ship’s engines. Fish samples intended for organic contaminants analysis have to be stored in metal containers for their transport to a stainless steel of aluminium clean working surface in the ship’s laboratory. Before starting the handling of fish

[3] UNEP (2019b) UNEP/MED WG.467/8. Data Standards and Data Dictionaries for Common Indicators related to pollution and marine litter.
[4] EC Council Regulation No 1967/2006 concerning management measures for the sustainable exploitation of fisheries resources in the Mediterranean Sea

samples, it is important to identify possible contamination sources in the ship and the samples handling area, in order to take appropriate measures to avoid contamination.

14. In case fish transport to the laboratory is done in less than 24 hours, samples can be stored on ice. However, for longer periods, fish samples have to be frozen (-20 ºC) and transported frozen to the laboratory for further processing. Each sample should be labelled with the sample's identification number, the type of tissue, and the date and location of sampling.

2.2 Protocol for the collection of bivalves for heavy metal and organic contaminants analysis

15. *Mytilus galloprovincialis* and *Donnax trunculus* are the bivalve species suggested to be analysed for heavy metals and organic contaminants in the framework of CI17 (UNEP/MAP, 2019; UNEP/MAP 2019a). If the Contracting Party decides to analyse other bivalve species, it has to provide UNEP/MAP the rationale behind its decision. To facilitate reporting a list of available reference species (Code list) is provided in the document UNEP/MED WG.467/8 (UNEP, 2019b).

16. In line with the IMAP Monitoring Protocols for CI17 (UNEP, 2019a), 3-5 parallel composite samples of bivalves (10 specimens for each bivalves sample) are collected yearly from the same size class at each trend monitoring. Minimum bivalves sampling is once per year, although twice per year may be applied if possible to be in line with CI18 sampling frequency. The most adequate sampling period is during the post winter months, but before the spawning period. Usually, in most Mediterranean coastal areas, April-June is an appropriate sampling period, but local climatic characteristics have to be taken into consideration for the fixing of the sampling period.

17. The bivalves' size to be collected should be 4-5 cm, to be in line with the sampling protocol for CI18. However, a length-stratified sampling could be applied, which is generating data that can also be used in monitoring programmes for temporal trends of contaminants in biota (HELCOM, 2012a. The HELCOM methodology requires that at least 20 mussels in the largest length interval can easily be found and the length stratification should be determined in such a way that it can be maintained over many years for the purposes of temporal trend monitoring. It is also requiring that the length interval shall be at least 5 mm in size. The length range should be split into at least three length intervals (small, medium, and large) which are of equal size after log transformation and the number of specimens selected for analysis depends on their length, e.g. 80-100 individuals are necessary to suffice material within the length range 4-5 cm (HELCOM, 2012a).

18. Bivalves sampling sites should host an abundant population of the targeted species in order to take appropriate size of sample and to be reasonably accessible in order to easily and rapidly transport biota samples to the laboratory. Bivalves growing on metal structures (i.e. underwater pipes) or substrates, which may be enriched in metals or organic contaminants, should be exempted from collection. Divers will collect manually the mussels living at a 4-5 m under the water surface. Mussel byssus threads should be cut from the substrate, since pulling the animals from the rocks (threading) can result in damage to internal tissues. Using mussels living at the water/air interface, the physical contamination by lipophilic contaminants present on the water surface may alter the evaluation of the chemical's content in mussel soft tissues.

19. Detailed guidelines for bivalves' collection for analysis and samples processing are presented in the recommended methods developed by UNEP/FAO/IOC/IAEA (1987) (Annex I) and UNEP/FAO/IOC/IAEA (1988) (Annex II). Also, similar guidelines are published by OSPAR (2008) (Annex VII) and HELCOM (2012a) (Annex III.).

20. In places were no wild bivalves populations are found, caged bivalves can be used as an alternative option for monitoring (UNEP, 2019a). Adult mussels (4-5 cm) are collected from a mussel farm, transported to the marine area under investigation and re-immersed for 10 days to permit them to re-cluster and reduce mortality risk during transplantation at the sampling site. Then cages with mussels are transported to the sampling site, where cages are suspended at 6m to 8m from the sea surface, anchored at the bottom with a 30 kg ballast, and exposed for 12 weeks. During recovery of cages, the biometric parameters shell height and wet weight (w.w.) of soft tissues are measured at least in 15 mussels per each cage. Details on the protocol for using caged bivalves in monitoring heavy

metals and organic contaminants in the marine environment are presented in Galgani et al. (2011)[5] and Galgani et al (2014)[6].

21. The undamaged bivalves are transported to the laboratory moist and alive in appropriate closed containers to avoid contamination (i.e. plastic containers for organisms to be analysed for heavy metals and metals containers for organisms to be analysed for chlorinated hydrocarbons and PAHs), at temperatures between 5 °C and 15 °C (24 hours is the maximum transport time in these conditions). Bivalves should be kept moist using clean seawater from the sampling site without submerging them. For a transportation time of more than 24 hours, bivalves should be placed in appropriate container and frozen. Frozen, samples can be stored in a deep freezer at temperatures of -20ºC. Each sample should be labelled with the sample's identification number, the type of tissue, and the date and location of sampling.

2.3 Protocol for the dissection of fish to collect muscle and liver

i) Dissection

22. Muscle and liver tissues of fish have to be dissected while they are in good condition, otherwise the decay of the tissues will affect the concentration of contaminants. Therefore, it is preferable to dissect collected fish on board, by experienced personnel able to perform the dissection and remove the fish tissues to be analysed (muscle and liver). The on-board dissection should be done in a clean area free from possible contamination of the sample by metals or organic contaminants respectively. If no on-board dissection capability is available (because of lack of experienced personnel and/or lack of adequate clean dissection area), collected fish should be transferred to the laboratory taking care to prevent tissue decay. If the laboratory is reachable within 24 hours, fish could be preserved on ice during the transfer. For longer periods, fish should be frozen immediately and transferred frozen to the laboratory, where they will be thawed before dissection.

23. According to the UNEP (2019a) Monitoring protocols for Common Indicators related to pollution, the fish tissues to be collected are muscle and liver. Detailed guidelines for the dissection of fish and collection of samples for further analysis is presented in the UNEP/FAO/IOC/IAEA Reference Method No 6 (1987) (Annex I.) and UNEP/FAO/IOC/IAEA Reference Method No 7 (1988) (Annex II).

24. HELCOM (2012a) proposes a similar procedure for fish dissection and removal of muscle for further analysis. (Annex III) The method requires the removal of the epidermis and the collection of a sample from the right side dorso-lateral muscle in order to ensure uniformity of samples (Figure 1). It is also suggested to take the entire right dorsal lateral filet as a uniform sample, from which subsamples can be taken after homogenizing for replicate dry weight and contaminant determinations. If the amount of material obtained by this procedure is too large to be easily handled, a specific portion of the dorsal musculature should be chosen for the sample. It is recommended that the portion of the muscle lying directly under the first dorsal fin should be utilized in this case. It is important to obtain the same portion of the muscle tissue for each sample, because both fat and water content vary significantly in the muscle tissue from the anterior to the caudal muscle of the fish.

25. In case fish samples are frozen for their transfer from the field to the laboratory, they have to rest until thawed. It is often suggested that the dissection of fish is easiest when the material, at least the surface layers of the muscle tissue, is half frozen. However, for the dissection of other organs, the thawing must proceed further. Extreme care has to be demonstrated during dissection because any loss of liquid or fat due to improper cutting or handling of the tissue makes the determinations of dry

[5] Galgani, F., Martínez-Gómez, C., Giovanardi, F., Romanelli, G., Caixach, J., Cento, A., Scarpato, A., BenBrahim, S., Messaoudi, S., Deudero, S., Boulahdid, M., Benedicto, J., Andral, B. (2011). Assessment of polycyclic aromatic hydrocarbon concentrations in mussels (Mytilus galloprovincialis) from the western basin of the Mediterranean Sea. Environ. Monit. Assess. 172 (1–4), 301–317. https://doi.org/10.1007/s10661-010-1335-5.

[6] Galgani, F., Chiffoleau, J.F., Barrah, M., Drebika, U., Tomasino, C., Andral, B. (2014). Assessment of heavy metal and organic contaminants levels along the Libyan coast using transplanted mussels (Mytilus galloprovincialis). Environ. Sci. Pollut. Res. 21, 11331–11339. https://doi.org/10.1007/s11356-014-3079-1.

weight and fat content less accurate, which is also affecting the accuracy of the reported contaminants' concentrations.

26. In all cases fish dissections should be undertaken by trained personnel.

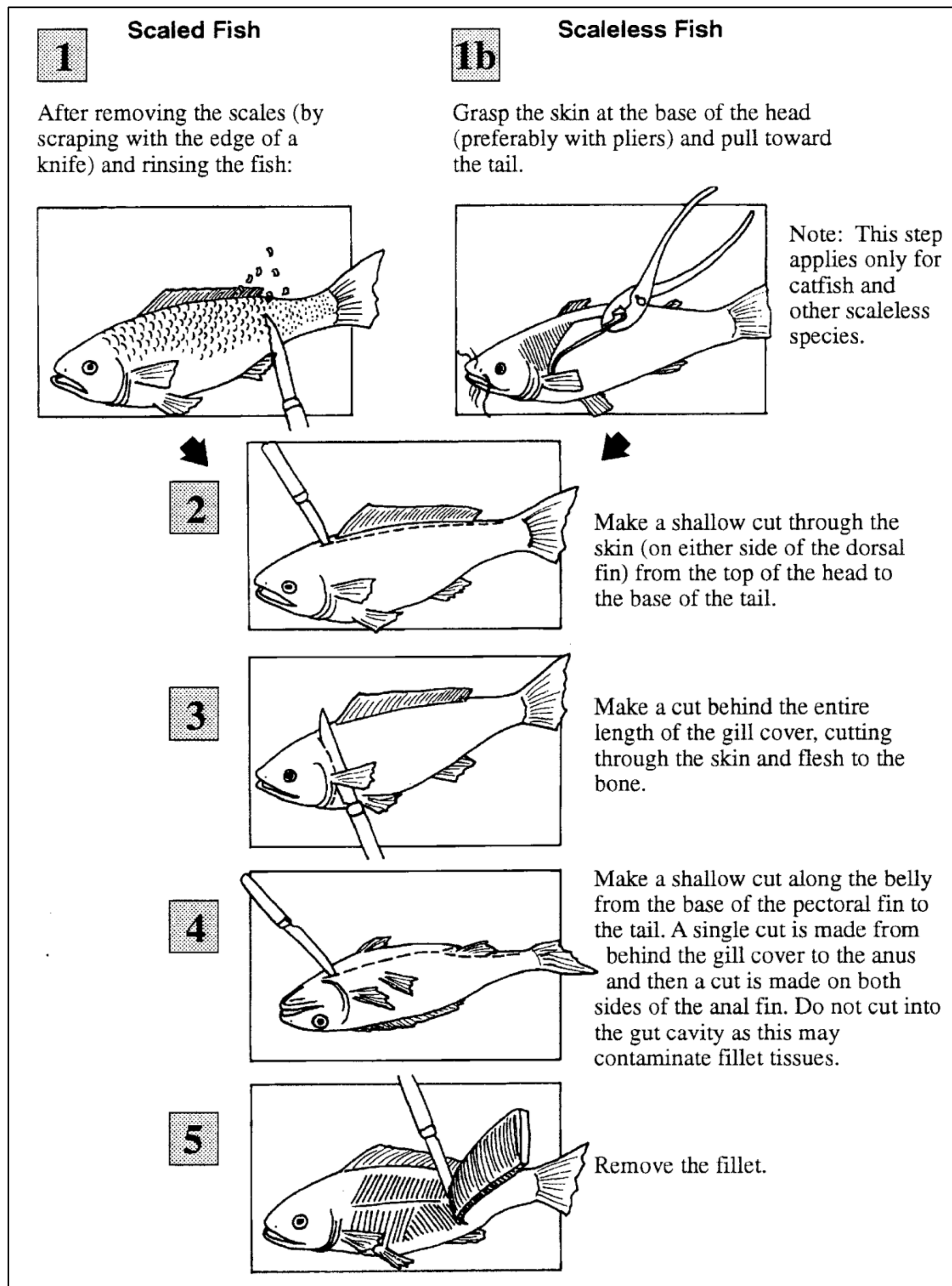


Figure 1. Fish filleting procedure (from US EPA, 2000[7])

27. In case liver tissue is sampled for analysis, HELCOM guidelines underline that "the liver must be identified in the presence of other organs such as the digestive system or gonads. After opening the

[7]US EPA (2000). Guidance for Assessing Chemical Contaminant Data for Use in Fish Advisories Volume 1 Fish Sampling and Analysis. Third Edition.

body cavity with a scalpel, the connective tissue around the liver should be cut away and as much as possible of the liver is cut out in a single piece together with the gall bladder. The bile duct is then carefully clamped and the gall bladder dissected away from the liver."

b) Avoiding contamination

28. For metal analysis, handling of fish should be made on a metal-free bench, using plastic knives and tweezers for holding tissues during dissection. After each sample has been prepared, all tools and equipment (such as homogenizers) should be cleaned.

29. For organic contaminants analysis, handling of fish should be made on a metallic (stainless steel or aluminium) bench, using stainless steel knives and tweezers for holding tissues during dissection. After each sample has been prepared, all tools and equipment (such as homogenizers) should be cleaned.

30. After the removal of a tissue sample from a fish, the tools have to be cleaned before being used to remove another organ (i.e. liver) of the same individual or being used on a different individual.

31. HELCOM (2012a) recommends the following procedures for cleaning tools used for preparing samples:

For analysis of heavy metals, tools should be:

i) Washed in acetone or alcohol and high purity water.

ii) Washed in HNO_3 diluted (1+1) with high purity water. Tweezers and haemostates should be washed in diluted (1+6) acid.

iii) Rinsed with high purity water.

For analysis of organochlorine pesticides

i) Washed in acetone or alcohol and rinse in high purity water.

32. The glass plate used during dissection should be cleaned in the same manner. The tools must be stored in a dust-free area when not in use. Also, the dissection room should be kept clean and the air should be free from particles. If clean benches are not available on board the ship, the dissection of fish should be carried out in the land-based laboratory under conditions of maximum protection against contamination.

2.4 Protocol for the dissection of bivalves

a) Depuration

33. Collected bivalves should be left to void the gut contents and any associated contaminants before freezing or sample preparation, because gut contents may contain significant quantities of contaminants associated with food and sediment particles which are not truly assimilated into the tissues of the mussels (HELCOM, 2012a). Bivalve's depuration over a period of 24 hours is usually sufficient and should be undertaken under controlled conditions and in filtered sea water in the laboratory. The aquarium should be aerated, and the temperature and salinity of the water should be similar to that from which the animals were removed.

b) Bivalve dissection

34. According to the UNEP (2019a) UNEP/MED WG.463/6. Monitoring Protocols for IMAP Common Indicators related to pollution, the whole soft tissue of bivalves has to be collected for analysis. Detailed guidelines for the dissection of fish and collection of samples for further analysis is presented in the UNEP/FAO/IOC/IAEA Reference Method No 6 (1987) (Annex I) and UNEP/FAO/IOC/IAEA Reference Method No 7 (1988) (Annex II). Guidelines for sampling and processing of bivalves is also prepared by HELCOM (2012a) (Annex III)

35. In general, foreign materials attached to the outer surface of the shell have to be removed using a clean plastic/stainless steel knife and a strong plastic/metal brush. Handle the mussels as little as possible. Rinse each mussel with clean seawater and let the water drain off. Then pull out the byssus

which extrudes from between the closed shells on the concave side of the shells; weigh the whole mussel and note the weight.

36. For removing the soft tissue for analysis, bivalves should be shucked live and opened with minimal tissue damage. Insert a clean plastic/stainless steel knife into the opening from which the byssus extrudes and cut the adductor muscles. Avoid forcing the mussel to open, if the abductor muscle is cut, the bivalve will open easily (Figure 2). Rinse the soft part of the mussel in its shells with clean seawater. The soft tissues should be removed and homogenized as soon as possible, frozen and kept in plastic containers (for metal analysis) or in metal containers at -20°C until analysis. Homogenization can be done using stainless steel blades (for organic contaminants analysis) or using an agate mortar, following the drying of the sample.

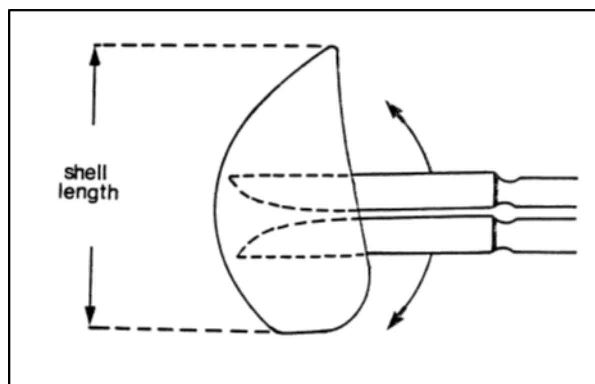


Figure 2. Cutting the abductor muscle

37. For metal analysis, the handling of bivalves should be made on a metal-free bench, using plastic knives and tweezers for holding tissues during dissection. After each sample has been prepared, all tools and equipment (such as homogenizers) should be cleaned with a tissue and rinsed with clean water.

38. For organic contaminants analysis, the handling of bivalves should be made on a metallic (stainless steel or aluminium) bench, using stainless steel knives and tweezers for holding tissues during dissection. After each sample has been prepared, all tools and equipment (such as homogenizers) should be cleaned with tissue and rinsed with solvent

39. In all cases bivalve dissection should be undertaken by trained personnel.

3 Technical note for the sample preservation of marine biota for the analysis of heavy metals and organic contaminants

40. Once the biota samples arrive at the laboratory, additional processing is required to dry and homogenize the samples and to store the dried samples in appropriate conditions. During the processing of the samples it is important to avoid any cross contamination (metal or organic contaminants) from the equipment and the containers used to store the dried samples. Analysis may be performed at a later stage, it is therefore important to avoid any alteration of the contaminants' concentrations in the samples during storage.

41. Under the Technical Note, this Guidelines for Sampling and Sample Preservation of Marine Biota for IMAP Common Indicator 17 provides the following Protocols:

- Protocol for the treatment of biota samples prior to analysis of heavy metals;
- Protocol for the treatment of biota samples prior to analysis of organic contaminants.

3.1 Protocol for the treatment of biota samples prior to analysis of heavy metals

a) Storage of wet samples on board

42. Upon collection wet samples have to be stored on board in such a way as to preserve them from deterioration that will affect the subsequent analysis of contaminants. When the fish transport to the laboratory is done in less than 24 hours, samples can be stored on ice. However, for longer periods, fish samples have to be frozen (-20 ºC) and transported frozen to the laboratory for further processing. Each sample should be labelled with the sample's identification number, the type of tissue (if already dissected), and the date and location of sampling.

b) Drying of biota tissues

43. Drying biota tissues is a procedure to establish the dry/wet weight (dw/ww) ratio of the tissues, in order to express metal concentrations accordingly enabling comparisons between different data sets. Dried biota tissues can then be digested for heavy metal analysis, although biota tissues can also be digested wet, without prior drying (HELCOM, 2012b[8]).

44. Frozen biota samples are placed in clean wide-mouth glass or plastic containers suitable for freeze-drying and are freeze-dried for 24 hours taking care to protect them from cross-contamination from particles and vapours. A possible way to protect samples from contamination is to cover the sample containers with a filter paper perforated with a small hole (HELCOM, 2012b). Then the containers with the samples are weighted and freeze-dried again for another 24 hours and weighted. If the difference between the 2 weighing is less than 0.5%, drying is completed and the dw/ww ratio can be calculated. Otherwise the drying cycle can be repeated (24 hours) until the difference between successive weighing is less than 0.5%.

45. Freeze dried biota tissues are then grinded and homogenized using a metal-free ball mill.

46. Guidelines for processing biota samples for metal analysis is also provided by HELCOM (2012b) (Annex V).

c) Storage of dried biota tissues

47. Freeze-dried tissue samples can be stored in pre-cleaned wide-mouth bottles with a screw cap. Samples intended for the analysis of metals can be stored in plastic or glass containers. For mercury analysis, samples must be stored in acid-washed borosilicate glass or quartz containers, as mercury can move through the walls of plastic containers (EC, 2010[9]).

48. Containers with biota tissue samples should be archived and kept in storage after the completion of the analysis, in order to be used as a replicate sample in case crosschecking of the results are required or additional determinations are needed in the future. Freeze-dried biota tissues remaining after analyses could be stored in the original sample bottle, closed with an airtight lid to protect against moisture and stored in a cool, dark place. Under these conditions, samples may be archived and stored for 10-15 years. (EC, 2010).

3.2 Protocol for the treatment of biota samples prior to analysis of organic contaminants

a) Storage of wet samples on board

49. Upon collection wet samples have to be stored on board in such a way as to preserve them from deterioration that will affect the subsequent analysis of contaminants. When the fish transport to the laboratory is done in less than 24 hours, samples can be stored on ice. However, for longer periods, fish samples have to be frozen (-20 ºC) and transported frozen to the laboratory for further processing. Each sample should be labelled with the sample's identification number, the type of tissue, and the date and location of sampling.

b) Drying of biota tissues

50. Drying biota tissues is a procedure to establish the wet/dry ratio of the tissues, in order to express organic contaminants concentrations, accordingly, enabling comparisons between different

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[8] HELCOM (2012b). COMBINE - Guideline for the determination of heavy metals in biota. Annex B-12, Appendix 4: Technical note on the determination of trace metallic elements in biota.

[9] EC (2010). Guidance Document No: 25 Guidance on chemical monitoring of sediment and biota under the Water Framework Directive

data sets. Dried biota tissues can then be digested for further analysis, although wet biota tissues can also be used, without prior drying, for organic contaminants extraction prior to analysis. UNEP/MAP Recommended methods for biota processing and analysis propose analytical schemes using alternatively wet or freeze-dried biota tissues for the analysis of chlorinated hydrocarbons (IAEA, 2011)[10] (Annex IV) and PAHs (IAEA, 2013[11] Annex VI.).

51. Frozen biota samples are placed in clean wide-mouth glass containers suitable for freeze-drying and are freeze-dried for 24 hours taking care to protect them from cross-contamination from particles and vapors. A possible way to protect samples from contamination is to cover the sample containers with a filter paper perforated with a small hole (HELCOM, 2012c[12]). Then the containers with the samples are weighted and freeze-dried again for another 24 hours and weighted. If the difference between the 2 weighing is less than 0.5%, drying is completed and the dw/ww ratio can be calculated. Otherwise the drying cycle can be repeated (24 hours) until the difference between successive weighing is less than 0.5%.

52. Freeze dried biota tissues are then grinded and homogenized using a plastic-free ball mill.

c) Storage of dried biota tissues

53. Freeze-dried tissue samples can be stored in pre-cleaned wide-mouth bottles with a screw cap. Samples intended for the analysis of organic contaminants should be stored in glass containers.

54. Containers with biota tissue samples should be archived and kept in storage after the completion of the analysis, in order to be used as a replicate sample in case crosschecking of the results are required or additional determinations are needed in the future. Freeze-dried biota tissues remaining after analyses could be stored in the original sample bottle, closed with an airtight lid to protect against moisture and stored in a cool, dark place. Under these conditions, samples may be archived and stored for 10-15 years. (EC, 2010).

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[10] IAEA (2011). Sample work-up for the analysis of selected chlorinated hydrocarbons in the marine environment. Reference Methods for Marine Pollution Studies No 71.
[11] IAEA (2013). Recommended methods for the determination of petroleum hydrocarbons in biological samples.
[12] HELCOM (2012c). Manual for marine monitoring in the COMBINE programme. Annex B-12, Appendix 3. Technical note on the determination of chlorinated biphenyls and organochlorine pesticides in biota.

Annex I

UNEP/FAO/IOC/IAEA (1987). Reference methods No 6 (Rev. 1): Guidelines for monitoring chemical contaminants in marine organisms (2.1.1)

UNITED NATIONS ENVIRONMENT PROGRAMME SEPTEMBER 1992

Guidelines for monitoring chemical contaminants in the sea using marine organisms

Reference Methods For Marine Pollution Studies No. 6

Prepared in co-operation with

FAO IOC IAEA

UNEP 1992

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PREFACE

The Regional Seas Programme was initiated by UNEP in 1974. Since then the Governing Council of UNEP has repeatedly endorsed a regional approach to the control of marine pollution and the management of marine and coastal resources and has requested the development of regional action plans. The Regional Seas Programme at present includes ten regions and has over 120 coastal States participating in it (1),(2).

One of the basic components of the action plans sponsored by UNEP in the framework of the Regional Seas Programme is the assessment of the state of the marine environment and of its resources, and of the sources and trends of the pollution, and the impact of pollution on human health, marine ecosystems and amenities. In order to assist those participating in this activity and to ensure that the data obtained through this assessment can be compared on a world-wide basis and thus contribute to the Global Environment Monitoring System (GEMS) of UNEP, a set of Reference Methods and Guidelines for marine pollution studies are being developed as part of a programme of comprehensive technical support which includes the provision of expert advice, reference methods and materials, training and data quality assurance (3). The Methods are recommended to be adopted by Governments participating in the Regional Seas Programme.

The methods and guidelines are prepared in co-operation with the relevant specialized bodies of the United Nations system as well as other organizations and are tested by a number of experts competent in the field relevant to the methods described.

In the description of the methods and guidelines the style used by the International Organization for Standardization (ISO) is followed as closely as possible.

The methods and guidelines, as published in UNEP's series of Reference Methods for Marine Pollution Studies, are not considered as final. They are planned to be periodically revised taking into account the development of our understanding of the problems, of analytical instrumentation and the actual need of the users. In order to facilitate these revisions the users are invited to convey their comments and suggestions to:

Marine Environmental Studies Laboratory
IAEA Marine Environment Laboratory
19, Avenue des Castellans
MC 98000 MONACO

which is responsible for the technical co-ordination of the development, testing and intercalibration of Reference Methods.

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(1) UNEP: Achievements and planned development of the UNEP's Regional Seas Programme and comparable programmes sponsored by other bodies. UNEP Regional Seas Reports and Studies No. 1 UNEP, 1982.

(2) P. HULM: A Strategy for the Seas. The Regional Seas Programme: Past and Future, UNEP 1983.

(3) UNEP/IAEA/IOC: Reference Methods and Materials: A Programme of comprehensive support for regional and global marine pollution assessments. UNEP 1990.

The present document was prepared at the initiative of FAO, the Food and Agriculture Organization of the United Nations as part of its contribution to the Regional Seas Programme and in particular the Mediterranean Action Plan. The assistance of Dr. G. Topping with this work is particularly appreciated. The document was subsequently edited at IAEA's Marine Environmental Laboratory and reviewed by GEMSI, the IOC/UNEP Group of Experts on Methods, Standards and Intercalibration. The assistance of all those who participated in this work is gratefully acknowledge.

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1. SCOPE AND FIELD OF APPLICATION

This publication provides guidlines for monitoring chemical contaminants in the sea using measurements in marine organisms. It describes strategies for applying such measurements to the protection of public health, the assessment of the geographical distribution of contaminants and the evaluation of time trends in contamination which in turn can demonstrate the effectiveness of measures designed to control potential sources of pollution.

2. REFERENCES

The following are useful publications to consult in relation to the design, planning and conduct of marine pollution monitoring programmes using marine organisms:

BRYAN, G.W., LANGSTONE, W.J. and HUMMERSTONE, L.G. (1980). The use of biological indicators of heavy metal contamination in estuaries. Marine Biological Association of the United Kingdom, Occasional publication Number 1, June 1980, 73 p.

BURNS, K.A. and SMITH, J.L. (1981). Biological monitoring of ambient water quality: the case for using sentinel organisms for monitoring petroleum pollution in coastal waters. Estuar. Coastal Shelf Sci., 13: 433-443.

DAVIES, I.M. and PIRIE, J.M. (1980). Evaluation of a "Mussel Watch" project for heavy metals in Scottish Coastal waters. Mar.Biol., 57: 87-93.

GOLDBERG, E.D., BOWEN, V.T., FARRINGTON, J.W., HARVEY, G., MARTIN, J.H., PARKER, P.L., RISEBOROUGH, R.W., ROBERTSON, W., SCHNEIDER, E. and GANBLE, E. (1978). The "Mussel Watch". Environ.Conserv., 5: 101-125.

GORDON, M., KNAUER, G.A. and MARTIN, J.H.. (1980). Mytilus californianus as a bio-indicator of trace metal pollution: variability and statistical considerations. Mar.Pollut.Bull., 11: 195-198.

PHILLIPS, D.J.H., (1980). Quantitative aquatic biological indicators: Their use to monitor trace metal and organochlorine pollution. Pollution Monitoring Series, London, Applied Science Publishers Ltd, 488 p.

PHILLIPS, D.J.H. and SEGAR, D.A. (1986). Use of bio-indicators in monitoring conservative contaminants: Programme design imperatives. Mar.Pollut.Bull., 17(1): 10-17.

SEGAR, D.A. and STAMMAN, E. (1986). Fundamentals of marine pollution monitoring programme design. Mar.Pollut.Bull., 17(5): 194-200.

TOPPING, G. (1983). Guidelines for the use of biological material in the first order pollution assessment and trend monitoring. Dept. of Agriculture and Fisheries for Scotland, Marine Laboratory, Scottish Fisheries Research Report No 28. ISSN 0308 8022, 28 p.

3. INTRODUCTION

Marine organisms can accumulate contaminants from seawater, suspended particulate matter, sediments and their food. It has also been demonstrated, through field observations and experimental studies, that the concentration of some contaminants in tissues are related to the concentrations in the surrounding environment. This process, termed bio-accumulation, has been used by scientists to assess the marine contamination which has been caused by man's activities (eg. marine disposal of wastes by pipeline discharges and dumping from ships).

There are however certain difficulties in using bio-accumulators, or bio-indicators as they are sometimes known, for this purpose. For example, individuals of the same species exposed to the same concentration of contaminants for the same period of time will not accumulate the substances at the same rate. This is related to such factors as age, sex, size and physiological state of the individual. Similarly, different species do not bio-accumulate to the same level when they are exposed to the same concentration of contaminant in sea water, and often have different rates of contaminant elimination.

Therefore, careful consideration must be given to the above factors when a monitoring programme is designed in order to reduce (or allow for) the effects of natural variability.

This document provides guidance on the design of such programmes and is intended for scientists who are responsible for marine pollution monitoring programmes. It is particularly aimed at programmes which fall under the auspices of the UNEP, IOC and FAO.

The guidelines presented in this report cover the following aspects of marine pollution monitoring programmes:

- aims
- pilot studies
- criteria for the selection of contaminants, organisms and locations to be studied
- size of sample
- frequency of sampling operations
- tissue selection.

Although an important component of these programmes is the analysis of contaminants in samples, this matter will not be addressed in detail in this document since other UNEP Reference Methods For Marine Pollution Studies cover this topic. Readers of this document are therefore advised to have the relevant analytical documents to hand (see UNEP/IOC/IAEA 1990); particularly "Contaminant monitoring programmes using marine organisms: Quality Assurance and Good Laboratory Practice" Reference Method No 57, since this deals with all aspects of work which influence the quality of data.

4. DEFINITIONS

Before discussing the programmes for which these guidelines may be used, it is necessary to define some of the more important terms which are used in this report.

Term	Definition
Accuracy, precision limit of detection	See definitions in Appendix 2 of Reference Method No 57.
Anthropogenic	Derived from human activity
Contamination	in the context of the marine environment this term describes a situation where either the concentrations of some natural substances (eg. metals) are clearly above normal values, or the concentrations of man-made substances (eg. DDT) is detectable but which do not necessarily cause deleterious effects (referred to as pollution, see definition below).
Bio-indicator	A species which accumulates a contaminant in its tissue in amounts that are proportional to the levels of the contaminant in the local environment (ie. water, sediment and food).
Hot spot	An area of the sea where there is a significantly high level of contamination
Pollution	The Group of Experts on Scientific Aspects of Marine Pollution defines pollution as "the introduction by man, directly or indirectly, of substances or energy into the marine environment (including estuaries) which results in such deleterious effects as harm to living resources, hazards to human health, hindrance to marine activities including fishing, impairment of quality for use of seawater and reduction of amenities."
Monitoring	A programme of repeated measurements of contaminants in marine samples which is carried out for a specific purpose eg. annual measurements of mercury in the edible tissue of fish to provide information on the potential annual intake of mercury by consumers. A study of mercury in fish which examines levels in different species would not be classed as monitoring. If, however, this study was repeated in subsequent years these sets of data would be classed as `monitoring data'.

Pilot study Measurements of contaminants in marine samples, in an area not previously studied, to investigate the current levels of contamination. This work is a prerequisite to a monitoring programme since the information collected in this study enables the investigator to design the sampling programme suited to the specific aims of the monitoring work. Without such information the investigator may be unable to judge which contaminants, organisms and locations to select for the monitoring programme.

Quality Assurance All procedures that are carried out by a laboratory to ensure that it produces data of the appropriate quality to meet the defined aims of its monitoring programme. Quality Assurance essentially consists of two elements - quality control and quality assessment. Definitions of these latter terms are given in UNEP Reference Method No 57.

5. AIMS OF MONITORING PROGRAMMES

There are three principal aims of monitoring programmes which involve the collection and analysis of marine organisms; they are:

- to compare contaminant levels in the edible tissues of marine organisms against national limits and to provide data to calculate the potential amount of contaminant taken in by consumers (ie Public Health monitoring).

- to compare the levels of contamination in different geographical areas (Spatial Monitoring). Such measurements are often made to assess whether the current discharges of wastes are producing unacceptable levels of contamination ie they are causing, or likely to cause, marine pollution problems.

- to measure the levels of contaminants over time at particular locations to judge whether they are changing in relation to the inputs of contaminants (ie Trend Monitoring). Such measurements are made to assess the efficiency of measures taken to reduce pollution.

Investigators should write down the specific aims of each monitoring programme before commencing any field measurements. These aims are needed to narrow the list of parameters, species and sites to be investigated). There are two distinct aspects of aims:

Environmental management - Are standards complied with? What is the spatial extent of contamination? What are the changes of levels with time in relation to changes in inputs of contaminants?

Environmental science - Statistical significance of differences in levels of contaminants - representative sampling of the population - selection of analytical methods with the required accuracy and precision.

6. PILOT STUDY

This assists the investigator in the design of an efficient monitoring programme for each specific aim. Provided a pilot study is carefully planned (see Appendix 1 for guidance), it can provide the following information:

a) In relation to public health studies, it can identify the relevant edible species, particularly the ones which contain elevated levels of regulated contaminants and therefore merit further investigation to determine the need for additional regulatory action, such as input controls or restriction on the harvesting or consumption of fish/shellfish.

b) It can identify which areas of the marine environment are sufficiently contaminated to warrant monitoring.

c) It can provide an indication of the variability of contaminant levels in individuals of the same species from the same population and location. This information is essential to an investigator wishing to establish a programme of trend monitoring. Without it, he may not be able to judge whether his sampling and analytical work will be sufficiently detailed to detect changes in contaminant levels with time against the natural fluctuations that may exist in any population of organisms.

d) It can identify which tissues of organisms, particularly fish and large shellfish, are the most appropriate ones to use in specific monitoring programmes since not all tissues reflect changes in the levels of contaminants in the environment to which the organism is exposed.

e) It can identify, and sometimes quantify, inputs of contaminants to the study area. This will help the investigator to select which contaminants should be given priority, if the resources for monitoring are limited, and in which areas contaminated organisms are likely to be found.

A pilot study can easily be expanded in order to accommodate measurements of biological effects. These effects may include changes in community structure and populations or adverse changes in the biochemistry of organisms (for example, acetyl cholinesterase depression by organophosphorus pesticides). Linkage of "levels" with "effects" is an important step in a complete pollution assessment. When effects are noted on a pilot scale, associated with specific contaminants or groups of contaminants, a strong case can be made for incorporating such contaminants in a full-scale monitoring programme and for taking immediate measures for their control and abatement. Details of some biological effects measurements are included in the Reference Method Series (see UNEP/IOC/IAEA, 1990).

Once a pilot study has been successfully completed, and the results evaluated, the investigator should prepare a protocol for each specific monitoring programme for the collection and analysis of samples. This protocol will specify what information is required to meet the specific aims, and the criteria to obtain the required quantity and quality of data. Time spent on the planning of a statistically significant sampling and analytical programme, will inevitably produce a more efficient programme which makes the best use of the laboratory's most important resource (ie staff time). Initially, it is generally sensible to conduct a programme which satisfies essential, rather than very ambitious, aims. It is relatively easy to expand this basic programme if extra resources become available. Finally, it is necessary to review the monitoring programme on a regular basis, to assess how well the aims are being met. This review may result in a reduction of effort on sampling and analyses, and the time gained can be usefully employed on other aspects of marine pollution studies. However, it might identify the need to put in more effort.

7. DESIGNING A MONITORING PROGRAMME

There are a number of factors to be considered in the planning of a monitoring programme which is to meet specific aims:

a) Which contaminants should be measured?

b) Which organism(s) should be selected?

c) Where should the samples be collected?

d) When should the sampling be done and how frequently should it be carried out?

e) How many individual organisms should be collected on each sampling occasion and which size(s) should be included in each sample?

f) Which tissue(s) of the organism(s) should be selected for analysis?

It is the principal investigator, together with a knowledgeable statistician and biologist, who will have to do this evaluation, design and plan the sampling work, prepare the necessary instruction sheets for the field staff, discuss with the analysts the precautions to be taken by staff in the storage and processing of samples prior to their analysis.

Specifically, the investigator will have to do the following:

(i) Design a sampling programme for the organisms of interest; selecting sufficient numbers, and sizes, of individuals at each site at appropriate intervals of time to take into account the inherent variability of contaminant levels in the organisms. This work will be done on the basis of the results obtained from the pilot study and any relevant information from other similar studies. Sampling must be designed to provide a statistically sound basis on which to judge changes in contaminant levels. Once this sampling programme has been designed, instruction sheets should be prepared and issued to the field staff.

(ii) Ensure that samples are collected, stored and transported to the laboratory in a way which minimizes losses and gains of contaminants prior to analysis. Guidance on this can be obtained by consulting the relevant documents in the UNEP Reference Methods series. Again it will be necessary to prepare instruction sheets for field and laboratory staff.

(iii) Arrange for the processed samples to be analyzed using methods which have the required accuracy and precision. Experience has shown that close collaboration between the principal investigator and the analysts is essential if this work is to be successful. The investigator and the principal analyst should consult the UNEP Reference Method No 57 which gives guidelines on Quality Assurance, if they are in any doubt about how to achieve and maintain the required quality of analytical data.

(iv) Ensure that there is an adequate system of documentation to allow samples to be traced from the time of collection to the recording of analytical data. The investigator should ensure that all relevant staff are aware of, and comply with, the system of documentation (see Appendix 2 for more details on this matter).

Each of the factors a - f will now be considered in more detail.

8. SELECTION OF CONTAMINANTS

The selection of substances to be monitored will be determined by a) the aims of the monitoring programme, b) the findings of the pilot study (ie which contaminants, present at significant levels above the background values, justify further study), and c) the ability of the analyst to measure these substances with the required accuracy and precision. In practice the last factor will often determine whether a particular contaminant or group of contaminants can be included in the monitoring programme.

It is essential that the principal investigator and the principal analyst agree to the required accuracy, precision and limit of detection for the measurements to ensure that the necessary standards of analysis are achieved eg. it would be inappropriate to consider measurements of specific changes in contaminant levels using an analytical method which had an inadequate level of precision.

If the analytical method used in the pilot study does not meet the required standard for the specific monitoring purposes, the analyst must select another method which meets the required standard. If for any reason this is not possible (eg. there is a statutory requirement to use a particular method) the investigator should abandon the proposed monitoring programme. Any other action will merely result in wasted effort, since the aims will not be met using an inadequate analytical method. However, it must be stressed that the use of an analytical method which, in theory, has the required performance characteristics to meet the aims does not necessarily guarantee success. Other factors have to be taken into account in obtaining the required quality of analytical data. These are discussed in some detail in "Quality Assurance and Good Laboratory Practice in relation to Marine Pollution Monitoring Programmes", UNEP Reference Method No 57. Investigators are strongly advised to obtain a copy of this document for analysts at the outset of the work.

In addition to selecting contaminants to meet the aims of the laboratory's marine pollution programme, it may be appropriate to include other contaminants which meet regional and international needs. This should only be considered if the additional data is useful to the laboratory, or if it is part of the laboratory's commitment to Regional Studies, and does not jeopardize the main aims of the laboratory's monitoring programme. A list of contaminants, identified by some organizations (International Council for the Exploration of the Seas, Oslo and Paris Commission's Joint Monitoring Group) for monitoring work in the North Sea and adjacent waters as well as those recommended (category I and II substances) for the MED POL programme are given, for information, in Appendix 3.

The final selection of contaminants should also be related to knowledge of their likely sources (eg. an extensive monitoring programme for pesticides along a desert coastline would be unwarranted) and information from scientific literature on their transport and persistence in the environment. Such information will also help to identify which environmental compartment should most usefully be monitored. As an example, organophosphorus pesticides are rapidly metabolized by many marine organisms but are rather persistent in sediments. It would be pointless to monitor them in biota but highly relevant to monitor their biological effects.

9. SELECTION OF ORGANISMS

9.1 Spatial and trend monitoring

Experience has shown that the most reliable data on contaminant trends in organisms are obtained by sampling organisms which have the following characteristics:

- A simple relationship exists between contaminant residues in the organisms and the average concentrations in the surrounding seawater or sediments.
- The organism accumulates the contaminant without being affected by the levels encountered.
- The organism is sedentary and thus representative of the area of collection.
- The organism is widespread in the study region, to allow comparisons between different areas.
- The organism is sufficiently long-lived, to allow sampling of more than one year class if desired.
- The organism is of a reasonable size, to give adequate tissue for analysis.
- The organism is easy to sample and robust enough to survive in the laboratory, allowing (if desired) depuration before analysis and, if needed, studies of uptake of contaminants.
- The organism exhibits high concentration factors, to allow direct analysis without pre-concentration.
- The organism is tolerant of brackish water, to allow comparisons to be made between estuarine and offshore sites.

These characteristics restrict the useful organisms to a range of fairly large, abundant, widespread, inter-tidal organisms, mainly molluscs. Filter-feeding molluscs are more likely to reflect contaminants in the water column, whilst deposit feeders will also be influenced by sediment chemistry. The working of the sediments both by organisms and water currents will cause an averaging of short-term variations in contaminant loading. Water chemistry, however, will more rapidly respond to effluent discharges and dispersal conditions at the time of sampling. Filter-feeders are therefore more likely to provide the information required to fulfill the objectives of a monitoring programme concerned with water quality. In Appendix 4, lists are given of organisms which some scientists in the United Kingdom have suggested may be used for monitoring a range of metals and organochlorine compounds in either rocky or muddy inter-tidal areas in UK waters.

In practice the selection of an organism, for monitoring purposes, is determined by its availability in the study area and its known ability to act as a bio-indicator. If this latter information is not known it must be obtained from either the scientific literature (eg. Phillips 1980), or the pilot study. Final selection should be made in consultation with a knowledgeable biologist. Common mussels, (Mytilus edulis, M. californianus and M. galloprovincialis), that are used in global mussel watch programmes are generally suitable for spatial and trend monitoring programmes in coastal waters.

Other species of shellfish, and fish, can be used for spatial and trend monitoring purposes provided the organism can be shown to accumulate the specific contaminant(s) and that the concentrations of the contaminant(s) are in proportion to the concentrations in either water or sediment or food.

9.2 Public Health programmes

If the pilot study has revealed that edible species from the local fishery contain levels of contaminants which approach or exceed statutory limits for contaminants in foodstuffs, then these organisms should be included in any subsequent public health monitoring programme.

Since permissible limits of some contaminants (eg. Cd) in foodstuffs are extremely low, the analytical method for this work must be capable of producing the required data quality. A high degree of accuracy, and a detection limit which is ca 1/10 of the permissible concentration of the contaminant in the foodstuff, are essential for this work. These criteria enable the analyst to have confidence in the results that are provided to managers for regulatory purposes.

10. LOCATION OF SAMPLING SITES

10.1 Spatial and trend monitoring

Hot spots are usually found in estuarine and coastal areas where anthropogenic wastes are discharged. The offshore areas where hot spots are most likely to occur are those used for the dumping of wastes from ships or those in the vicinity of offshore oil platforms.

A decision to monitor contaminant levels in `hot spots' should be taken only after careful consideration of the discharges to these areas. If, as a result of the pilot study, the relevant authorities decide to reduce inputs then it would be appropriate to monitor to judge whether the new controls have been effective in reducing levels in organisms. If no action is to be taken on the regulation of discharges then monitoring is only justified if there is a good reason to update the information collected in the pilot study.

Other estuarine, coastal and offshore sampling sites may be included in the programme to provide coverage of both clean and moderately contaminated areas. All sampling should be done by scientific personnel operating from research or chartered vessels, rather than by fishermen, to ensure that contamination of the samples during and after collection is kept within acceptable limits.

For long-term monitoring programmes, the precise locality of sampling sites should be registered as very small spatial variation may strongly influence the final data (ie "mussels from the harbour wall" should specify which point in the harbour wall). In some cases it may be useful to photograph the sites, particularly where intertidal organisms are taken.

10.2 Public Health Programmes

In some countries there may be officials who are knowledgeable about the edible species of fish and shellfish caught by commercial fishermen. Investigators may find it helpful to discuss their proposed monitoring programme with such officials since they can often offer valuable advice in the design of the collection programmes.

Samples of fish and shellfish may be obtained from the fish markets or from fishing vessels or research ships which are operating in traditional fishing areas. The basic requirement is a representative sample of the species normally consumed by the general public. It should be noted, however, that some countries may specify the exact sampling procedures for public health monitoring.

Commercial fishermen do not usually take any special precautions during the collection, storage, transport and off-loading of their catches, other than to ensure that they are presentable enough for sale. The retailer and the consumer do not normally adopt any stringent dissection procedures, other than from a public health viewpoint. The scientist, however, will use careful sampling and pre-treatment procedures to ensure that contamination is kept within acceptable limits. These different approaches to sampling may lead to differences in the amount of contaminants found in the samples. In general the scientific samples will be less contaminated than those taken from fish markets, fishing boats and fish retailers.

The final decision on where and how to collect samples for public health monitoring will depend on whether information is required on actual contaminant intake by the consumer (in which case samples will be taken from the fish markets or fish retailers) or whether the aim is to determine which edible species and areas are exposed to contamination (in which case the sampling must be done by scientific staff).

11. PERIOD AND FREQUENCY OF SAMPLING

11.1 Spatial and trend monitoring

For spatial monitoring, collections should be made over a short interval of time (within weeks rather than months) to enable a synoptic comparison of concentrations of contaminants at different sites. This also helps to ensure that organisms are in the same physiological state. If major annual changes in the quantity and/or composition of inputs are anticipated it would be appropriate to conduct an annual or biennial sampling. Experience has shown that the effects of changes in inputs of contaminants are often confined to the area in the immediate vicinity of the discharge. It is these areas where more frequent monitoring should be conducted.

For trend monitoring, the frequency of sampling will a) reflect the time scales over which the changes are required to be detected, b) the degree of confidence required in the measurement of these changes, and c) the available laboratory resources. Investigators should note that there is nothing more frustrating and time-wasting than a programme in which the proposed work is well below the minimum standard required to detect the desired changes in contaminant levels. If, for any reason, the resources are insufficient to meet the specific aims of the programme, then the programme should be canceled and replaced with one which has less ambitious aims but which can be carried out successfully with available resources.

If no changes in inputs are expected, then it would be sensible to restrict sampling to ca 5 yearly intervals. A more frequent sampling programme can only be justified if there is a need to provide more regular data for other purposes eg. to reassure the general public that levels of contaminants are not changing.

Seasonal variations in food supply, and the spawning cycle, are known to cause changes in total body weight, as well as lipid concentration and composition and, these may influence contaminant levels in the tissues of some organisms. In order to minimize these variations, it is suggested that sampling be undertaken at the pre-spawning period.

11.2 Public Health monitoring

Unless there is a seasonal fishing pattern for some species, samples may be taken at any time of the year. Ideally all species should be sampled at the same time so that a synoptic picture of the contaminant levels can be obtained. A typical monitoring programme might consist of a survey every 5 years. A more frequent sampling programme (ie annual) will be needed if the results of the pilot programme show that concentrations of contaminants in foodstuffs approach or exceed permissible limits for foodstuffs. Increased sampling should be confined to the particular species and contaminants which give cause for concern.

12. SIZE OF SAMPLE

12.1 Spatial and trend monitoring

Ideally, the investigator will have established the relationship between contaminant levels and size of organisms from the results of the pilot study. It is good practice to select a particular size or size range to minimize the variance of contaminant levels from sample to sample. The number of individuals required for each sample will be determined by the magnitude of the change that is considered to be significant in relation to the specific aims. The smaller the difference the greater the number of individuals required for each sample. (See Appendix 5 for further guidance)

If the relationship between size of organism and contaminant level has not been obtained from the pilot study then a sufficient number of individuals should be collected at one of the sampling sites to cover the size range of organisms in the population, to establish the variability of contaminant levels with size. This is a minimum requirement since, ideally, this sampling procedure should be done at all sites. The information on variability at one site will allow the investigator to make comparisons with other sites where individuals of a limited size range are collected.

If either analytical resources or sample material is limited it may not be practical for the individuals from each site to be analyzed separately. In this case, individuals should be combined to make one sample (often referred to as `pooled' samples). For `pooled samples', no information will be obtained on the variation of contaminant levels with size but the data can be used to assess site to site differences with some level of confidence, provided that a number of replicate analyses are done on each of the `pooled' samples, and the `pooled' samples consist of individuals from the same size range.

12.2 Public Health monitoring

The size(s) of organisms to be sampled should be based on information on consumption patterns. If a range of sizes is sold, then these different sizes should be analyzed. The number of individual organisms in each sample will be influenced by the importance of the species as a foodstuff, the availability of scientific manpower and the need to sample sufficient numbers of each species and of each size category to cover the range of values encountered in a typical population or catch. Generally, a sample of 5-10 individuals from each size range of fish and large shellfish (crabs, lobsters) and ca 50 individuals for smaller shellfish (eg. mussels, shrimps) would be sufficient.

13. SELECTION OF TISSUE

13.1 Spatial and trend monitoring

For invertebrates, whole soft tissue (less viscera) should be taken for analysis.

For fish, muscle is the most useful tissue for most purposes. However, liver and kidney tissues have been used for studies of fish and the digestive gland of large crustaceans. In general, whole soft tissue is taken for smaller shellfish.

13.2 Public Health monitoring

Only edible tissue need be analyzed for contaminants - usually this means muscle tissue for fish and large crustaceans and whole soft tissue (less viscera, ie guts, gills and gonad) for small shellfish.

Every opportunity should be taken to collect data on the size (or length) and age of the species. This may be relevant to subsequent decisions on regulatory action.

13.3 Normalization procedures

It is usual to report all tissue data on a dry weight basis (ie. g contaminant/g (dry weight)). However, some literature values use wet weight which may be required for public health studies. Since drying is a common part of most analytical protocols (see RM. No. 7 "Sampling of selected marine organisms and sample preparation for trace metal analysis" and RM. No. 12 "Sampling of selected marine organisms and sample preparation for the analysis of chlorinated hydrocarbons"), the reader is advised to record wet/dry weight ratios on a routine basis.

In the case of lipophilic contaminants, such as chlorinated hydrocarbons, contaminant concentrations are often expressed in terms of g contaminant/g HEOM (where HEOM is Hexane Extractable Organic Matter, principally lipid). This procedure enables a certain degree of normalization for seasonal or spatial variations in the lipid content of sentinel organisms and facilitates the comparability of data.

Appendix 1

GUIDANCE ON THE PLANNING OF A PILOT STUDY

Desk Study

It is important to determine what is known about contaminants in the proposed study area, before any field work is done. Some of this information can be found by reviewing the relevant scientific journals and other published material (eg. books, conference proceedings).

Annual reports of other marine institutes, local and central government and industrial research laboratories are also useful sources of data, as are unpublished scientific reports from these organizations. If these latter sources provide useful data, it is good practice to contact scientists from the relevant organizations to identify whether there is any other unpublished data or information, which might be useful to the investigator.

This review can often provide data on the current levels of contamination in water, sediments or biota and occasionally information on inputs of contaminants to the area via rivers, pipelines or dumping from ships. It may also reveal the type of industry and agriculture located in the coastal region, the range and scale of potentially toxic substances used by them, and possibly information on their discharges to the rivers and sea. These latter data should be verified by contacting the local or national authority, which has responsibility for regulating discharges to rivers and coastal waters. This authority should also be approached for information on the past and present discharges to the area.

For public health work, the investigator should identify which fish and shellfish species are caught for human consumption, and whether there are relevant permissible limits for contaminants in marine foodstuffs. Information on commercial catches can be obtained from either the local fishermen or their representative organizations or the local or central government fisheries department. Information on food standards can be obtained from the local environmental health department or the central government department responsible for food safety. It is difficult to be more specific about the exact sources of the above information in each country since they do vary from country to country.

This review should enable the principal investigator to identify the group of contaminants, and specific fish and shellfish, which should be given priority in the pilot study for public health purposes. It will also give some general guidance on the species to be selected for spatial and trend monitoring purposes. However, before the principal investigator can plan this latter work he needs to do some additional desk work to identify the locations where samples should be collected.

Identifying sampling sites

It is essential that the pilot study covers the areas which are likely to be contaminated and the areas which, from a hydrographic and input viewpoint, are unlikely to be significantly affected (ie sites located well offshore from industrialized areas or those located in inshore areas next to less populated and industrialized areas).

The level and extent of contamination in coastal and estuarine waters is determined by:

- the rate of input of contaminants
- the location of the individual inputs
- the composition of the waste - whether the contaminants are in solution, attached to solids or associated with mixtures of solid and liquid
- the dilution and dispersion of wastes following discharge, and in the case of discharges containing solids, the settlement of solid material to the sea bed sediments
- the physical and chemical processes in the sea (ie adsorption and desorption of substances between dissolved and particulate phases of seawater).

Unless the principal investigator has a good working knowledge of hydrography of the local area, it will be necessary to seek the help of an hydrographic expert to determine the optimum locations for sampling in relation to known inputs.

Assuming the principal investigator can provide the hydrographer with the relevant information on inputs, and that his colleague has a good understanding of the hydrographic characteristics of the area (direction, speed and variability of currents, salinity and temperature of the water masses, and the freshwater flows to the sea) it should be possible to calculate the theoretical dilution and dispersion of wastes at estuarine and coastal sites. This information can then be used to identify the locations where organisms are exposed to contamination and the adjacent areas where they will probably not be subject to contamination (ie clean or control areas).

If expert hydrographic advice is not available, the principal investigator should establish a sampling grid along the likely gradient of contamination; with sampling sites located at progressively increasing distances from the input (100m, 300m, 1000m, 3000m etc.). If a river is the principal source of contamination to the study area, the investigator can establish his sampling grid along the salinity gradient. It is relatively easy to calculate the dilution of river water, and the corresponding dilution of contaminants, by measuring the salinity at locations in an estuary and comparing these measurements with the salinity values of the water entering the estuary. For this calculation, the investigator assumes that river water has zero salinity and that the contaminants behave conservatively during mixing of freshwater and seawater.

Sample size

The concentration of some contaminants can vary with size of the organisms. It is important in spatial and trend monitoring to reduce this source of variability in the data to detect differences in contaminant levels between sites and with time (see Appendix 5). If this relationship is not known by the investigator prior to the commencement of monitoring, it will be necessary to establish it during the pilot study.

To do this, the investigator must collect a representative sample of each population of species at each sampling site. This sample should include sufficient numbers of individuals to cover the range of sizes/ages/lengths of individuals in each population. The investigator should consult a knowledgeable biologist for guidance on the range of sizes that might be expected for each species.

Selection of tissue

Although there is considerable scientific literature on the accumulation of contaminants by different tissues (eg. Phillips, 1980), it is advisable for the investigator to check this aspect for the specific organisms to be examined in the pilot study. It is also advisable to consult a biologist to determine the best procedure for dissection of organisms into their constituent parts, to ensure that there is no possibility of one tissue being contaminated by another.

Ideally, the investigator should investigate the relationship between the contaminant level, tissue and size of organism by analyzing tissue from individuals of different sizes rather than by analyzing pooled samples; even if the latter consist of a number of individuals of the same size or size range. However, if analytical resources are limited, it may be necessary for him to establish this relationship by analyzing pooled samples.

Appendix 2

DOCUMENTATION OF DATA

The adoption of the following guidelines by a laboratory should provide adequate documentation to allow it to trace samples from the collection stage to the completion of its analyses by providing a record of the appropriate data in logbooks or in computer files.

Documentation

(i) Descriptions of the sampling strategy, methods of sample collection, procedures for storage, and pre-treatment and analytical procedures, plus a list of ancillary site observations;

(ii) Sample documentation (description of organisms, numbers of individuals collected for each sample, weights of tissue taken for analysis (individual tissue or homogenate) plus ancillary data on organisms (length, weight and age);

(iii) Description of analytical procedures, including details of accuracy, precision and limit of detection;

(iv) Description of quality control and quality assessment and evidence that these procedures have been applied and have provided acceptable data;

(v) Description of working standards used on each occasion and calculations of results;

(vi) A secure system for the long term storage of data either in logbooks or computer files is essential. It is also advisable to have a duplicate set of records in case one is lost, mislaid or accidentally destroyed;

Advice should be sought on the correct method of storing computer tapes and/or discs to ensure the long-term stability of data files.

Storage of data

It has been shown that even the most experienced personnel can make simple arithmetic errors in calculating results. Thus, a check should be made for such errors before compiling tables of results. Once this check has been MADE it is appropriate to carry out a preliminary assessment of the quality of the data, prior to its evaluation and publication, to ensure that no erroneous results are included. This assessment can include a comparison of the results with existing data (ie data for the study area either previously collected by the laboratory or data published in the literature). Before consigning data to long term storage, a final check should be made to ensure that no errors have been made in transcribing the data (ie the re-typing of data sets by typists or data processors can sometimes lead to such errors).

Appendix 3

EXAMPLES OF CHEMICAL SUBSTANCES MEASURED IN MARINE ORGANISMS FOR MONITORING PURPOSES (SOURCE:

Trace metals

Arsenic (As), Cadmium (Cd), Chromium (Cr), Copper (Cu), Lead (Pb), Mercury (Hg), Nickel (Ni), Tin (Sn), and Zinc (Zn).

DDT and its metabolites

o,p'-DDD, p,p'-DDD, o,p'-DDE, o,p'-DDT, and p,p'-DDT.

Chlorinated pesticides other than DDT

Aldrin, Alpha-Chlordane, Trans-Nonachlor, Dieldrin, Heptachlor, Heptachlor epoxide, Hexachlorobenzene, Lindane (gamma-BHC), and Mirex (+ Endosulfan ?)

Polychlorinated biphenyls (PCBs)

Measurements are usually restricted to either a small number of individual compounds (known as congeners) or to the total concentration of PCBs.

Polyaromatic hydrocarbons

These can include:

2-ring compounds	Naphthalene, 1-Methylnaphthalene, 2-Methylnaphthalene, 2,6-Dimethylnaphthalene, and Acenaphthene.
3-ring compounds	Fluorene, Phenanthrene, 1-Methylphenanthrene and Anthracene.
4-ring compounds	Fluoranthrene, Pyrene, and Benz(a)anthracene
5-ring compounds	Chrysene, Benzo(a)pyrene, Benzo(e)pyrene, and Dibenz(a,h)anthracene.

For the purposes of the Long-term programme for pollution monitoring and research in the Mediterranean sea (MED POL - Phase II) the following chemical contaminants were identified for analysis in marine organisms.

category I (mandatory)	category II (optional)
total mercury	total arsenic
organic mercury	radionuclides
cadmium	polynuclear aromatic hydrocarbons
halogenated hydrocarbons	

Appendix 4

A. LIST OF MED-POL SPECIES

For the purposes of the Long-term programme for pollution monitoring and research in the Mediterranean sea (MED POL - Phase II) the following species (nearly all edible), representing different ecotypes, are recommended for the monitoring of chemical contaminants in marine organisms.

a) Bivalves

Mytilus galloprovincialis, or
Mytilus edulis, or
b) Demersal fish
Perna perna, or
Donax trunculus

M. edulis, P. perna or D. trunculus can only be monitored as alternative species if Mytilus galloprovincialis does not occur in the area.

Mullus barbatus, or
Mullus surmuletus, or
Upeneus molluccensis

M. surmuletus or U. molluccensis can only be monitored as alternative species if Mullus barbatus does not occur in the area.

c) Pelagic carnivore fish

Thunnus thynnus, or
Thunnus alalunga, or
Xiphias gladius

d) Pelagic plankton feeding fish

Sardina pilchardus
Other clupeids should only be monitored as alternative species if S. pilchardus does not occur in the area.

e) Crustaceans

Parapenaeus longirostris, or
Nephrops norvegicus, or
Penaeus kerathurus

N. norvegicus or P. kerathurus can only be monitored as alternative species if P. longirostris does not occur in the area.

B. LIST OF POSSIBLE ORGANISMS FOR THE ASSESSMENT OF CONTAMINATION IN THE NORTH ATLANTIC REGION

	Cd	Hg	Cu	Cr	Pb	Zn	HH	PHC
Rocky substrate								
Mytilus edulis (common mussel)	+		?	+	+	+	+	+
Littorina littorea (gastropod)	+		+	?	+	+		
Patella vulgata (limpet, gastropod)	+		+		+	+		
Muddy substrate								
Scrobicularia plana (da Costa) (peppery furrow bivalve)	+	+	?	+	+	+		
Macoma balthica (bivalve)	+	+	?	+	+	+		
Nereis diversicolor (annelid)	+	+	+	+	+	+		

Key: + = appears to act as good indicator
? = doubt about use as indicator
HH = halogenated hydrocarbons
PHC = petroleum hydrocarbons

NOTES: The organisms listed for muddy substrates are all deposit feeders, whilst those for rocky substrates are filter feeders or herbivores. It is unlikely that contaminant levels in the tissues of the two groups will reflect contaminat levels in the same part of the marine environment.

Annex II:

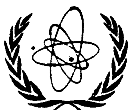
UNEP/FAO/IOC/IAEA (1988). Reference methods No 7 (Rev. 2): Sampling of selected marine organisms and sample preparation for trace metal analysis (2.1.2)

UNITED NATIONS ENVIRONMENT PROGRAMME 12 November 1984

Sampling of selected marine organisms and sample preparation for trace metal analysis

Reference Methods for Marine Pollution Studies No. 7 Rev. 2

Prepared in co-operation with



FAO IAEA IOC

UNEP 1984

Note: This document has been prepared in co-operation between the Food and Agriculture Organization of the United Nations (FAO), the International Atomic Energy Agency (IAEA), the Intergovernmental Oceanographic Commission (IOC) of UNESCO and the United Nations Environment Programme (UNEP) under projects FP/ME/0503-75-07, ME/5102-81-01, FP/5102-77-03 and FP/5101-84-01.

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PREFACE

The Regional Seas Programme was initiated by UNEP in 1974. Since then the Governing Council of UNEP has repeatedly endorsed a regional approach to the control of marine pollution and the management of marine and coastal resources and has requested the development of regional action plans. The Regional Seas Programme at present includes ten regions and has over 120 coastal States participating in it. 1/ 2/

One of the basic components of the action plans sponsored by UNEP in the framework of Regional Seas Programme is the assessment of the state of marine environment and of its resources, of the sources and trends of the pollution, and the impact of pollution on human health, marine ecosystems and amenities. In order to assist those participating in this activity and to ensure that the data obtained through this assessment can be compared on a world-wide basis and thus contribute to the Global Environment Monitoring System (GEMS) of UNEP, a set of reference methods and guidelines for marine pollution studies are being developed and are recommended to be adopted by Governments participating in the Regional Seas Programme.

The methods and guidelines are prepared in co-operation with the relevant specialized bodies of the United Nations system as well as other organizations and are tested by a number of experts competent in the field relevant to the methods described.

In the description of the methods and guidelines the style used by the International Organization for Standardization (ISO) is followed as closely as possible.

The methods and guidelines, as published in UNEP's series of Reference Methods for Marine Pollution Studies, are not considered as final. They are planned to be periodically revised taking into account the development of our understanding of the problems, of analytical instrumentation and the actual need of the users. In order to facilitate these revisions the users are invited to convey their comments and suggestions to:

International Laboratory of Marine
Radioactivity
International Atomic Energy Agency
c/o Musée Océanographique
MC98000 MONACO

which is responsible for the technical co-ordination of the development, testing and intercalibration of reference methods.

1/ UNEP: Achievements and planned development of UNEP's Regional Seas Programme and comparable programmes sponsored by other bodies. UNEP Regional Seas Reports and Studies No. 1 UNEP, 1982.

2/ P. HULM: A Strategy for the Seas. The Regional Seas Programme: Past and Future UNEP, 1983.

This issue (Rev.2) of the Reference Method for Marine Pollution Studies No. 7 was prepared in co-operation with the Food and Agriculture Organization of the United Nations (FAO), the International Atomic Energy Agency (IAEA) and the Intergovernmental Oceanographic Commission (IOC) of UNESCO. It includes comments received from IOC's GIPME Group of Experts on Methods, Standards and Intercalibration (GEMSI), from the FAO/UNEP/IAEA Experts Consultation Meeting on Reference Methods for the Determination of Chemical Contaminants in Marine Organisms (Rome, 4-8 June 1984) and from a number of scientists who reviewed and tested the method. The assistance of all those who contributed to the preparation of Revision 2 of this reference method is gratefully acknowledged.

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1. SCOPE AND FIELD OF APPLICATION

This publication describes the sampling and sample preparation procedures suitable to obtain uncontaminated samples of mussels (total soft tissue), shrimps (muscles), and fish (muscles) for trace metal analysis by atomic absorption spectrophotometry.

2. REFERENCES

BERNHARD, M. (1976) Manual of methods in aquatic environment research. Part 3 Sampling and analyses of biological material. FAO Fish.Tech.Pap. No. 158 (FIR1/T158), pp. 124. FAO, Rome.

UNEP/FAO/IAEA (in preparation). Guidelines for monitoring chemical contaminants in marine organisms. Reference methods for marine pollution studies No. 6. UNEP, Geneva.

3. PRINCIPLES

Specimens of organisms selected and collected according to UNEP/FAO/IAEA (in preparation) are enclosed in plastic containers and transported to the analytical laboratory either as cooled (-2 to 4°C) or as deep-frozen (-18°C) samples There the specimens are dissected under "clean conditions" and subsamples are prepared for the analyses of trace metals.

4. REAGENTS

4.1 Demineralized distilled water or glass distilled water of equivalent quality, with a trace metal content below detection limits when checked with this reference method.

4.2 Uncontaminated "open-ocean" subsurface (1 m below the surface) sea water.

4.3 Detergent recommended for laboratory use.

5. APPARATUS

5.1 Plastic thermo-insulated boxes (camping equipment) cooled with commercially available cooling bags. For storage and transport of mussels the boxes must be equipped with a grid in the bottom in order to avoid the mussels being submerged when moistened during transport and storage.

5.2 Refrigerator (required for 6.2, 6.3, 6.4).

5.3 Deep-freezer (-18°C).

5.4 Heavy duty, high-density polythylene bags or suitable plastic containers for storage of specimens.

5.5 Plastic length-measuring board, length-measuring scale (ruler) or transparent Pyrex dish (cooking utensil) with centimetre scale attached underneath (for small and medium-size specimens).

5.6 Two or more plastic knives made out of high-density and purity polyethylene or similar material. Alternatively, quartz knives can be used.

5.7 Pyrex dishes or porcelain dishes (cooking utensils) as working surface for sample preparation.

5.8 Two or more pairs of plastic, commercially available or "home-made", tweezers (see Appendix A).

5.9 High density and purity polyethylene bags and airtight plastic containers with screw caps, for preservation of samples in deep-freezer, cleaned with detergent (4.3) and rinsed with distilled water (4.1) or uncontaminated sea water (4.2).

5.10 High-density polyethylene sheets for covering working bench.

5.11 Smaller polyethylene sheets to be used as "weighing plastic".

5.12 Balance (100-200 g) with a precision of 0.001 g or better, for weighing specimens and subsamples; preferably a "top-loading" balance.

5.13 Plastic wash bottle containing glass-distilled water (4.1).

5.14 Scraper (figure 1), a strong rust-free knife or similar for collecting mussels.

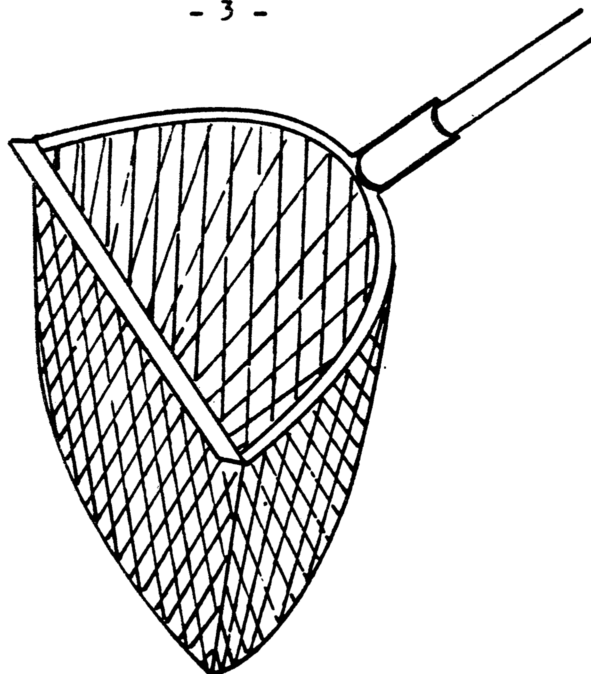

FIGURE 1 : SCRAPER FOR COLLECTING MUSSELS

5.15 Plastic tank or bottle (20 - 50 l) for the sea water (4.2) needed to moisten live mussel samples during storage and transport.

5.16 Large rust-free metal knife for cutting portions from large fishes.

5.17 Stainless steel blender or other tissue homogenizer made from glass and/or teflon. Stainless steel equipment should be tested for trace metal contamination by homogenizing reference (standard) material and comparing the analytical result obtained with same material which was not homogenized with stainless steel equipment.

5.18 Strong plastic brush for removing foreign material attached to the surface of mussels.

6. SAMPLING AND TRANSPORT

6.1 Presampling preparations

Clean the thermo-insulated boxes (5.1), the high density polyethylene bags or containers (5.4), the length-measuring board (5.5), the large rust-free knife (5.16), the scraper or the knife (5.14) with detergent (4.3) and rinse them with distilled water or, alternatively, with clean open-ocean sea water (4.2).

6.2 Sampling of mussels

Remove mussels from their attachments with the clean scraper or the rust-free knife (5.14).

Transfer a suitable number (UNEP/FAO/IAEA (in preparation)) of undamaged mussels into clean thermo-insulated boxes with grid on the bottom (5.1). Collect, from the sampling site, a clean sea water sample in a suitable container (5.15) to keep the mussels moist if a long transport (more than 2 hours in hot climates) is envisaged. Keep the mussels moist with the clean sea water without submerging them.

If the mussels have to be transported and stored before sample preparation (7) for more than 24 hours place a suitable number of mussels in plastic bag (5.9). Squeeze out the air and close the bag airtight with a knot, thermoseal, or similar. Place the bag into another bag (5.4) together with a sample identification note (see Appendix B), close airtight the second bag and deep-freeze.

This represents the "specimen sample".

NOTE: The transport of mussels collected near the laboratory will not present special transport and storage problems. Mussels should be kept exposed to air and moistured with clean sea water during the transport to the laboratory. When gathered from the intertidal zone, they will survive aerial exposure for 24 hours. Mussels submerged in sea water during transport will open their valves, start pumping water and excreting waste products, while during aerial exposure their valves will remain closed and their metabolic rate is greatly reduced; therefore their submersion in sea water during transport should be avoided.

6.3 Sampling of shrimps and small to medium-size fish

Place in a clean plastic bag (5.4) a suitable number of the undamaged specimens (select according to UNEP/FAO/IAEA (in preparation)) collected from a fishing vessel, fish market, etc., taking care that the legs, spines, etc. will not puncture the plastic. Squeeze out the air and close the bag airtight with a knot, thermoseal, or similar. Place the bag into another bag (5.4) together with a sample identification note (see Appendix B), and close the second bag airtight also. Deep-freeze (5.3) the bag whenever possible. Use a refrigerator (5.2) or a cooled thermo-insulated box (5.1) only if the storage period is not too long (48 hours in hot climates).

This represents the "specimen sample".

6.4 Sampling of large-size fish

Determine and note the fork-length, the body weight and sex of the collected specimen.

Separate with a clean rust-free metal knife (5.16) a portion of at least 100 g of muscle tissue. This portion must be at least 5 cm thick so that during sample preparation (7.3) contaminated and dirty tissue can be sliced off. Place each portion into a separate clean bag (5.4), squeeze out the air and close the bag airtight. Place it together with the sample identification note (see Appendix B) into a second bag (5.4) and close it airtight also. Deep-freeze

(5.3) the bag whenever possible, otherwise use a refrigerator (5.2) or a cooled thermo-insulated box (5.1) if the storage period is not too long (48 hours in hot climate).

This represents the "specimen sample".

7. SAMPLE PREPARATION

7.1 Preparatory activities

If necessary, partially thaw deep-frozen samples (6) by placing them overnight in a refrigerator at -2°C to 4°C (partially frozen samples are easier to cut than completely thawed or even fresh samples).

Clean the knives (5.6), the dishes (5.7), the tweezers (5.8), the length-measuring board (5.5) and "weighing plastics" (5.11) with detergent (4.3), rinse with distilled water (4.1) or clean sea water (4.2). Cover the working area with pre-cleaned plastic sheets (5.10). Clean hands carefully with detergent (4.3) and rinse them with distilled water (4.1) or clean sea water (4.2).

NOTE: If hands are cleaned and precautions are taken not to touch the dissected part with hands, bare hands are preferred to hands covered with gloves, since the operator has a much better control of instruments, etc. If possible a clean room should be used for preparatory activities.

7.2 Sample preparation of mussels

Scrape off all foreign materials attached to the outer surface of the shell with a clean plastic knife (knife no. 1) (5.6), to be used only for this purpose or with a strong palstic brush (5.18). Handle the mussels as little as possible.

Rinse each mussel with distilled water (4.1) or alternatively with clean sea water (4.2) and let the water drain off.

Pull out the byssus which extrudes from between the closed shells on the concave side of the shells.

Weigh (5.12) the whole mussel and note the weight.

Insert a second clean plastic knife (knife no. 2) (5.6) into the opening from which the byssus extrudes and cut the adductor muscles by turning the knife as indicated in figure 2 and open the mussel. Do not try to break the mussel open with the knife; if the muscles are cut, the mussel will open easily. Check if the byssus has been eliminated completely; if not, remove the remainder with clean tweezers (5.8).

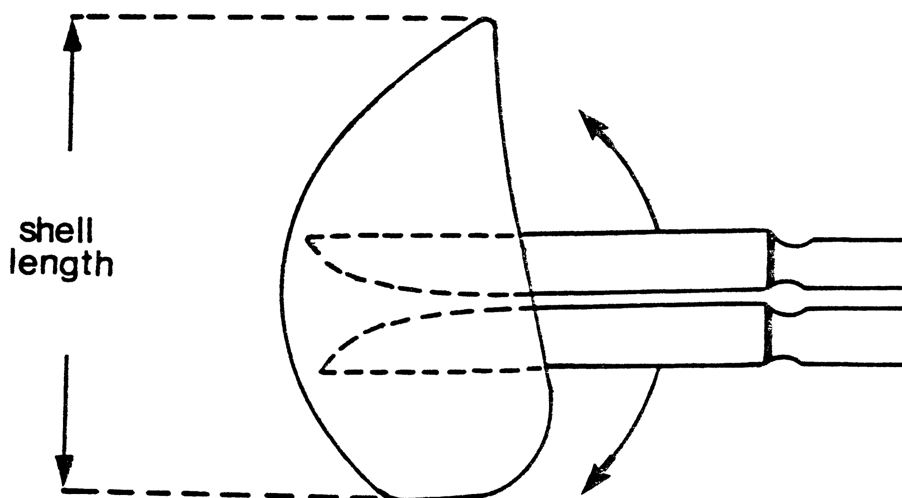

FIGURE 2 : CUTTING THE ADDUCTOR MUSCLE

Rinse the soft part of the mussel in its shells with distilled water (4.1) or clean sea water (4.2).

Loosen all tissue with the second clean knife (knife no. 2) (5.6), remove the soft tissue from the shell with a pair of clean plastic tweezers (5.8) without touching the outer part of the shells, and let all the water drain off.

(a) Single specimen sample: Weigh a clean empty container (5.9) on the balance and note the weight. Then put the soft part of the mussel in it and reweigh. Note the fresh weight of the soft part. Close the container airtight, label it with the sample preparation code. Determine the length of the mussel's shell (figure 2) by placing it with the inner part facing the cm scale (5.13). Note the length of the shell and the weight of the soft part of the mussel.

(b) Composite sample: Fill a container (5.9) of known weight with at least 10 soft parts of mussels prepared as described above. Reweigh the plastic container and note the composite fresh weight of the mussels. Homogenize the specimens in a cleaned blender (5.17), and return the homogenate in the plastic container. Note the total weight again and recalculate the fresh weight of the homogenate. Lable the plastic container with the sample code.

NOTE: When preparing composite samples, use mussels of similar size. The length and weight of each specimen should be determined separately before the soft parts are pooled.

Place several plastic containers in a clean plastic bag (5.4), include an identification note with the containers sample codes, seal the bag airtight and deep-freeze.

This represents the "tissue sample".

7.3 Sample preparation of shrimps

(a) Single specimen sample: Determine the length of the shrimp from rostrum to uropod (see figure 3) using the appropriate length measuring device (5.5). Weigh the shrimp after placing a clean "weighing plastic" (5.11) on the balance (5.12) and note its length and fresh weight.

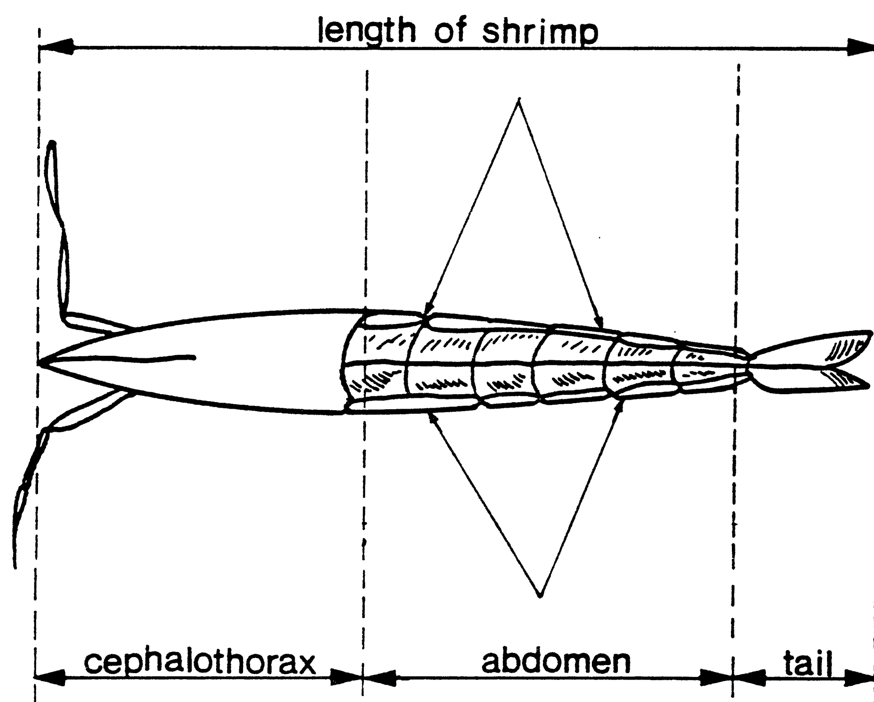

FIGURE 3 : SCHEMATIC DIAGRAM OF A SHRIMP
(arrows indicate where to cut after the legs have been removed)

Separate the abdomen from the cephalothorax and the "tail" (telson and uropod) with a first plastic knife (knife no. 1) taking care that no viscera remain in the abdomen (figure 3). Cut off all legs. Turn the abdomen with the ventral side up and cut with a plastic knife along the edges of the sterinites (ventral exoskeleton); lift the sterinites off with a pair of plastic tweezers and discard.

Loosen with a second clean knife (knife no. 2) the abdomen muscle and lift it from the exoskeleton with a clean pair of tweezers.

Determine and note the sex by examining the gonads.

Transfer the muscle with a clean pair of plastic tweezers (5.8) into a preweighed plastic container (5.9), determine and note the fresh weight of the muscle. Close the container airtight, label it with the sample code, place a suitable number of containers in a plastic bag, add a sample identification note to the containers, and close the bag airtight and deep freeze the samples.

(b) Composite sample: Start sample preparation as described for single specimen sample above taking care to record length, fresh weight, tail muscle weight and the sex of each specimen separately. Reduce the tail muscle(s) of the large specimens to the weight of the smallest tail muscle. A composite sample should not contain less than 6 tail muscles from 6 different specimens of

the same sex and size. Homogenize the tail muscles in a blender (5.17). Transfer the homogenate into a suitable clean container (5.9) which has been weighed empty. Close the container airtight, label it and weigh the container with the homogenate. Note the weight of the homogenate together with the other data in a protocol. Place a suitable number of containers in a plastic bag (5.4), add a sample identification note, close the bag airtight and deep-freeze (5.3) the containers.

This represents the "tissue sample".

NOTE: The concentration of trace metals in a composite sample should represent the mean value of metal concentrations of single specimens. In order to avoid overrepresentation of large specimens, only shrimp of similar size (age) should be used for the preparations of composite samples. In addition, the weight of the tail muscles of all specimens to be included in the composite sample should be reduced to that of the tail muscle of the smallest specimen. As there might be considerable differences in the trace metal content of male and female specimens, use them in separate composite samples.

7.4 Sample preparation for small and medium size fish

(a) Single specimen sample: Determine the fork-length (from tip of snout when the mouth is closed to the apex of the fork of the tail) of fish (figure 4) to the nearest mm on the length-measuring board (5.5). Weigh the fish on a clean "weighing plastic" (5.11) with an accuracy of 0.1% of its total weight and note both the fork-length and the fresh weight of the specimen.

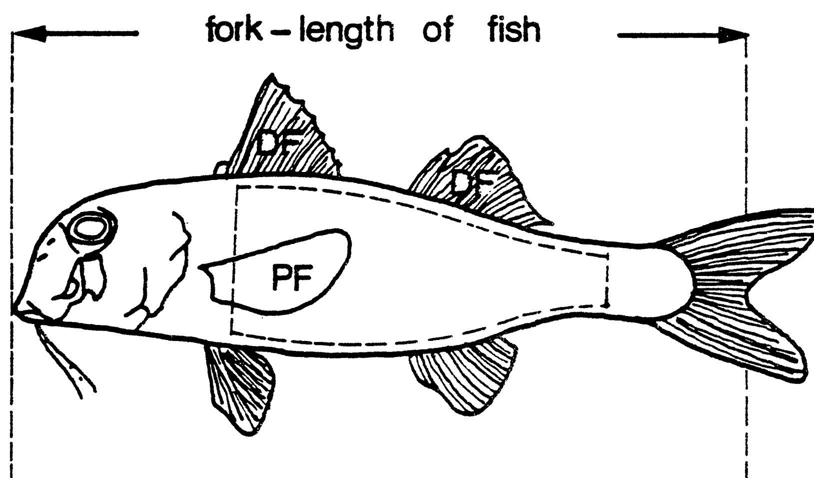

FIGURE 4 : SCHEMATIC DIAGRAM OF A FISH
(PF=pectoral fin, DF=dorsal fins, dashed line shows where the cuts should be made)

Rinse the fish with distilled water (4.1) or clean sea water (4.2) and place it on a clean working surface (5.7). Remove the pectoral fin and cut the skin of the fish with a first knife (5.6) near the dorsal fins, starting from the head to the tail (figure 4).

Cut near the gills across the body, along the ventral edge from the gills to the tail and finally across the body near the tail. These four cuts should be carried out first on one side only taking care not to cut too deep in order to avoid cutting into the viscera and thus contaminating the fillet. It is advisable that a second person hold the fish by the head and tail during this operation.

Pull the skin from the fillet with a pair of tweezers (5.8), taking care that the outer skin does not contaminate the fillet.

With a second clean knife (5.6), cut the fillet from the vertebral column (backbone) starting from the cut near the gills. Lift the fillet with a second clean pair of tweezers (5.8), so that the fillet will not touch the working surface (e.g. the Pyrex dish) or other parts of the fish.

Weigh the fillet in a clean plastic container (5.9) and note its fresh weight.

If one fillet does not yield enough material for analysis, put the fish, skin side upwards, on a clean portion of the working surface (5.3) or on a new working surface and remove the second fillet from the other side of the same fish as described above, add it to the first sample and record their total weight.

Close the container airtight. Identify the container with a code number and/or label, record all data in the protocol and deep-freeze (5.3).

This represents the "tissue sample".

Determine and note the sex of fish by examining the gonads.

NOTE: Comparing the weight of the container holding the fillet sample(s) determined at this point with the weight of the container before the digestion step will show if the tissues have lost moisture during prolonged storage.

(b) Composite sample: Start sample preparation as described for single specimen sample above taking care to record the length, the fresh weight and fillet (sample) weight of each fish separately. Determine and note by examining the gonads the sex of each specimen separately.

Reduce the fillet(s) of the large specimens to the weight of the smallest fillet. A composite sample should not contain less than 6 fillets from 6 different specimens of the same sex and size. Homogenize the fillets in a blender (5.17). Transfer the homogenate into a suitable clean container (5.9) which has been weighed empty. Close the container airtight, label it and weight the container with the homogenate. Note the weight of the homogenate together with the other data in a procotol and deep-freeze (5.3) the container.

This represents the "tissue sample".

NOTE: The concentration of trace metals in a composite sample should represent the mean value of metal concentrations of single specimens. In order to avoid overrepresentation of large specimens, only fish of similar size (age) should be used for the preparation of composite samples. In addition, the weight of the fillets of all specimens to be included in the composite sample should be reduced to that of the fillet of the smallest fish. As there might be considerable differences in the trace metal content of male and female specimens, use them in separate composite samples.

7.5 Sample preparation of large-size fish

If necessary, thaw partially, e.g. overnight in a refrigerator (-2 to 4°C), the subsample taken in the field during sampling (6.4).

Rinse the subsample with distilled water (4.1) or clean sea water (4.2) and place it on a clean working surface (5.7). Remove any skin and bone that may be present. Cut off thin slices from all surfaces with clean plastic knife (5.6) and discard them. Repeat the operation with a second clean knife (5.6) in order to obtain a clean uncontaminated block of homogeneous tissue.

NOTE: It has been recognized that differences in trace metal concentrations may exist between different muscles in large fish, therefore as much information as possible on the actual sample should be recorded.

Transfer the tissue into an airtight container (5.9), close and label it, weigh it, note all data together with data of the subsample in the protocol, and deep-freeze (5.3).

This represents the "tissue sample".

8. SAMPLING AND SAMPLE PREPARATION PROTOCOL

Fill in the sampling and sample preparation protocol (table 1) giving full details in every column. This protocol should be attached to the test report on the determination of trace metals in the analyzed sample.

The following guidelines should be kept in mind when completing the protocol (the numbers refer to those used in table 1):

1.1 Use the scientific name for the species sampled. If necessary indicate subspecies or variety.

1.2 Indicate the name under which the species is known locally.

1.3 Use any code adopted by your institution. Never use the same sample code for more than one sample.

3.2 For samples obtained on fish market, indicate the town (village) where the market is. For samples taken at standard sampling stations or areas, indicate the name (code) of the station or area.

3.3 If the sampling point does not coincide with a standard sampling station or area, it may be advisable to code (name) it, in particular when the sampling point is used more frequently (e.g. a particular fish market). Never use the same sampling point code for more than one sampling point.

3.4 and 3.5 Always indicate the longitude and latitude of the sampling point to the nearest minute. For samples obtained from fish market, enquire about their provenience and try to reflect it also as geographic co-ordinates. Circle either E or W and N or S, as appropriate.

3.6 Give any additional information which may be relevant for the interpretation of the results (e.g. sampling point in vicinity of outfalls or similar).

4.1 Indicate the difference between data given under 2 and 5.

4.2 Mark the storage conditions used. If none of them applicable, give additional explanations in 4.3.

6.2 Identify sex of the specimen whenever possible. As for specimen length, determine shell length for mussels, fork length for fish and total length for shrimp as indicated in figures 2, 3, and 4. Specimen weight always refers to the fresh weight of the whole mussel, of the whole shrimp and of the whole fish. Note that sample weight, in the case of mussels, refers to the total weight of soft tissues. In the case of shrimp, the sample weight refers only to the fresh weight of the muscle, and in the case of fish, to the fresh weight of the fillet or of the combined weight of fillets removed from the same fish.

6.3 Whenever possible use six or more specimens of the same sex and size (age) in preparing composite samples. Mean length and weight refers to the arithmetical mean of the weight and length of individual specimens, as explained above. Always calculate the standard deviations.

Table 1: Sampling and Sample Preparation Protocol

1. Sample (specimen)

1.1 Scientific name:________________________________

1.2 Common name:________________________________

1.3 Sample code:________________________________

2. Date of sampling: day________; month________; year_____

3. Sampling point

3.1 Country:________________________________

3.2 Type of sampling point:__________________fish market;

____________________________sampling area/station

3.3 Sampling point code:________________________________

3.4 Longitude: ______________° ______________' E or W

3.5 Latitude: ______________° ______________' N or S

3.6 Conditions at sampling point which may be relevant for the interpretation of results:

____

____

____

4. Sample storage

4.1 Duration of storage:___________hours; ___________days

4.2 Storage: deep-freezing________; cooling___________

4.3 Factors relevant to sample storage which may be important for the interpretation of results:

____

____

____

5. Date of sample preparation: day_____; month_____; year_____

6. Sample preparation

6.1 Tissue type (kind)____________________

6.2 Single specimen sample: sex____; specimen length______cm;

specimen weight____________g; sample weight____________g

6.3 Composite sample:

- number of specimens ____________________; sex__________
- mean length of specimens________cm; stand. dev.________
- mean weight of specimens________g; stand. dev.________
- total weight of composite sample__________g
- total net weight of homogenate____________g

6.4 Factors relevant to sample preparation which may be important for the interpretation of results:

____

____

____

7. Full address of the institution carrying out the sampling and sample preparation:

____

____

____

____

8. Name(s) and signature(s) of the person(s) who carried out the sample preparation:

____

____

Date:____________________

Appendix A

Preparing plastic tweezers

Methylmetacrylate of 4 mm thickness has been found to be very useful as it has the right elasticity. If thinner or thicker material has to be used, either the strips from which the tweezers are to be made are cut wider or narrower. The easiest way to heat the plastic and bend it is with a hot air blower used for forming plastics. A drying oven can be used also. However, it is much more difficult to make tweezers by heating the plastic in an oven since the plastic twists easily.

Materials:

- sheets of acrylic (methylmetacrylate) resin; 4 mm thick (trade names: e.g. Perspex, Flexiglas, Lucite);
- a plastic tube, about 40 mm in diameter.

Equipment:

- hot air blower (300-350°C) used for molding plastics, or Drying oven (135-140°C).

Procedure:

(a) With a hot air blower

- cut from the sheet with an electric or a hand saw strips of about 10 mm width and 250 mm length;
- heat about a 60 mm long part in the middle of the strip so that it bends easily. Bend it around the plastic tube carefully in order to make both ends meet. Cool the plastic with cold water;
- sharpen the ends with a file and roughen the inside of the tweezers so that they grip well;
- wash the tweezers carefully with detergents and rinse them with distilled water.

(b) With a drying oven

- place the plastic strip on a clean piece of wood in a drying oven (135-140°C) until it becomes soft;
- lift the strip at one end with a pair of tweezers and bend it around the plastic tube without letting the tweezer tips meet;
- cool the tips by dipping them in a beaker of clean cold water and afterwards bend the ends of the tweezer so that the tips meet;
- prepare the ends of the tweezers as described earlier.

Appendix B

Sample indentification note

A standard sample identification note should contain the following data:

- sample code (the same code should be used in 1.3 of the Sampling and Sample Preparation Protocol; see table 1);
- species name (important in particular whenever storage of sample may create difficulties in determining the species);
- sampling date;
- sampling location (given as sampling point code, if possible; see 3.3 of table 1);
- collector's (sampler's) name;

Example:

AN 435
Mytillus galloprovincialis
3 March 1982
F 17
D. Degobbis

Issued and printed by:

Regional Seas Programme Activity Centre
United Nations Environment Programme

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United Nations Environment Programme
Palais des Nations
GENEVA
Switzerland

Annex III:

HELCOM (2012). Annex B-12, Appendix 1. Technical note on biological material sampling and sample handling for the analysis of persistent organic pollutants (PAHs, PCBs and OCPs) and metallic trace elements (2.1.3)

HELCOM Manual for marine monitoring in the COMBINE programme

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

1. GENERAL PRINCIPLES

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

1. The work must be carried out by personnel capable of identifying and removing the desired organs according to the requirements of the investigations; and

2. There must be no risk of contamination from working surfaces or other equipment.

2. TOOLS AND WORKING AREA

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

Colourless polyethylene tweezers are recommended as tools for holding tissues during the dissecton of biological tissue for metallic trace element analysis. Stainless steel tweezers are recommended if biological tissue is dissected for analysis of chlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) and polunuclear aromatic hydrocarbons (PAHs).
After each sample has been prepared, including the samples of different organs from the same individual, the tools should be changed and cleaned.

The following procedures are recommended for cleaning tools used for preparing samples:

1) for analysis of metallic trace elements

• a) Wash in acetone or alcohol and high purity water.
• b) Wash in HNO_3 (p.a.) diluted (1+1) with high purity water. Tweezers and haemostates in diluted (1+6) acid.
• c) Rinse with high purity water.

2) for analysis of CBs and OCPs

• a) Wash in acetone or alcohol and rinse in high purity water.

The glass plate used during dissection should be cleaned in the same manner. The tools must be stored dust-free when not in use.

The dissection room should be kept clean and the air should be free from particles. If clean benches are not available on board the ship, the dissection of fish should be carried out in the land-based laboratory under conditions of maximum protection against contamination.

3. FISH MUSCLE AND LIVER SAMPLES DISSECTION

For fish analysis, commercial catches can be used if fish transport to the laboratory does not take longer than 24 hours. The fish must be transported on ice. The dissection then takes place at the laboratory.

For analysis of **fish muscle**, the epidermis and subcutaneous tissue should be carefully removed from the fish. Samples should be taken under the red muscle layer. In order to ensure uniformity of samples, the **right** side **dorso-lateral muscle** should be taken as the sample. If possible, the entire right dorsal lateral filet should be used as a uniform sample, from which subsamples can be taken after homogenizing for replicate dry weight and contaminant determinations. If, however, the amount of material obtained by this procedure is too large to handle in practice, a specific portion of the dorsal musculature should be chosen for the sample. It is recommended that the portion of the muscle lying directly under the first dorsal fin should be utilised in this case. As both fat and water content vary significantly in the muscle tissue from the anterior to the caudal muscle of the fish (Oehlenschläger, 1994), it is important to obtain the same portion of the muscle tissue for each sample.

To sample **liver tissue**, the liver must be identified in the presence of other organs such as the digestive system or gonads (Harms and Kanisch, 2000). The appearance of the gonads will vary according to the sex of the fish and the season. After opening the body cavity with a scalpel, the connective tissue around the liver should be cut away and as much as possible of the liver is cut out in a single piece together with the gall bladder. The bile duct is then carefully clamped and the gall bladder dissected away from the liver.

When fish samples which have been frozen at sea are brought to the laboratory for analysis, they should be dissected as soon as the tissue has thawed sufficiently. The dissection of fish is easiest when the material, at least the surface layers of the muscle tissue, is half frozen. For dissection of other organs, the thawing must proceed further, but it is an advantage if, for example, the liver is still frozen. It must be noted that any loss of liquid or fat due to improper cutting or handling of the tissue makes the determinations of dry weight and fat content, and consequently the reported concentrations of determinands, less accurate.

After muscle preparations, the liver should be completely and carefully removed while still partly frozen to avoid water and fat loss. Immediately after removing it from the fish, the liver should be returned to the freezer so that it will be completely frozen prior to further handling. This is particularly important for cod liver.

4. SHELLFISH SAMPLING

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

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

For shellfish, the upper limit of shell length should be chosen in such a way that at least 20 mussels in the largest length interval can easily be found. The length stratification should be

determined in such a way that it can be maintained over many years for the purposes of temporal trend monitoring. The length interval shall be at least 5 mm in size. The length range should be split into at least three length intervals (small, medium, and large) which are of equal size after log transformation.

Mussels are collected by a bottom grab and selected onboard. The number of specimens selected for analysis depends on their length, e.g. 80-100 individuals are necessary to suffice material within the length range 4-5 cm.

5. STORAGE OF FISH AND MUSSEL SAMPLES

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

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

Liver tissue can deteriorate rather rapidly at room temperature. Consequently, samples should be frozen as soon as possible after packaging. They can be frozen rapidly by immersion in liquid nitrogen or blast freezing, but both these techniques need care. Whatever system is used, freezing a large bulk of closely packed material must be avoided. The samples in the centre will take longer to cool and will therefore deteriorate more than those in the outer layer.
Once frozen, samples can be stored in a deep freezer at temperatures of -20$_{o}$C or below. Frozen liver tissue should not be stored longer than six months, while lean muscle tissue can be stored up to two years. Each sample should be carefully and permanently labelled. The label should contain at least the sample's identification number, the type of tissue, and the date and location of sampling.

Mussels should be shucked live and opened with minimal tissue damage by detaching the adductor muscles from the interior of at least one valve. The soft tissues should be removed and homogenised as soon as possible, and frozen in glass jars at -20 °C until analysis. Mussel tissue for trace metal determination is homogenised and decomposed in a wet state while for persistent organic pollutants determination it is homogenised and water is removed by freeze-drying. Frozen liver tissue should not be stored longer than six months, while lean muscle tissue can be stored up to two years. Each sample should be carefully and permanently labelled. The label should contain at least the sample's identification number, the type of tissue, and the date and location of sampling.

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- Harms, U.; Kanisch, G. (2000): Intra-Individual Variability of levels of lead detected in the liver of Baltic cod (Gadus morhua). Mar. Poll. Bull., 40 / 8, 710-712
- Oehlenschläger, J. 1994. Quality assurance during sampling onboard. *In* ICES/HELCOM Workshop on Quality Assurance of Chemical Analytical Procedures for the Baltic Monitoring Programme. Ed. by G. Topping and U. Harms. Baltic Sea Environment Proceedings No. 58: 82-84.

Annex IV:

IAEA (2011). Sample work-up for the analysis of selected chlorinated hydrocarbons in the marine environment. Reference Methods for Marine Pollution Studies No 71 (4.2.1)

UNITED NATIONS ENVIRONMENT PROGRAMME **November 2011**

Sample work-up for the analysis of selected chlorinated hydrocarbons in the marine environment

Recommended Methods For Marine Pollution Studies 71

Prepared in co-operation with

NOTE: This recommended method is not intended to be an analytical training manual. Therefore, the method is written with the assumption that it will be used by formally trained analytical chemists. Several stages of this procedure are potentially hazardous; users should be familiar with the necessary safety precautions.

For bibliographic purposes this document may be cited as:

UNEP/IAEA: Sample work-up for the analysis of selected chlorinated hydrocarbons in the marine environment. Reference Methods for Marine Pollution Studies No 71, UNEP, 2011.

PREFACE

The Regional Seas Programme was initiated by UNEP in 1974. Since then, the Governing Council of UNEP has repeatedly endorsed a regional approach to the control of marine pollution and the management of marine and coastal resources and has requested the development of regional action plans. The Regional Seas Programme at present includes thirteen regions and has over 140 coastal States participating in it (1).

One of the basic components of the action plans sponsored by UNEP in the framework of the Regional Seas Programme is the assessment of the state of the marine environment, its resources and the sources and trends of the pollution and its impact on human health, marine ecosystems and amenities. In order to assist those participating in this activity and to ensure that the data obtained through this assessment can be compared on a world-wide basis and thus contribute to the Global Environment Monitoring System (GEMS) of UNEP, a set of Reference Methods and Guidelines for marine pollution studies are being developed as part of a programme of comprehensive technical support which includes the provision of expert advice, reference methods and materials, training and data quality assurance (2). The Methods recommended for adoption by Governments participating in the Regional Seas Programme.

The methods and guidelines are prepared in co-operation with the relevant specialised bodies of the United Nations system as well as other organisations and are tested by a number of experts competent in the field relevant to the methods described.

In the description of the methods and guidelines, the style used by the International Organisation for Standardisation (ISO) has been followed as closely as possible.

The methods and guidelines published in UNEP's series of Reference Methods for Marine Pollution Studies are not considered as definitive. They are planned to be periodically revised taking into account the new developments in analytical instrumentation, our understanding of the problems and the actual need of the users. In order to facilitate these revisions, the users are invited to convey their comments and suggestions to:

Marine Environmental Studies Laboratory
IAEA Environment Laboratories
4, Quai Antoine 1er
MC 98000 MONACO

which is responsible for the technical co-ordination of the development, testing and inter-calibration of Reference Methods.

References:

(1) www.unep.org/regionalseas (2011)

(2) UNEP/IAEA/IOC: Reference Methods and Materials: A Programme of comprehensive support for regional and global marine pollution assessment. UNEP, 1990.

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Explanations sent to all laboratories with sets of standard provided by IAEA-EL/MESL. 36

1. SCOPE AND FIELD OF APPLICATION

This reference method is intended for use in monitoring programmes and pilot research studies. The document describes procedures for the isolation of purified fractions amenable for the determination of DDTs and PCBs in marine sediments and marine organisms by capillary GC/ECD. It is assumed that most of the participants in the UNEP Regional Seas Programmes are equipped with advanced high resolution capillary gas chromatographs and will be able to implement most, if not all, of the procedures described in Reference Method No 40, "Determination of DDTs and PCBs by capillary gas chromatography and electron capture detection" (UNEP 1988). Assuming consistent results are routinely being obtained with these methods by the analytical laboratory, the determination of specific compounds (as opposed to generic mixture of PCBs) opens up the possibility not only of identifying environmental "hot spots", but also for characterising sources, elucidating transport pathways and developing data of greater toxicological relevance. The organisation and content of this document, however, deserves further comment. Under the sections devoted to SEDIMENTS and ORGANISMS, subsections are provided relating to procedures for: 1) Sampling, 2) Extraction and 3) Clean-up and fractionation. In each subsection, several alternative procedures are described. These various procedures have been previously tested and are provided to accommodate the range of capabilities in participating laboratories. For example, laboratories which have access to an HPLC may consider the benefits of using HPLC fractionation procedures in lieu of more conventional low pressure column chromatographic method. Participants are generally encouraged to implement the most effective procedures within the constraints of their individual laboratories.

Several other halogenated pesticides and other electron capturing organic compounds may be present in environmental samples and many of these compounds could also be isolated by the methods described here. However, not all residues will be stable to the clean-up procedures applied for the determination of PCBs and DDTs. Consequently, every analyst must test for analyte recovery and analytical reproducibility prior to applying these methods for other analytes on a routine basis. Primary emphasis should be placed on obtaining the cleanest possible purified fraction for capillary GC/ECD analysis so that interferences and misidentification are minimised, if not eliminated.

2. PRINCIPLES

Following collection of sediment or biota samples using appropriate techniques, samples are stored in trace organic free vessels at -20°C until analysis. For analysis, the samples are prepared for solvent extraction. To achieve a satisfactory recovery of the chlorinated hydrocarbons, samples are dried by either desiccation with anhydrous sodium sulphate or by freeze-drying. Lipids are then Soxhlet extracted from sediments using hexane and dichloromethane, and from biota using hexane or petroleum ether. Following initial clean-up treatments (removal of sulphur from sediment extracts and treatment of biota extracts with concentrated sulphuric acid to destroy some interfering lipids), extracts are fractionated using column chromatography. Detailed protocols for absorption chromatographic fractionation are described for both low and high pressure systems, using Florisil and silica gel respectively. (Additional information concerning alternative techniques including gel permeation chromatography is provided).

3. REAGENTS, SOLVENTS, STANDARDS

3.1. Reagents

3.1.1. List of reagents

- Demineralized distilled water produced by distillation over potassium permanganate (0.1 g/l $KMnO_4$) or equivalent quality, demonstrated to be free from interfering substances.
- Detergent.
- Potassium dichromate.
- HCl. 32%.
- Concentrated H_2SO_4 (d 20°C: 1.84 g/ml).
- Sulfochromic cleaning solution made from concentrated sulphuric acid and potassium

dichromate.
- KOH.
- Anhydrous sodium sulphate.
- Copper fine powder (particle size 63µm).
- Carborundum boiling chips.
- Hg.
- Glass wool
- Alumina (200-240).
- Silica gel (60-100).
- Florisil PR (60-100).
- Bio-Beads SX-3 (200-400).
- Sephadex LX-20.

Solvents:
- Hexane, Dichloromethane, Methanol, Pentane, Cyclohexane, Toluene and Ethyl Acetate, all "distilled in glass" quality.

Standards:
- PCB congeners: 29, 30, 121, 198.
- ε HCH.
- Endosulfan Id_4.
- n-C_{14} d_{30}, n-C_{19} d_{40}, n-C_{32} d_{66}.
- Naphthalene d_8.
- Hexamethylbenzene.
- Cadalene: 1, 6-dimethyl-4-(1-methylethyl)naphthalene.
- DDT reference solutions - Prepare a stock solution of the DDT series (pp' DDT, op DDT, pp' DDD, op DDD, pp' DDE, op DDE) by dissolving 50 mg of each compound in 100 ml of hexane. Store stock solution in sealed glass ampoules.
- Other reference solutions - should be prepared if other residues are to be quantified in these procedures.

NOTES:

Working solutions obtained from the stock reference solutions should be prepared on a regular basis depending on their use and stored in clean glass volumetric flasks tightly capped with non-contaminating materials such as Teflon or glass. Extreme care must be taken to ensure that the concentrations of the standards have not altered due to solvent evaporation.

In order to achieve acceptable accuracy for the standard solutions, at least 50 mg of pure individual compound should be weighed and dissolved into 100 ml of hexane. This will give stock solutions of 500ng/µl.

Example of preparation of stock solutions:

Preparation of a stock solution of pp' DDE at approximately 500ng/µl:

The pp' DDE stock solution is prepared by dissolving approximately (but weighed accurately) 50 mg of pp' DDE in hexane in a 100 ml volumetric flask and bringing the volume to exactly 100 ml with hexane. If the actual weight of pp' DDE is 52 mg, then

$$\frac{52\,\text{mg DDE}}{100\,\text{ml solvent}} \times \frac{1000\,\mu g}{\text{mg}} \times \frac{\text{ml}}{1000\,\mu l} = \frac{52\,\text{mg DDE}}{100\,\text{ml of solution}}$$

52 mg/100 ml ⇒ 0.52 mg/ml⇒ 520 µg/ml ⇒ 520 ng/µl

The concentration of the stock solution will be: 520ng/µl

Preparation of an intermediate solution:

Use the stock solution to prepare the intermediate solution. The concentration of pp' DDE intermediate solution should be approximately 5ng/µl. To prepare the 5ng/µl intermediate solution, transfer 1 ml of the pp' DDE stock solution into a 100 ml volumetric flask and dilute with hexane to 100 ml.

$$\frac{1\,\text{ml DDE stock solution}}{100\,\text{ml final volume}} \times \frac{520\,\text{ng DDE}}{\mu l} = \frac{5.2\,\text{ng}}{\mu l\ \text{intermediate solution}}$$

The concentration of the intermediate solution will be: 5.2 ng/µl

Preparation of the working solution:

Use the intermediate solution to prepare the working solution. The concentration of pp' DDE in the working solution could be approximately 50pg/µl.

To prepare the 50 pg/µl working solution, transfer 1 ml of the pp' DDE intermediate solution into a 100 ml volumetric flask and dilute with hexane to 100 ml.

$$\frac{1\,\text{ml DDE intermediate solution}}{100\,\text{ml final volume}} \times \frac{5.2\,\text{ng}}{\mu l} \times \frac{1000\,\text{pg}}{\text{ng}} = \frac{52\,\text{pg}}{\mu l\ \text{working solution}}$$

The concentration of the working solution will be: 52 pg/µl

3.1.2. Cleaning of solvents

All reagents, including the distilled water should be of analytical quality. Commercially available solvents like acetone, acetonitrile, dichloromethane, hexane and pentane are invariably contaminated with ECD-active substances; their concentrations vary from batch to batch and with supplier. Reagent quality should be checked by injection of 2 µl of a 100 ml batch of solvent, after concentration to 50 µl in a rotary evaporator. No peak in the GC-ECD chromatogram (90 - 250 °C) should be larger than that for 1pg of lindane. Otherwise, the solvent must be distilled. The following procedure has been found to be both efficient and cost effective, as it allows the use of technical grade solvents as the basic material (reducing the cost by one order of magnitude). 130 - 150 cm height columns are required; the packing material must be glass (to allow subsequent cleaning with an oxidising acid). The entire equipment is cleaned prior to use by 2 consecutive distillation procedures with 500 ml water in each case. It is essential that a current of nitrogen gas (15 ml/min) flows from the distillation flask during distillation of the organic solvents: the condenser serves as exhaust. Ambient air is not in contact with the solvent in this way. Problems are associated with other methods of excluding room air (e.g., active carbon or molecular sieves), the most important one being discontinuity. The condensate is distilled into a 1 litre flask at a 1:20 ratio. This large volume allows for direct transfer into the appropriate solvent containers which should be made of glass and of a sufficient size to provide solvent for not more than 6 analyses. A bottle with sufficient solvent for 10 - 15 analysis has to be opened and closed many times and even when kept closed, when not in use, contamination from the surrounding atmosphere takes place. For more detailed information, consult the Reference Method No 65: UNEP/IOC/IAEA: Reagent and laboratory ware clean-up procedures for low-level contaminant monitoring.

3.1.3. Cleaning of reagents and adsorbents

3.1.3.1. Cleaning of reagents

Powdered or crystalline reagents, such as anhydrous sodium sulfate (Na_2SO_4)*, potassium hydroxide (KOH), glass wool * and carbon or carborundum boiling chips *, must be thoroughly cleaned before use. They should be extracted with hexane in a Soxhlet apparatus for 8 hours and then with methanol or dichloromethane for another 8 hours. For those items indicated by an *, this will require pre-combustion in a muffle furnace at approximately 400°C.

3.1.3.2. Cleaning of adsorbents

Silica gel, alumina and Florisil have to be solvent extracted. Each reagent is first refluxed with methanol or dichloromethane in a Soxhlet apparatus for 8 hours, then with n-hexane for the same period. The solvent is removed by a rotary evaporator operating at low speed, until the sorbent starts falling down as fine particles. Reagents are dried in a drying oven at 0.01 mbar. If this is not available, they are dried in a normal oven at 120°C for 4 hours. This serves to activate silica and alumina. Florisil has to be activated at 130°C for 12 hours. The sorbent is allowed to cool in the oven (if possible under vacuum to avoid uptake of contaminants from the atmosphere) or alternatively, in a dessicator. As active sorbents attract water and contaminants from the atmosphere, controlled deactivation should be carried out by adding water to the fully active sorbent (5% by weight to silica, 2% by weight to alumina, and 0.5% by weight to Florisil). The deactivation procedure should be carried out by adding the water to the sorbent and mixing by gentle shaking for a few minutes. The equilibration takes one day. The activity can be maintained for longer periods of time by sealing the required amount of sorbent in glass ampoules. Otherwise, the activation/deactivation has to be done the day before use.

3.2. Apparatus and equipment

The laboratory used for organic trace analysis must be a dedicated facility, isolated from other projects that could be sources of contamination. It must be properly constructed with fume hoods and benches with electric sockets that are safe for use with flammable solvents. The laboratory must have extractors and rotary evaporators cooling water to run the stills. In tropical regions and in dry climates, a refrigerated re-circulating system should be used to reduce temperatures to the required levels and/or to conserve water. Stainless steel or ceramic tiles make good non-contaminating surfaces. If necessary, benches can be coated with a hard epoxy resin and walls can be painted with epoxy paint. A sheet of aluminium foil on the workbench provides a surface which can be cleaned with solvent. A vented storage facility for solvents is essential. Benches must be fitted with frames to hold stills, extractors, etc. The emergency cut-off switch should be accessible from both inside and outside the laboratory. Fire fighting equipment should be mounted in obvious places and laboratory personnel trained in their use.

3.2.1. List of materials

- A coring device with liners and plunger or a grab sampler (thoroughly cleaned with detergents and solvents before use).
- Glass jars and aluminium foil, stainless steel knives, scoops, forceps, labels, marking pens, logbook.
- Insulated plastic boxes for transporting samples. Ice or dry ice.
- Deep freezer (-18 to -20°C) for sample preservation (frost free type freezers heat to above zero during frost removal cycles and they cannot be used for long term storage).
- Rotary evaporator.
- Kuderna-Danish (or similar) concentrator and heater.
- Soxhlet extraction apparatus and heaters.
- Glassware including boiling flasks, ground glass stoppers, beakers, Erlenmeyer flasks, separatory funnels, centrifuge tubes, weighing bottles, pipettes, tissue grinders.
- Drying oven (temperature range up to at least 300°C) for determining sample dry weights, baking of contaminant residues from glassware and reagents.

Note: A muffle furnace is better for baking materials at greater than 300°C, if required.

- Centrifuge and tubes.
- Freeze-dryer and porcelain pestle and mortar.
- Analytical balance with an accuracy of 0.1 mg and an electro-balance with an accuracy of at least 1 µg.
- Stainless steel tweezers and spatulas.

- Dessicator - completely free of organic contamination and with no grease applied to sealing edges.
- Supply of clean, dry nitrogen.
- Columns for silica gel, alumina and Florisil chromatography.
- Mechanical blender (food mixer).
- Vacuum pump (water-jet air pump).

3.2.2. Cleaning of glassware

Scrub all glassware vigorously with brushes in hot water and detergent. Rinse five times with tap water and twice with distilled water. Rinse with acetone or methanol followed by hexane or petroleum ether. Bake overnight in an oven at 300 °C. All glassware should be stored in dust free cabinets and tightly sealed with pre-cleaned aluminium foil when not in use. Ideally glassware should be cleaned just before use.

For more detailed information, consult Reference Method No 65: UNEP/IOC/IAEA: Reagent and laboratory ware clean-up procedures for low level contaminant monitoring.

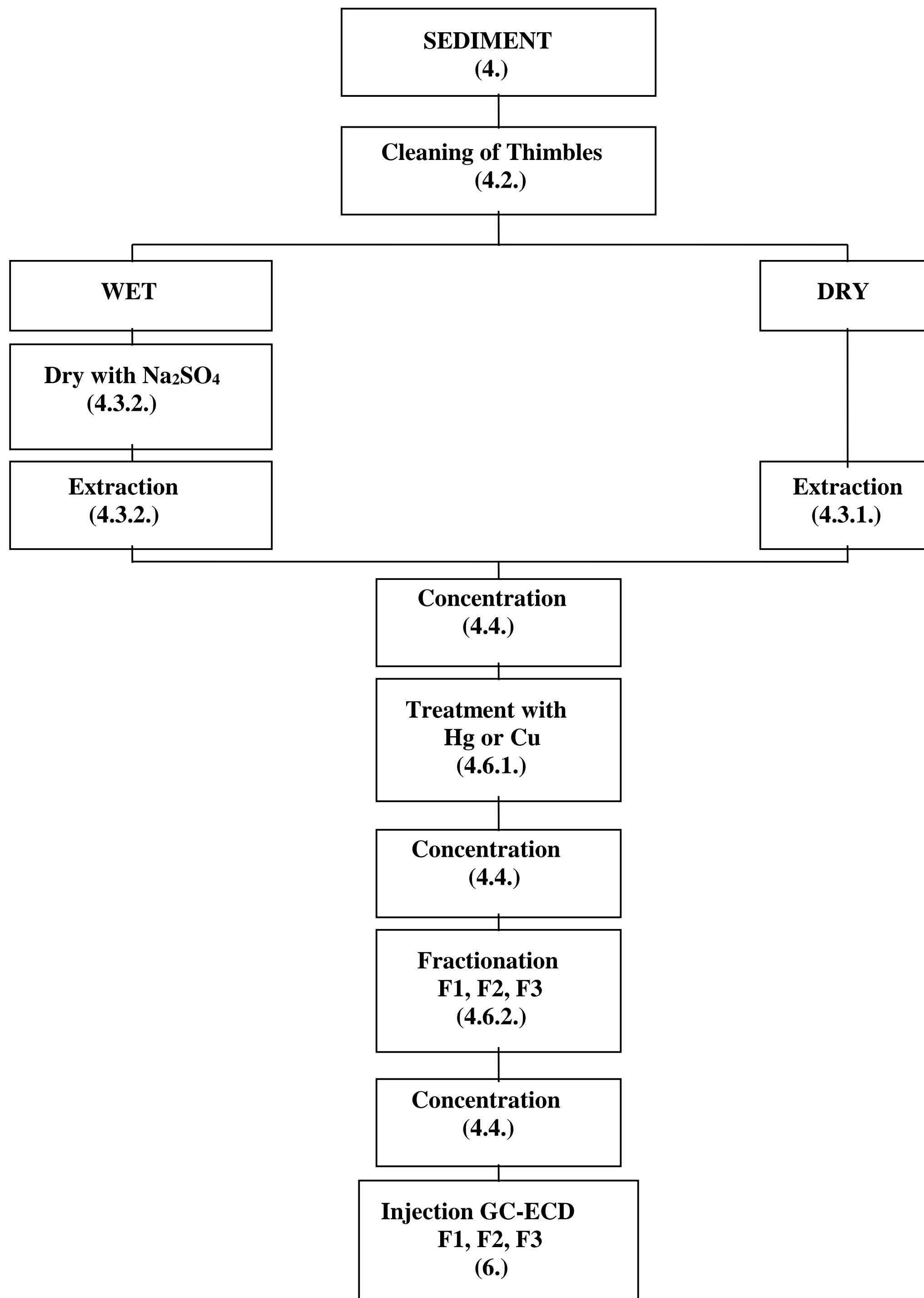

Diagram of the extraction procedure for sediment samples.

4. SEDIMENTS

4.1. Sampling

For the preparation of the samples (including selection of sites, collection of samples and storage) the reader should refer to the Reference Method Nº 58: Guidelines for the use of sediments for the marine pollution monitoring programmes, to the Reference Method Nº 20: UNEP/IOC/IAEA: Monitoring of petroleum hydrocarbons in sediments and to UNEP(DEC)/MEDW.C282/Inf.5/Rev1: Methods for sediment sampling and analysis (2006).

4.2. Cleaning of extraction thimbles

Paper extraction thimbles should be cleaned prior to sample extraction. For use in the extraction of sediment samples, the extraction can be performed in the Soxhlet apparatus with 250 ml of a mixture hexane / dichloromethane (50:50) for 8 hours cycling the solvent through at a rate of 4 to 5 cycles per hour. Add into the solvent a few carborundum boiling chips to get a regular ebullition.

The use of disposable paper thimbles for the extraction procedure rather than re-usable glass fibre thimbles is recommended due to the difficulties encountered in cleaning the latter.

4.3. Extraction of sediments

4.3.1. Extraction of freeze-dried samples

Select a 50-100 g sub-sample of the sediment, weigh this sub-sample and freeze-dry it. When dried, re-weigh it and calculate the dry to wet ratio. Then pulverise the sample using a pestle and mortar and sieve it using a 250 µm stainless steel sieve. Accurately weigh about 20 g of ground sample and place it in the pre-cleaned extraction thimble. Add 1 ml of a solution of 25 pg/µl of 2,4,5 trichlorobiphenyl (PCB Nº 29), 20.9 pg/µl of 2,2',3,3',4,5,5',6 octachlorobiphenyl (PCB Nº 198), 20 pg/µl of ε HCH and 21 pg/µl of Endosulfan Id_4 as internal standards and extract for 8 hours in a Soxhlet apparatus with 250 ml of a mixture hexane / dichloromethane (50:50), cycling the solvent through at a rate of 4 to 5 cycles per hour, add into the solvent a few carborundum boiling chips to get a regular ebullition. Alternatively (or in addition), PCB congeners No 30, 121, or octachloronaphthalene and PCB congeners can be used as internal standards. Prepare a procedural blank by extracting an empty thimble using the same procedure as for the samples.

4.3.2. Extraction of wet samples

The sediment is thawed, sieved at 250 µm and homogenised manually with a stainless steel spatula or clean glass rod. A sub-sample of 1-2 g is weighed into a flask and placed in a drying oven at 105 °C for 24 hours, then allowed to cool to room temperature and re-weighed. Calculate the dry to wet ratio and discard the dry sediment (unless it is being used for other analysis e.g. TOC, total organic carbon).

Place a 30-40 g sub-sample of thawed, homogenised sediment into a blender. Slowly, add 100g of anhydrous sodium sulphate (desiccant) and blend the mixture at high speed for 10 minutes. Transfer the dried sample quantitatively to the pre-cleaned extraction thimble in the Soxhlet apparatus, add the internal standard solution (see above) and apply the same extraction procedure as above. Extract the same amount of sodium sulphate as a procedural blank, making sure to add an appropriate amount of internal standard solution.

4.3.3. Example of determination of percent moisture

Many environmental measurements require the results to be reported on a dry weight basis. The percent moisture or water content in the sample is determined by weighing an aliquot, not used for analysis, of the sample before and after drying. The drying can be done by heating a few grams (1-2 g) of the sample in an oven to constant weight.

Weigh an empty glass beaker that will be used to hold the sample while it is dried.
Empty beaker weight = 10.4417 g

Add the wet sample to the beaker and reweigh. Calculate the wet weight of the sample.
Empty beaker weight + wet sample = 12.2972 g
Wet sample weight = 12.2972 g - 10.4417 g = 1.8555 g

Dry the sample to constant weight: dry the sample for 24 hours, weigh it, dry again for 12 hours, re-weigh it, when the difference in weight is less than 5%, it means that the sample is dried.
Empty beaker weight + dry sample weight = 10.9396 g
Dry sample weight = 10.9396 g - Empty beaker weight
Dry sample weight = 10.9396 g - 10.4417 g = 0.4979 g

Calculate the percent dry sample weight.

$$\% \text{ Sample weight} = \frac{\text{Sample dry weight}}{\text{Sample wet weight}} \times 100$$

$$= \frac{0.4979}{1.8555} \times 100 = 26.8\ \%$$

Calculate the percent moisture.

Water content = wet weight - dry weight
= 1.855 g - 0.4979 g = 1.3576 g

$$\% \text{ Moisture} = \frac{\text{Sample water weight}}{\text{Sample wet weight}} \times 100$$

$$\% \text{ moisture} = \frac{1.3576}{1.8555} \times 100 = 73.2\ \%$$

4.4. Concentration of the extract

For both extraction procedures, the extracts are concentrated in a rotary evaporator to about 15 ml. Under good vacuum conditions the temperature of the water bath must not exceed 30 °C. Dry the extract with anhydrous sodium sulphate (when the sodium sulphate moves freely in the flask it means that the extract is dried). Collect the dried extract in the graduated tube of a Kuderna-Danish concentrator. Concentrate the extract to approximately 5 ml with the Kuderna-Danish concentrator and adjust the volume to exactly 1 ml by evaporating excess solvent under a gentle stream of clean dry nitrogen. The sample extract will be analysed gravimetrically for extractable organic matter (EOM) content at the 1 ml volume as a starting point. If measurements of the EOM are outside the calibration range of the balance, the total volume of the extract is adjusted accordingly using either dilution with hexane or evaporating under a stream of nitrogen gas.

4.5. Extractable organic matter

Before carrying out the clean-up procedure, it is advisable to determine the extractable organic matter.

The EOM is determined in the following manner. On the weighing pan of an electro-balance, evaporate a known volume of the sediment or biota extract (up to 100 µl) and weigh the residue with a precision of about ± 1 µg. If the residue is less than 2 µg, pre-concentration of the original extract is required. The quantity of EOM is:

$$\text{EOM } (\mu g/g) = \frac{\text{Weight of residue } (\mu g) \text{ x volume of the extract (ml) x } 1000}{\text{Volume evaporated } (\mu l) \text{ x quantity of sample extracted (g)}}$$

Note that extreme care must be taken to ensure balance and pans are clean, dry and stable to obtain accurate readings at the ± 1 µg level. A small hot plate is used to warm pans and forceps and thus keep these instruments dry after solvent cleaning. If no electro-balance is available, a known volume of the extract can be transferred into a clean pre-weighed beaker. The solvent is evaporated with dry and clean nitrogen until a constant weight of about 1 mg is reached. Calculate the amount of "lipids" in the sample taking into account the volume of the lipid extract which was dried.

Example of calculation of E.O.M.

The extractable organic matter content of a sample is operationally defined as the weight of material extracted with the solvent employed (H.E.O.M. in case hexane is used as solvent). An aliquot of the sample extract is taken (few µl), the solvent is evaporated and the residue is weighed to determine the quantity of lipids extracted in the aliquot and from it to the total sample. The results are normally reported in mg lipids per gram dry weight extracted.

A 1 µl aliquot is removed from a 2.5 ml sample extract for determination of E.O.M. The 1 µl aliquot is evaporated on the pan of an electro-balance and the residue is weighed. Three determinations are made and the average taken.

Measurements:
Sample dry weight extracted: 4.443 g
Total volume of the extract: 2.5 ml
Sample aliquot removed: 1 µl
(1) Weight of a 1 µl aliquot after solvent evaporation: 32.2 µg
(2) Weight of a 1 µl aliquot after solvent evaporation: 32.1 µg
(3) Weight of a 1 µl aliquot after solvent evaporation: 32.3 µg
Average weight of a 1 µl aliquot : 32.2 µg

Total volume of the extract: 2.5 ml

Total quantity of lipids in the sample:

$$32.2 \ \mu g/\mu l \text{ x } 2.5 \text{ ml x } \frac{1000 \ \mu l}{\text{ml}} = 80500 \ \mu g \text{ or: } 80.5 \text{ mg}$$

With 4.443 g of sample extracted:
80.5 mg/ 4.443 g = 18.1 mg lipids/g

4.6. Clean-up procedure and fractionation

Purposes of the clean-up: removal of lipids, whenever present at a significant amount; removal of elementary sulphur and sulphur compounds. Both these compound classes can interfere with the gas-chromatographic separation.

4.6.1. Sulphur and sulphur compounds removal

Elementary sulphur and sulphur compounds such as mercaptans should be removed from the extract. This could be done by using either mercury or activated copper.

a) Mercury method.

Add one drop (a few ml) of mercury to the sediment extract and shake vigorously for one minute. Centrifuge and carefully recover and transfer the extract in another tube with a Pasteur pipette. If the mercury is still tarnished, repeat the treatment with another drop of mercury, shake, transfer the hexane into another tube. Repeat this treatment until the mercury stays brilliant in the extract. Rinse the mercury with 5 ml of hexane and combine the extracts. Then, concentrate the resulting solution to ca. 1 ml with a gentle stream of nitrogen.

Cleaning of mercury:

Caution: When removing mercury from the sample, always use a plastic tray to keep the glassware in and work under a fume hood.

Fit a folded filter paper in a 10 cm diameter conical glass funnel and fix the funnel over a 250 ml glass beaker. Using a needle, make a small hole in the bottom of the filter paper. Carefully put the mercury onto the funnel. The mercury flows through the small hole in the filter paper leaving the solid impurities on its surface. The mercury collected is washed three times by shaking it carefully with dichloromethane and by removing dichloromethane layer with the help of a clean glass syringe. Allow the rest of dichloromethane evaporate and store the clean mercury in a thick walled glass bottle with a ground glass stopper. In order to avoid escape of mercury vapour, store the mercury under methanol.

Another way of cleaning the mercury involves sucking the dirty mercury through a capillary tube, such as a Pasteur pipette, connected to a guard-flask and then to a vacuum pump. The mercury will pass through the Pasteur pipette and will be collected and cleaned in the guard-flask. Then it should be transferred into a thick wall glass bottle with a ground glass stopper. The mercury is covered with a layer of methanol to protect it from oxidation.

b) Activated copper method.

Transfer about 20 grams of the copper powder in an Erlenmeyer. Add enough concentrated HCl to cover the copper powder, agitate. Sonicate for 10 min., agitate, put again in ultrasonic bath and sonicate for 10 min. Throw the used HCl, add some fresh HCl, transfer in ultrasonic bath and sonicate for 20 min. repeat that procedure four times in total. Wash with distilled water, agitate, discard, add water again, transfer in ultrasonic bath and sonicate for 15 min., discard the used water, repeat that procedure again, up to pH neutral. Wash with acetone, agitate, transfer in ultrasonic bath and sonicate for 15 min. repeat that procedure four times in total. Then use the same procedure with hexane as a solvent.
Keep in hexane (use it immediately, avoids Cu to be in contact with air).

Transfer 3 to 4 Pasteur pipettes per sample in the flasks containing the hexane extracts. Let the copper react all night. The presence of sulphur compounds in the sample will be detected by the tarnishing of the copper powder. Then, concentrate the resulting solution to ca. 1 ml with a gentle stream of pure nitrogen.

4.6.2. Fractionation

An adsorption chromatography step is used to remove interfering lipids and to fractionate the extract into classes of compounds. Many variations of adsorption chromatography clean-up procedures have been published to date. Four procedures are reported here in order of increasing complexity.

Preparation of the columns: Glass burettes (1 cm diameter) with Teflon stopcocks make convenient adsorption columns. The column is plugged with pre-cleaned cotton or glass wool. Prepare separate columns for each sample and blank determination. The column is partially filled with hexane. The appropriate amount of sorbent is mixed with hexane in a small beaker to form a slurry. A glass funnel and a glass rod are used to pour the adsorbent into the column. Several rinses with hexane are necessary to fill the column to the desired height. Tap with a pencil or a hard silicone tube against the column in order to settle the adsorbent into an even bed. Flush the material adhering to the wall of the column down to the bed with solvent. Prepare each column freshly immediately before use. Never let the column get dry.

4.6.2.1. Florisil

A Florisil column is used for this fractionation, which is prepared in the following way. The Florisil should be pre-extracted in the Soxhlet apparatus to remove any contaminants, using methanol or dichloromethane for 8 hours, followed by hexane for another 8 hours. It is then dried in an oven. Activation is achieved by heating the dried Florisil at 130°C for 12 hours. It is then partially deactivated with 0.5% water by weight and stored in a tightly sealed glass jar with ground glass stopper. The water should be well mixed into the Florisil and the mixture should be allowed to equilibrate for one day before use. The activation/deactivation procedure should be carried out one day before use. A 1 cm burette with Teflon stopcock is plugged with pre-cleaned glass wool. A column with a sintered glass disk could also be used. 17 grams of Florisil are weighed out in a beaker and covered with hexane. A slurry is made by agitation and poured into the glass column. The Florisil is allowed to settle into an even bed and any Florisil adhering to the column is rinsed down with hexane. The solvent is drained to just above the Florisil bed. It should be rinsed with a further 5 ml of hexane; one gram of anhydrous sodium sulphate is added to the top of the column in order to protect the surface of the Florisil from any disturbance. The column should never run dry. Individual columns should be prepared immediately before use and a new column of Florisil used for each sample.

The extract, reduced to 1 ml, is put onto the Florisil column. It is carefully eluted with 65 ml of hexane and the first fraction collected. Then the column is eluted with 45 ml of a mixture containing 70 % of hexane and 30 % of dichloromethane and the second fraction collected. The third fraction will be eluted with 60 ml of pure dichloromethane.

Fraction one will contain the PCBs, pp' and op DDE and some other pesticides such as HCB, aldrin, heptachlor, DDMU.

Fraction two will contain the DDTs, DDDs, most of the toxaphene, and some pesticides such as the HCH isomers and chlordane components.

Fraction three will contain mainly dieldrin, endrin, heptachlor epoxide and endosulfan components. Typical chromatograms obtained are shown below.

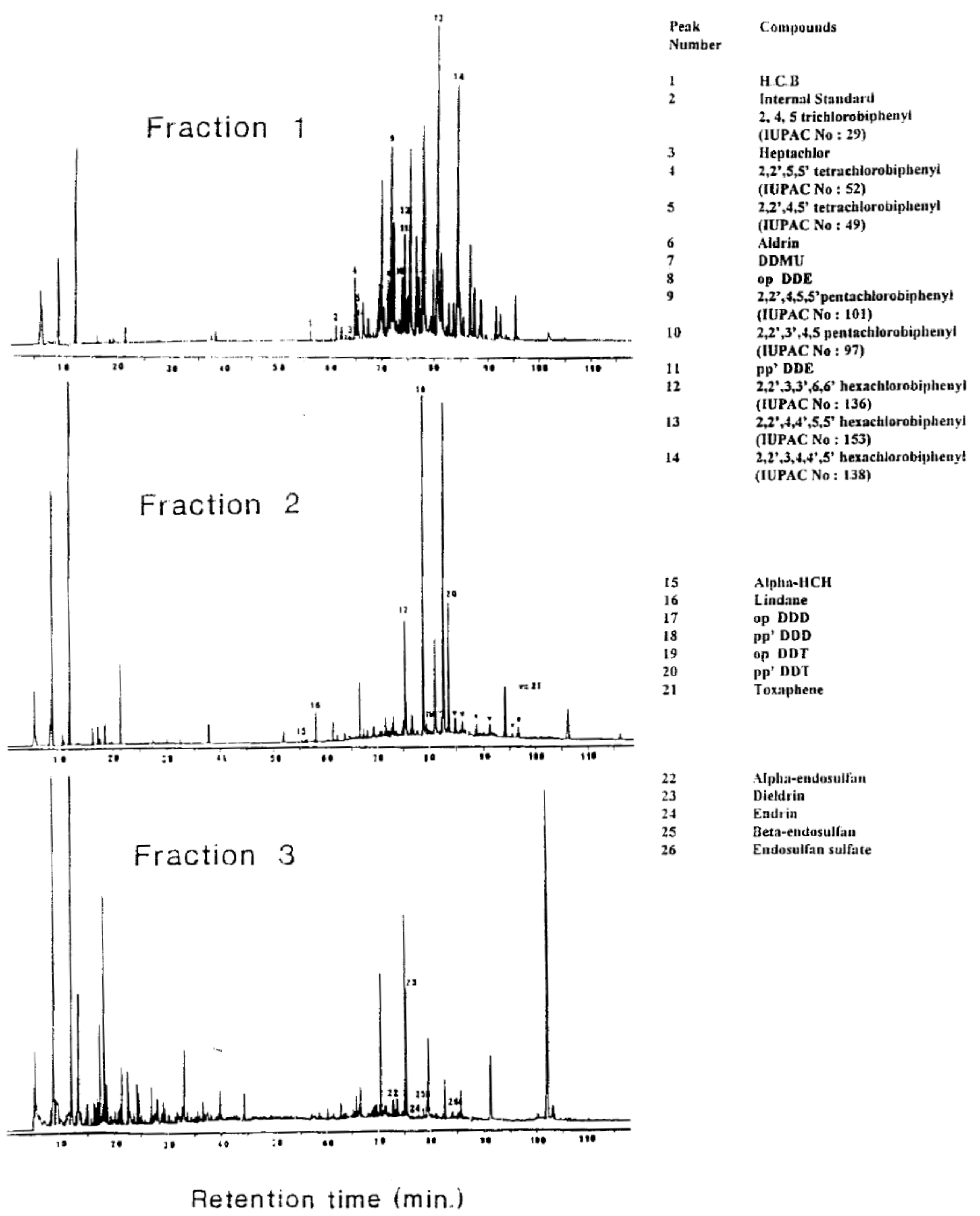

Figure 2: GC-ECD organohalogen analyses

4.6.2.2. Gel permeation chromatography

Low pressure GPC can be used as an alternative clean-up technique to remove high molecular weight co-extractable lipidic material from polycyclic aromatic compounds and halogenated aromatics. Concurrently, elemental sulphur could be also removed from the whole organic extract.

The main feature of the semi-preparative-GPC as a clean-up technique relies on the compatibility of this analytical procedure with labile components of the extract (i.e. DDTs, chlorinated cyclohexadiene derivatives), which are not stable in other types of extract clean-up procedures. Further, GPC as a clean-up technique has already been automated, enabling a high sample throughput, taking into account the short analysis time involved.

The GPC retention mechanism may involve adsorption, partition and size exclusion mechanisms. The predominance of one mechanism over the others is largely determined by the choice of the mobile phase and the pore size of the packing. In the case of GPC packings with large pore size (1000-2000 daltons) size exclusion and adsorption mechanisms prevail (Bio-Beads SX-3 using cyclohexane, dichloromethane-hexane, dichloromethane-cyclohexane, toluene-ethylacetate and ethylacetate-cyclohexane) (Ericksson *et al*., 1986). On the other hand, when smaller pore sizes (400 daltons) are used in combination with highly polar solvents, (THF, DMF) size exclusion predominates (Lee *et al*., 1981). While using the first approach, a chemical class fractionation could be obtained, however, if smaller pore sizes are used it should be combined with another fractionation technique (i.e. adsorption chromatography) to achieve this selectivity. It has yet to be demonstrated that using GPC as a single clean-up step produces a completely clean extract for GC-ECD determination. Nevertheless, taking into account the increasing availability of high-resolution low molecular weight exclusion packings, they could definitively integrate fractionation and clean-up in a single step.

Low resolution packing (Sephadex LH and Bio-Beads SX, 200-400 mesh size) are the most widely used because they are inexpensive and afford relatively high sample loading (500 mg in 10 mm i.d. columns). The implementation of low resolution GPC requires a solvent delivery system and a UV detector and may be useful. For method development, it is advisable to inject a broad range of standard compounds covering the whole range of molecular weights of the analytes to be determined in order to determine the cut-off points to fractionate real samples. Reported recoveries of PCBs and PAHs range from 60 to 80 % for the concentration level (ng) injected. (Fernandez and Bayona, 1992).

4.6.2.3. Alumina and HPLC (silica column)

The first step in this clean-up procedure is an adsorption step using an alumina column to remove most of the lipid material. Prepare an alumina column (4 x 0.5 cm i.d., made from a Pasteur pipette). Apply the concentrated extract to the top of the column and elute with 10 ml hexane. Concentrate the eluate to about 200 µl. It is followed by a second step to more completely remove interfering compounds and at the same time to separate the compounds of interest into different fractions, containing aliphatics, PCBs, PAHs, pesticides and toxaphene. Between 20 and 200 µl of the extract (after alumina clean-up) are eluted on a stainless steel column (200 x 4 mm i.d.), packed with Nucleosil 100-5 with n-pentane, 20 % dichloromethane in n-pentane and finally dichloromethane. The eluate is collected in fractions containing 1) n-hydrocarbons, 2) PCBs, 3) PAHs and toxaphene, 4) pesticides and toxaphene and 5) acids, etc. (polar compounds). The size of the fractions has to be determined with standard solutions containing the compounds of interest, collecting the eluate in 0.5 ml fractions. Each fraction is then analysed by GC-ECD. Full details have been given in the literature (Petrick *et al*., 1988 and IOC, 1993).

4.6.2.4. High pressure chromatography

High pressure liquid chromatography (HPLC) columns packed with microparticles are available and have the advantages of high reproducibility, low consumption of solvents, high efficiency and high sample loading capacity.

This method can be used to separate fractions containing aliphatic hydrocarbons, PCBs and aromatic hydrocarbons from interfering compounds. These fractions can then be analysed separately for their constituents by GC-FID and/or GC-ECD.

HPLC methods have been developed using synthetic solutions of n-alkanes, PAHs, pesticides, PCBs and toxaphene and have been applied to samples in which interfering substances were present in such high concentrations as to render the analysis of HC and PCBs extremely difficult without this clean-up procedure (e.g. sediments and biological tissues with OCs in the ng/g range). The samples are eluted with n-hexane, subjected to clean-up over alumina, concentrated down to 20-200 µl and treated by HPLC. With the use of n-hexane, n-pentane and 10 %, 20 % and 50 % dichloromethane in n-hexane, respectively, the following five fractions are obtained : 1) n-hydrocarbons and alkenes, 2) PCBs and alkylbenzenes, 3) PAHs and toxaphene, 4) pesticides, 5) acids, etc.(polar compounds). (Petrick *et al*. 1988).

5. BIOTA

5.1. Sampling

Organisms accumulate many contaminants from their environment (i.e., from sea water, suspended particulate matter, sediment and food). Field and laboratory studies have shown that contaminant concentrations in some marine plants and animals reflect concentrations in their environment. Scientists use this process (termed bio-accumulation) to assess marine contamination resulting from human activity (e.g., pipeline discharges, dumping from ships).

There are problems with using biota as bio-accumulators (bio-indicators). For example, tissues from individuals of a species exposed to the same contaminant concentration may contain different levels of contamination after the same exposure time. These deviations reflect individual differences in factors such as age, sex, size, and physiological and nutritional states. Also, various species show different contaminant concentrations following identical exposure; differences in elimination rates may partially account for this. These factors must be considered when planning a monitoring programme in order to control their effects on the precision of the analysis (by reducing the variances). Variance reduction is necessary in order to detect smaller differences in mean contaminant concentrations observed in monitoring programmes.

For proper sampling and sample preparation, refer to Reference Method No 6 “Guidelines for monitoring chemical contaminants in the sea using marine organisms” and Reference Method No 12 Rev.2 “ Sampling of selected marine organisms and sample preparation for the analysis of chlorinated hydrocarbons”.

5.2. Cleaning of extraction thimbles

As for extraction of sediment samples, thimbles should be extracted first with the same solvent used for the extraction of the sample. As the extraction of biota sample is achieved with hexane, a pre-extraction of these thimbles is made with 250 ml of hexane for 8 hours in the Soxhlet apparatus, cycling the solvent through at a rate of 4 to 5 cycles per hour. Add into the solvent a few carborundum boiling chips to get a regular ebullition.

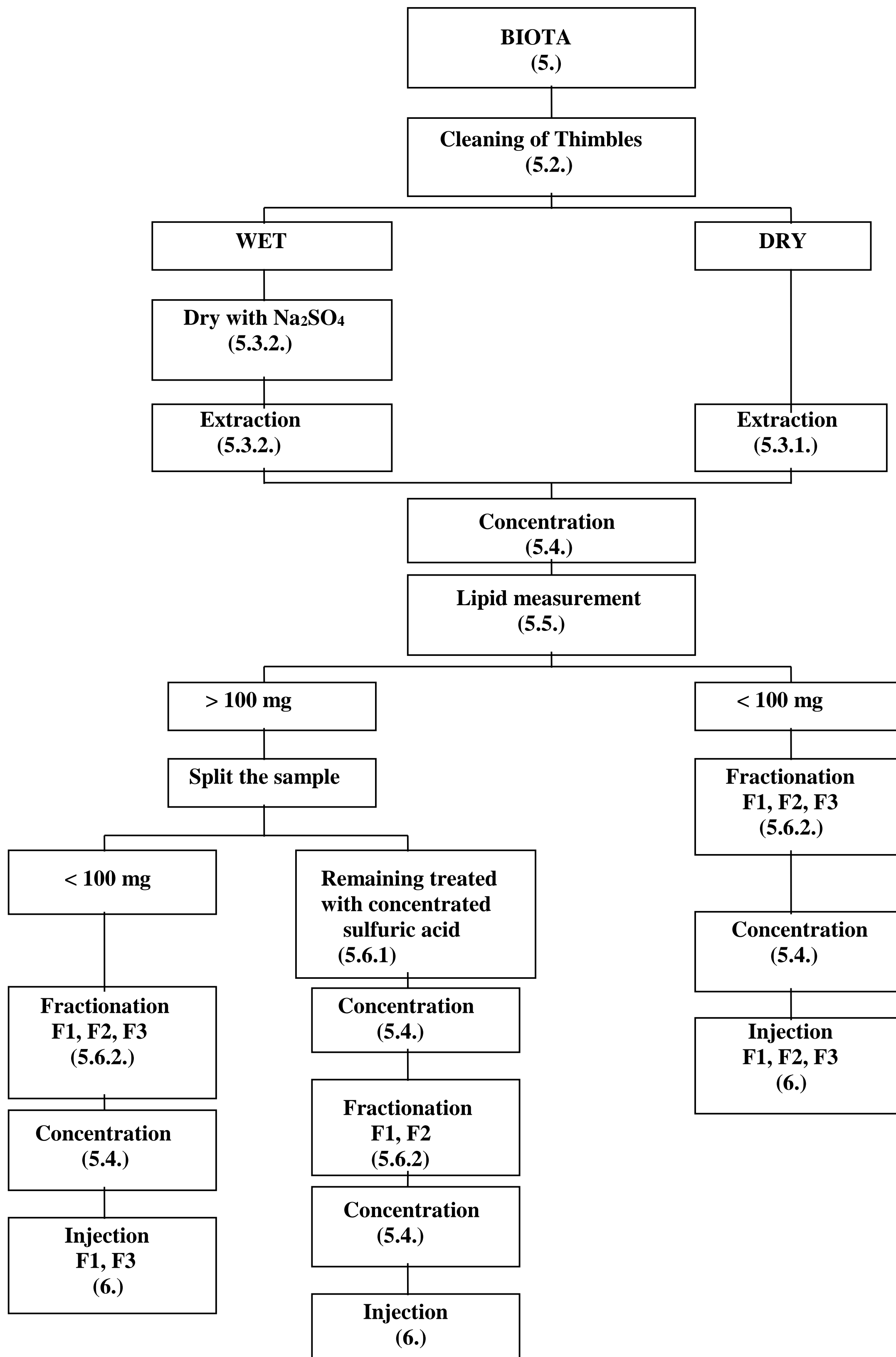

Figure 3: Diagram of the extraction procedure for biota samples.

5.3. Extraction of tissues

5.3.1. Extraction procedure for freeze-dried samples.

Take a 50 to 100 g fresh weight sub-sample from the sample. Weigh this sub-sample and freeze-dry it. When the sub-sample appears to be dry, re-weigh it and freeze-dry it for a further 24 hours and then re-weigh it. If the difference between the two dry weights is greater than 5%, continue the freeze-drying process. Special care must be taken to ensure that the freeze-drier is clean and does not contaminate the samples. The freeze drying procedure should be tested by drying 100 g Na_2SO_4 as a blank and extracting this as a sample. Pulverise the freeze-dried sub-sample carefully using a cleaned pestle and mortar. Accurately weigh about 5 to 10 g of this pulverised material, note the exact weight to be extracted, and place it into a pre-cleaned extraction thimble in a Soxhlet apparatus. The size of the sub-sample should be adjusted so that about 100 mg of extractable organic matter ("lipid") will be obtained. Smaller sub-samples should be used if residue concentrations are expected to be high. Add a known amount of internal standard to the sub-sample in the thimble before Soxhlet extraction. It is important to spike the sample at levels that are near to that of the analyte concentrations in the samples. If, in the end, the analyte and the internal standard concentrations do not fall within the established calibration range of the GC-ECD, the analysis must be repeated. Consequently, it may be advisable to perform range-finding analysis for samples of unknown character beforehand. Candidate internal standards are the same as for sediment samples (see 5.3.). Add about 200 ml of hexane or petroleum ether to the extraction flask with a few carborundum boiling chips, and extract the sample for 8 hours cycling the solvent through at a rate of 4 to 5 cycles per hour. Extract an empty thimble as a procedural blank, making sure to spike it with internal standards in the same fashion as the sample. If unacceptable procedural blanks are found, the source of contamination must be identified and eliminated rather than subtracting high blank values from the analytical results.

5.3.2. Extraction procedure without freeze-drying

Select a 25 to 100 g fresh weight sub-sample and place in a blender. Add anhydrous sodium sulphate to the sample, manually homogenise and determine whether the sample is adequately dried. If not, more sodium sulphate should be added until a dry mixture is obtained. Normally, 3 times by the sample weight used should be enough. Once this has been achieved, blend the mixture at high speed for 1 or 2 minutes until the mixture is well homogenised and the sample appears to be dry. Transfer the mixture to a pre-cleaned extraction thimble, add internal standards as described above and extract the dehydrated tissue with about 200 ml hexane or petroleum ether for 8 hours in a Soxhlet apparatus, cycling 4 to 5 times per hour. Extract the same amount of sodium sulphate as the procedural blank, making sure to add internal standards in the same fashion as the sample.

5.4. Concentration of the extract

Refer to section (4.4.)

5.5. Extractable Organic Matter (EOM)

Refer to section (4.5.)

5.6. Clean-up procedure and fractionation

5.6.1. Removal of lipids by concentrated sulphuric acid

If the lipid content of the extracts is higher than 100-150 mg, a preliminary step for the removal of the lipids is necessary before further sample purification. This can be carried out by using concentrated sulphuric acid. Treatment with sulphuric acid is used when chlorinated hydrocarbons are to be determined. However, sulphuric acid will destroy dieldrin and endrin so that an aliquot of the untreated extract must be set aside for the determination of these compounds.
CAUTION: During all this procedure it is very important to wear safety glasses.

Take an aliquot of the concentrated extract, containing about 200 mg of “lipids”, transfer into a separatory funnel and add to this extract enough hexane in order to dilute the sample (40 to 50 ml should be enough), this will allow recovery of the hexane after acid treatment, because if the sample is too concentrated, the destroyed “lipids” will become almost solid and it will be difficult then to recover the hexane from this solid mass. Add 5 ml concentrated sulphuric acid to the extract and tightly fit the glass stopper and shake vigorously. Invert the funnel and carefully vent the vapours out through the stopcock. Repeat this procedure for several minutes. Place the separatory funnel in a rack and allow the phases to separate. Four or five samples and a spiked blank are convenient to process at one time. The extract should be colourless. Recover the hexane phase into a glass beaker. Dry with sodium sulphate and transfer the hexane into a Kuderna-Danish concentrator. Reduce the volume of the extract by evaporating the solvent with a gentle stream of pure nitrogen to about 1 ml.

5.6.2. Fractionation

Refer to section (4.6.2.)

6. CAPILLARY GAS CHROMATOGRAPHIC DETERMINATIONS

6.1. Gas chromatographic conditions

- Gas chromatograph with a split/splitless injection system, separate regulation system for inlet and column pressures and temperatures; multi-ramp temperature programming facilities (preferably microprocessor controlled), electron capture detector interfaced with the column with electronic control unit and pulsed mode facilities. An integrator with a short response time (0.25 s) is essential.
- Narrow-bore (0.22 mm internal diameter), 25 m long, fused silica open tubular column, coated with SE-54 (0.17 µm film thickness, preferably chemically bonded) with sufficient resolution to separate the relevant peaks in the standards provided for PCB analysis.
- Carrier gas should be high purity H_2. If this is not available or if the GC is not equipped with a special security system for hydrogen leak, He may be used. Gas purification traps should be used with molecular sieves to remove oxygen, moisture and other interfering substances.
- High purity nitrogen gas (99.995 %) as ECD make-up gas can be used (Argon/methane high purity gas is another option).

Conditions:

- H_2 or He carrier gas at inlet pressure of 0.5 to 1 Kg/cm^2 to achieve a flow rate of 1 to 2 ml/min.
- Make-up gas N_2 or Ar/CH_4 at the flow rate recommended by the manufacturer (between 30 and 60 ml/min.).
- ECD temperature: 300°C

6.2. Column preparation

Fused silica columns are the columns of choice for their inertness and durability (they are extremely flexible). They are made of material that is stable up to 360 °C. The 5 % phenyl methyl silicone gum (SE-54) liquid phase, is present as a thin, (0.17 µm), uniform film which can tolerate temperatures up to 300 °C. SE-54 is relatively resistant to the detrimental effects of solvents, oxygen and water, at least at low temperatures. These columns are even more resistant and durable if the liquid phase is chemically bonded to the support by the manufacturer.

For GC/MS work, it is advised to restrict the film thickness to 0.17 µm because with thicker films some of the phase could be released, resulting in an increase of the noise signal in the GC/MS.

The flexible fused silica columns can be conveniently connected directly to the inlet and outlet systems without the transfer lines used in conventional glass capillary chromatography which often lead to increased dead volume. Low bleed graphite or vespel ferrules provide a good seal.

The presence of extraneous peaks and elevated baseline drift will result in poor detector performance. This can be caused by components which elute from the column, such as residual solvents and low molecular weight liquid phase fractions on new columns and build-up of later eluting compounds on old columns. Conditioning is a necessary step to remove these contaminants. New columns are connected to the inlet (while left unconnected to the detector). Columns are flushed with carrier gas at low temperature for 15 min. to remove the oxygen, then heated at 70-100 °C for 30 min. and finally at 170 °C overnight. The column can be then connected to the detector. Old columns can be heated directly to elevated temperatures overnight. The final temperature is selected as a compromise between time required to develop a stable baseline and expected column life. Thus, it may be necessary for older columns to be heated to the maximum temperature of the liquid phase resulting in shorter column life. The temperature of the ECD, when connected to the column, should always be at least 50 °C higher than the column, in order to avoid condensation of the material onto the detector foil. It is essential that carrier gas flows through the column at all times when at elevated temperatures. Even short exposure of the column to higher temperature without sufficient flow will ruin the column.

CAUTION: if H_2 is used as a carrier gas, position the column end outside of the oven to avoid explosion risk.

6.3. Column test

When the column has been connected to the detector, the carrier gas flow is set to 30 ml per minute for a column with 4 mm internal diameter. The column performance is then measured according to the criteria of the "number of theoretical plates" for a specific compound and can be achieved according to the following procedure.

- Set injector and detector temperatures at 200 and 300°C respectively and the column oven temperature at 180 °C.
- Inject pp' DDT standard and measure the retention time (Tr). Adjust the column temperature to get a pp' DDT retention time relative to Aldrin of 3.03.
- Measure the width of the pp' DDT peak at its half height ($b_{1/2}$), in minutes and the retention time (Tr) also in minutes.
- Calculate the number of theoretical plates using the formula:

$$\mathrm{N} = 5.54\left(\frac{Tr}{b_{1/2}}\right)^2$$

- A parameter which is independent of the column length is the height equivalent to a theoretical plate (HETP):

$$\mathrm{HEPT} = \frac{\mathrm{L}}{\mathrm{N}}$$

Where L is the column length. Adjust the flow rate of the carrier gas to obtain optimum performance. The HETP should be as low as possible (i.e. the number of theoretical plates should be as great as possible).

The column remains in optimum condition as long as the liquid phase exists as a thin, uniform film. The quality of the film at the inlet side may be degraded as a result of repeated splitless

injections. Decreased column quality may be remedied by the removal of the end of the column (10 to 20 cm) at the inlet side. Chemically bonded liquid phases require less maintenance.

6.4. Electron capture detector

High-energy electrons, emitted by a radioactive source within the detector (e.g. a ^{63}Ni foil), are subject to repeated collisions with carrier gas molecules, producing secondary electrons. These electrons, upon returning to their normal state, can be captured by sample molecules, eluting from a GC column. The resulting reduction in cell current is the operating principle of an electron capture detector. The detector current produced is actually a non-linear function of the concentration of electron-capturing material. However, the useful linear range of an ECD may be greatly improved if the instrument is operated at a constant current, but in a pulsed mode, i.e. with short voltage pulses being applied to the cell electrodes. The current in the cell is kept constant by varying the frequency of the pulses.

Contamination of the detector (and thus lower sensitivity) may result from high-boiling organic compounds eluting from the column. Periodic heating to 350°C may overcome this problem. The ^{63}Ni ECD can be used at 320°C under normal operational conditions, in order to limit such contamination.

The optimum flow for an ECD (30 to 60 ml/min.) is much higher than carrier gas flow through the column of one or two ml/min. Thus an additional detector purge flow is necessary (N_2 or Ar/CH_4). Once leaving the outlet of the column, the compounds have to be taken up into an increased gas flow in order to avoid extra-volume band broadening within the detector. Thus, the detector purge flow also serves as the sweep gas.

6.5. Quantification

The most widely used information for identification of a peak is its retention time, or its relative retention time (i.e., the adjusted retention time relative to that of a selected reference compound). Retention behaviour is temperature dependent and comparison of retention times obtained at two or more temperatures may aid in determining a peak’s identity. However, retention times are not specific and despite the high resolution offered by capillary columns, two compounds of interest in the same sample may have identical retention times.

One way of using retention indexes could be to inject di-n-alkyl-phthalates such as a mixture containing di-n-methyl-phthalate, di-n-ethyl-phthalate, di-n-propyl-phthalate, di-n-butyl-phthalate, di-n-hexyl-phthalate and di-n-heptyl-phthalate, which will cover the elution range from 70°C to 260°C. An arbitrary index of 100 is given to the di-n-methyl phthalate, 200 to the di-n-ethyl phthalate, and so on up to 700 to the di-n-heptyl phthalate; it is possible to identify all chlorinated pesticides by a proper retention index. This will be used also for unknown compounds which can be found easily on the GC/MS using the same index and so, identified. (Villeneuve J.P. 1986).

PCBs represent a complex mixture of compounds that cannot all be resolved on a packed column. Also there is no simple standard available for their quantification. Each peak in a sample chromatogram might correspond to a mixture of more than one individual compound. These difficulties have led to the recommendation of various quantification procedures. The usual method to quantify PCBs is to compare packed-column chromatograms of commercially available industrial formulations (Aroclors, Clophens, Phenoclors) with the sample chromatogram. Most commonly, it is possible to match one single formulation, such as Aroclor 1254 or Aroclor 1260 with the sample chromatogram. An industrial formulation (or mixture of formulations) should be chosen to be as close a match as possible and in the case of sample extracts from sediment or organisms, Aroclor 1254 and Aroclor 1260 are most frequently chosen.

For the second fraction obtained on Florisil separation, it is possible to quantify DDTs after comparison with the retention times of peaks in the sample chromatogram to those in the corresponding standard, the peak heights (or peak areas) are measured and related to the peak height (or peak area) in the standard according to the formula:

$$[\text{Concentration}] = \frac{h \times C \times V \times 1000}{h' \times V(inj) \times M \times R} \text{ ng/g (or pg/g)}$$

Where:

V = total extract volume (ml)
M = weight of sample extracted (g)
H = peak height of the compound in the sample
h' = peak height of the compound in the standard
C = quantity of standard injected (ng or pg)
V (inj) = volume of sample injected (µl)
R = Recovery of the sample

7. COMPUTERIZED GAS CHROMATOGRAPHY/MASS SPECTROMETRY (GC/MS)

7.1. Operating conditions

The chemical ionisation source of a mass spectrometer can be used to produce negative ions by electron capture reactions (CI-NI-MS) using a non-reactive enhancement gas such as methane or argon. CI-NI has the advantage of being highly selective, permitting the detection of specific compounds in complex matrices. Under CI-NI conditions, methane (99.99 %) is used as the reagent gas. Samples are introduced through a SE-54, 30 m x 0.25 mm i.d., fused silica column. The film thickness used is 0.17 µm in order to minimise the bleeding of the phase into the system. Helium is used as carrier gas with an inlet pressure of 13 psi, which gives a carrier flow of 1.5 ml/min. or a gas velocity of 44 cm/sec.

The temperature of the injection port is held at 250°C.

The temperature of the source is set at 240°C, the quadrupole at 100°C and the interface at 285°C.

Injections of 1-3 µl are made in the splitless mode.

The temperature programme of the oven starts at 70°C, for 2 minutes, then it is increased at 3°C/min. to 260°C and kept under isothermal conditions for 40 minutes.

```
File        : C:\HPCHEM\1\DATA\AR1254.D
Operator    : jpv
Acquired    : 12 Jul 95   8:02 am using AcqMethod OC
Instrument  :   5989B
Sample Name: standard ar1254
Misc Info   :
Vial Number: 1
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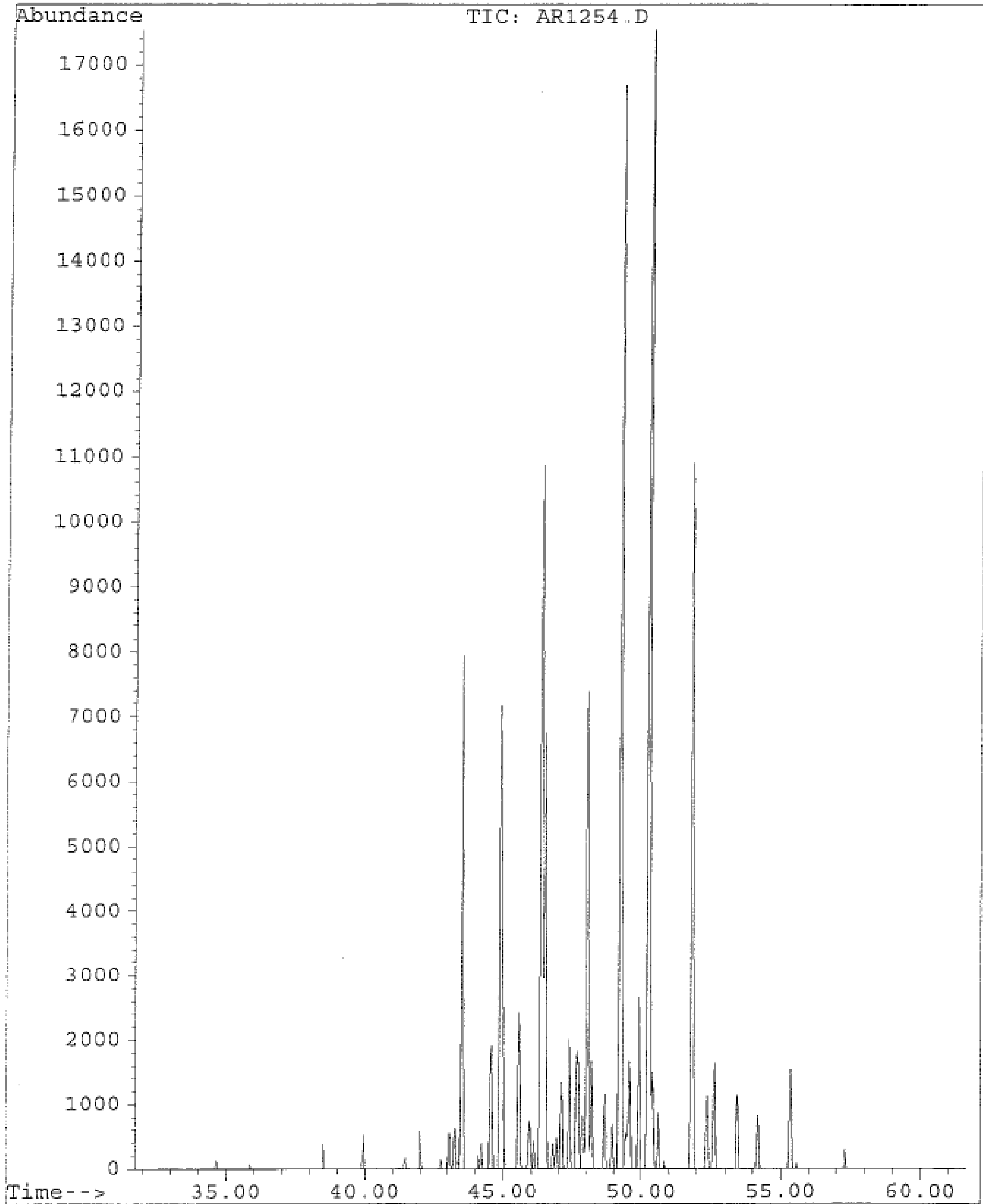

Figure 4: TIC of Aroclor 1254

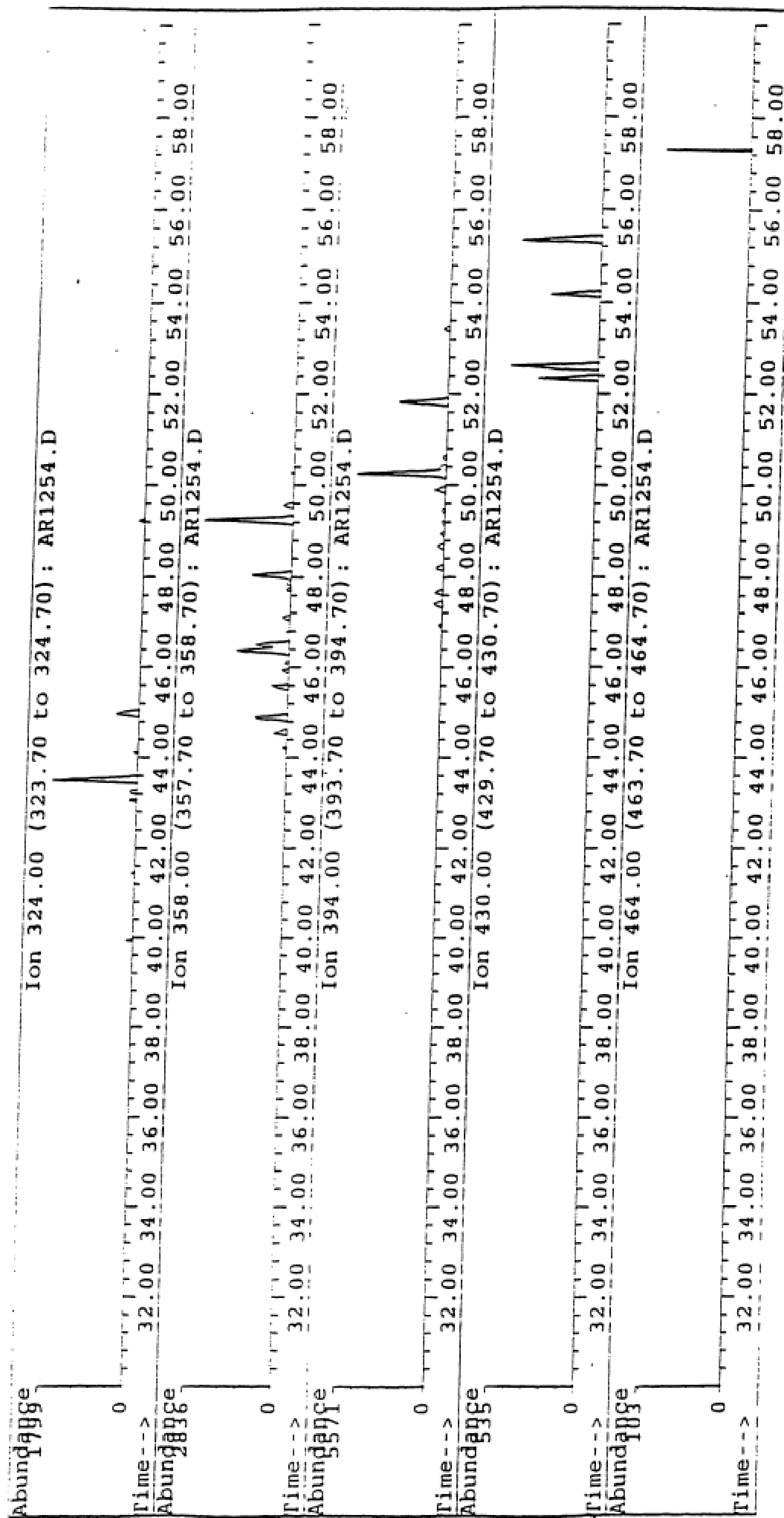

Figure 5: RIC of Aroclor 1254 main compounds

```
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Operator     : jpv
Acquired     : 12 Jul 95   9:42 am using AcqMethod OC
Instrument :   5989B
Sample Name: standard ar1260
Misc Info  :
Vial Number: 1
```

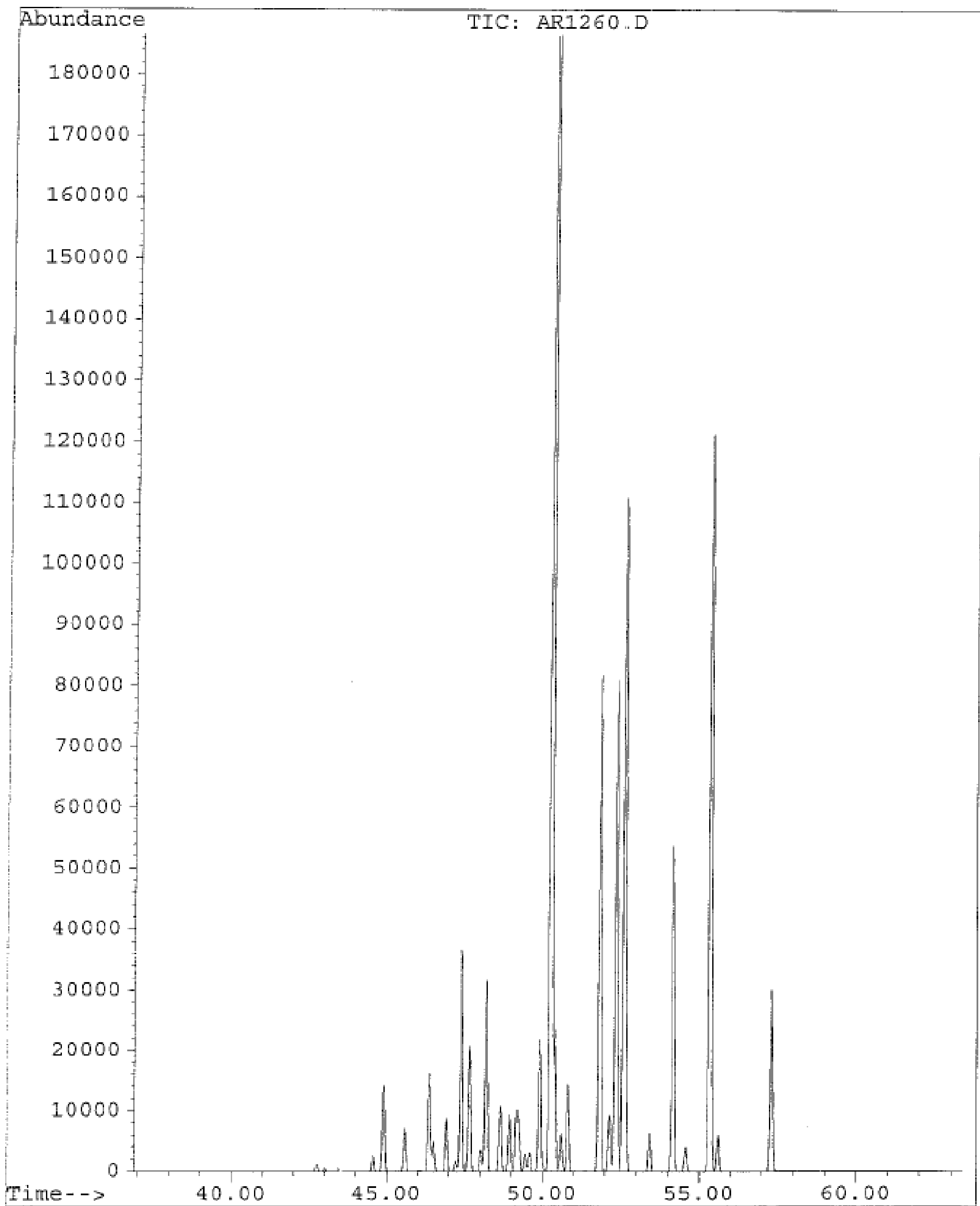


Figure 6: TIC of Aroclor 1260

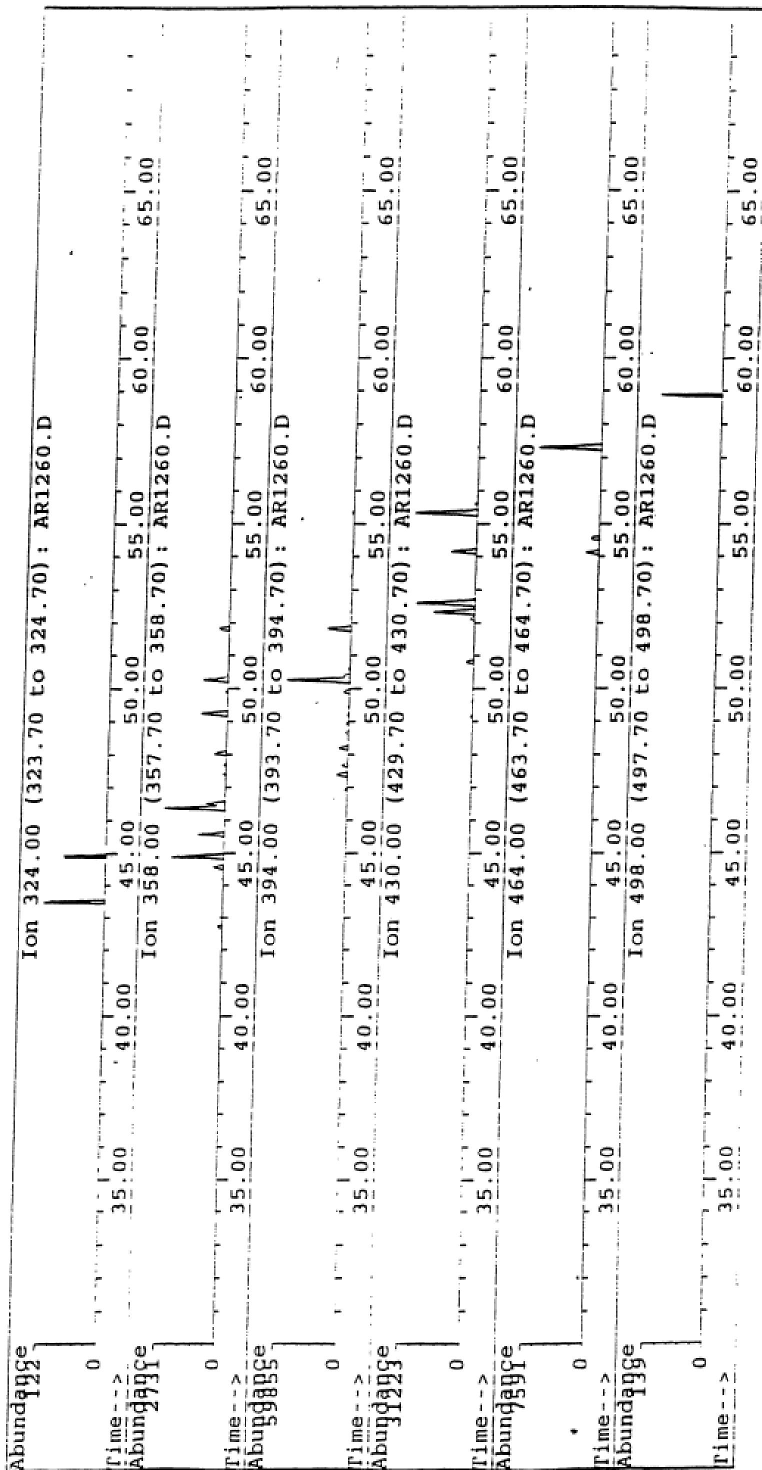


Figure 7: RIC of Aroclor 1260 main compounds

7.2. Example of a selected ion monitoring programme useful for quantitative analysis of chlorinated compounds.

Compounds	Fraction N° on Florisil	Retention Time (min.)	Target Ion (daltons)
HCB	1	37-38	284
Heptachlor	1	44-45	266
Aldrin	1	46-48	237
op DDE	1	51-53	246
Transnonachlor	1	52-54	444
pp' DDE	1	53-55	281
PCBs			
3 Cl	1		258
4 Cl	1		292
5 Cl	1	40-55	324
6 Cl	1	40-55	358
7 Cl	1	45-55	394
8 Cl	1	45-60	430
9 Cl	1	50-60	464
10 Cl	1	58-60	498
α HCH	2	37-39	255
β HCH	2	39-41	255
γ HCH (Lindane)	2	39-41	255
δ HCH	2	41-43	255
γ Chlordane	2	51-53	410
α Chlordane	2	52-54	266
op DDD	2	54-56	248
pp' DDD	2	56-58	248
op DDT	2	56-58	246
pp' DDT	2	58-60	283
Heptachlor epoxide	3	49-51	318
α Endosulfan	3	52-54	406
Dieldrin	3	53-55	346
Endrin	3	55-57	346
β Endosulfan	3	55-57	406
Endosulfan sulfate	3	58-60	386

8. NOTES ON WATER ANALYSIS

The levels of lipophilic compounds in tissues of aquatic organisms and organic fractions of sediments are determined to a large extent by the levels of these compounds in the surrounding water (marine mammals are an obvious exception). Data for CBs and hydrocarbons in sea water is therefore extremely useful for an understanding of the levels in organisms. However, the levels in sea water are extremely low and consequently, their determination needs considerable experience. Large volumes of water are required and extreme care has to be taken in order to avoid contamination during sampling, extraction and clean-up of the samples. Details are described in Manual and guide No 27 of IOC, 1993 and Villeneuve J.P. (1986).

9. ALTERNATIVE PROCEDURES

9.1. Combining sample preparation and extraction for chlorinated and petroleum hydrocarbons in sediment samples.

In the event that analyses for petroleum hydrocarbons and chlorinated compounds (and/or sterols) are of interest, the following extraction procedure can be used. To the freeze-dried sample introduce internal standards for each compound class. The following are suggested: 1) aliphatic hydrocarbons: - n-C_{14} d_{30}, n-C_{19} d_{40}, n-C_{32} d_{66}, 2) polycyclic aromatic hydrocarbons: Naphthalene d_8,

Hexamethylbenzene, Cadalene (deuterated PAHs are also useful), 3) organochlorine compounds: PCB congeners 29, 30, 121 or 198, ε HCH and Endosulfan Id_4, 4) sterols: 5 α (H)-androstan-3β-ol. These standards are used for quantifying the recovery of the total procedure. Samples are Soxhlet extracted for 8 hours with 250 ml of a mixture hexane / dichloromethane (50:50), cycling the solvent through at a rate of 4 to 5 cycles per hour. The solvent extract is concentrated by rotary evaporation down to 15 ml and transferred to a Kuderna-Danish tube. It is then further concentrated down to 5-6 ml under nitrogen gas. Following removal of sulphur and water, the extract is separated into aliquots: 1/3 for petroleum hydrocarbons and sterols and 2/3 for chlorinated hydrocarbons.

Note: Mercury method should be used only if chlorinated pesticides and PCBs are analysed. If the combined method is used for petroleum and chlorinated hydrocarbons, then the copper method should be used instead of mercury that will destroy some of the PAHs.

9.2. Supercritical fluid extraction (SFE) of marine samples

Sample preparation is probably the most time-consuming and labor-intensive analytical task performed in a laboratory. Studies shows that 60 % of the overall sample analysis time is spent in sample preparation which is the main source of error and of contamination. In addition, the amount of hazardous chemicals used for sample preparation is a continuous source of concern. Due to safe handling and disposal requirements, the reduction of their use is a priority for laboratories worldwide.

Supercritical fluids are gases (i.e. N_2O and CO_2) at room temperature and pressures above the critical point. The SFE technique allows an efficient extraction of a variety of contaminants with considerable reduction in the analysis cost, sample amount and allows the extraction of the thermal sensitive substances, reducing the amount of environmentally hazardous solvents.

A small change in the pressure of a supercritical fluid results in a big change in its density and the solvent strength of the fluid changes with changing density. As a result, one supercritical fluid easily performs the work of many solvents. If this is not enough, it is possible to add a modifier, such as methanol (a few per cent) to increase the solvating range of the fluid. Therefore, SFE should speed up the sample preparation process, minimising the wastes associated with the analysis.

Until now, the main fields of analytical applications of SFE are related to environmental studies and to the food-processing industry (Hawthorne, 1990, Bayona, 1993). A method using carbon dioxide (80°C-340 atm) for the extraction of total petroleum hydrocarbons has been approved as an EPA standard method. The extraction efficiency of modified CO_2 for the recovery of 41 organochlorine and 47 organophosphorus pesticides spiked on sand at different pressures and temperatures were higher than 80%. Furthermore, by increasing the extraction temperature up to 200°C, PCBs and PAHs can be extracted from naturally occurring samples with neat CO_2. Nam *et al.* (1991), have developed a method for rapid determination of polychlorinated organics in complex matrices. The method is based on direct coupling of supercritical fluid extraction with tandem supercritical fluid chromatography and gas chromatography. The on-line system permits simultaneous extraction and analysis with high reproducibility and accuracy.

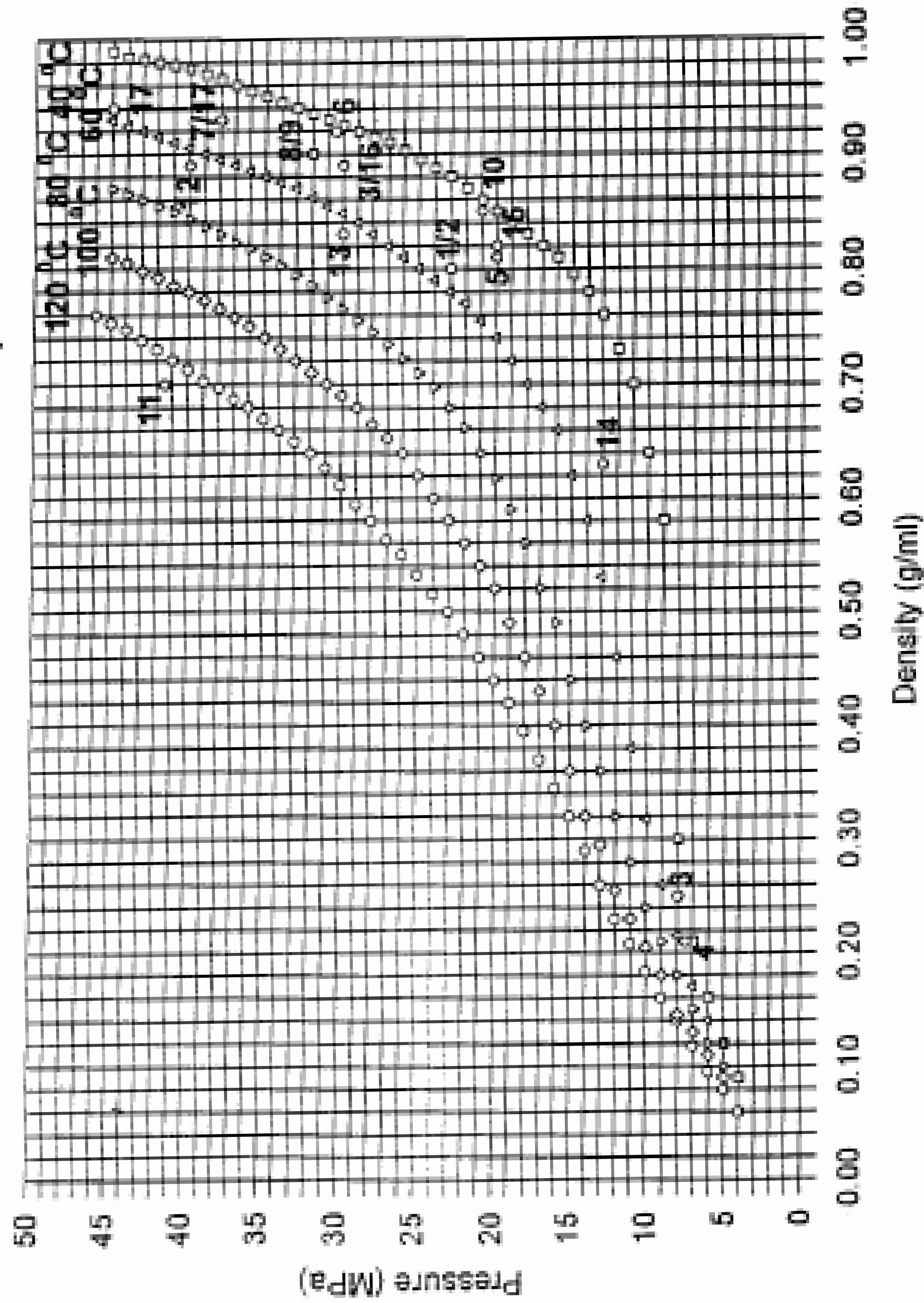


Figure 8: Guide for CO_2 extractions

9.3. Microwave assisted extraction for marine samples

9.3.1 Sediment

Another alternative method for the extraction of chlorinated hydrocarbons in sediment samples (or combined extraction for chlorinated hydrocarbons and petroleum hydrocarbons) is the use of the Microwave oven instead of the Soxhlet extractor. The main advantage of the microwave oven is the fact that, for one sample, only 40 ml of solvent mixture are used instead of 250 ml for clean-up of extraction thimbles and 250 ml for the extraction itself.

10 to 15 grams of freeze-dried sediment sample, ground and sieved at 250 µm, are put in the glass tube of the reactor. Appropriate internal standards (for OCs and/or PHs, see10.1.) are added to the sample for recovery and samples are extracted with 40 ml of a mixture of hexane / dichloromethane (50:50).

Extraction is realised within the following cycle:

- Power of the microwaves: 1200 watts
- Temperature increase to 115 °C in 10 minutes.
- Extraction maintained at 115 °C for 30 minutes
- Cooling to ambient temperature within one hour.

The carrousel containing 14 reactors, 12 samples could be extracted together with one blank and one Reference Material within 1 and half hour and with 10 times less solvent mixture than the standard Soxhlet extraction.

After cooling down to room temperature the solvent mixture is recovered in a 100 ml glass flask. The sediment is poured in a glass funnel containing a plug made of glass wool. The extracted sediment is washed with 10 - 20 ml of hexane. The extract follows then the procedure of clean-up and fractionation.

9.3.2 Biota

3 to 8 grams of freeze-dried biota sample is accurately weighted, the weight to be extracted is noted, and it is placed into the pre-cleaned glass tube of the reactor. A known amount of internal standard is added to the sub-sample in the tube before extraction. Candidate internal standards are the same than for sediment samples refers to section (5.3.1.)

Extraction is realized with 30 ml of a mixture hexane / acetone (90:10) within the following cycle:

- Power of the microwaves: 1200 watts
- Temperature increase to 115 °C in 10 minutes.
- Extraction maintained at 115 °C for 20 minutes
- Cooling to ambient temperature within one hour.

The carrousel containing 14 reactors, 12 samples could be extracted with one blank and one Reference Material within 1 and half hour and with 10 times less solvent mixture than the standard Soxhlet extraction.

After cooling down to room temperature the solvent mixture is recovered in a 100 ml glass flask. The powder of biota is poured in a glass funnel containing a plug made of glass wool. The extracted biota is washed with 10 - 20 ml of hexane. The extract is then concentrated with rotary evaporator and ready for E.O.M, clean-up and fractionation procedure.

10. DATA INTERPRETATION

10.1. DDT

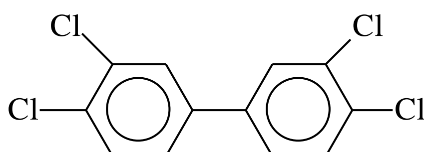
The residence time of total DDT in the environment is relatively short (t1/2 = 3-5 years), so, at least 75-80 % of the current total DDT should be in the form of DDE or DDD if it was introduced into the environment before the 1975 ban. Values of Henry's law constant indicate that these compounds can reach the troposphere as vapour. These vapours are little adsorbed by airborne particulate matter and represent the major component in atmospheric chlorinated hydrocarbon levels. Vapour movements of these pollutants suggest that restrictions and regulations operating in the more technically advanced countries could only be partially effective on a worldwide basis.

The presence of the op DDT together with anomalous pp' DDT values in environmental samples indicates a recent treatment with this insecticide.

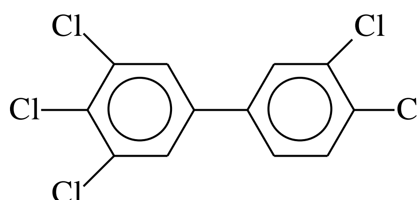
10.2. PCBs congeners

Among the 209 possible PCB congeners, seven of them: 28, 52, 101, 118, 138, 153 and 180, were selected as the most relevant because of their distribution in the chromatogram and in the chlorination range.

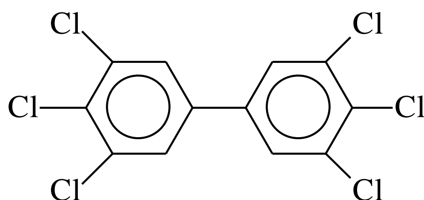
Recently, attention has been paid to congeners having 2 para-chlorines and at least 1 meta-chlorine. These congeners are called "coplanar" PCBs. Among the 209 congeners, 20 members attain coplanarity due to non-ortho chlorine substitution in the biphenyl ring. Three of these show the same range of toxicity as the 2,3,7,8 tetrachlorodibenzo-p-dioxin and the 2,3,7,8 tetrachlorodibenzofuran, these are the IUPAC Nº: 77, 126 and 169. These compounds should be identified and quantified in the environmental samples with high priority. They can be separated using fractionation with carbon chromatography (Tanabe *et al*., 1986).



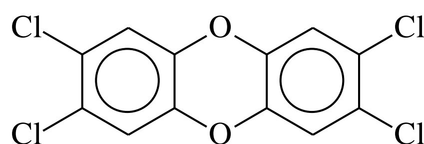
3,3',4,4' tetrachlorobiphenyl
IUPAC Nº: 77



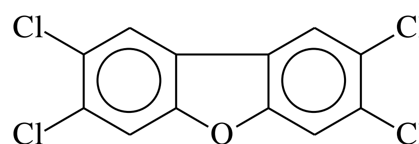
3,3',4,4',5 pentachlorobiphenyl
IUPAC Nº: 126



3,3',4,4',5,5' hexachlorobiphenyl
IUPAC Nº: 169



2,3,7,8 tetrachlorodibenzo-p-dioxin



2,3,7,8 tetrachlorodibenzofuran

10.3. Typical profiles of commercial mixtures

Formulations available in different countries are slightly different in their composition (Aroclor in USA, Kanechlor in Japan, Clophen in Germany, Phenoclor in France, Fenclor in Italy or Sovol in Russia). For the same global composition, such as Aroclor 1254, KC-500 or Phenoclor DP-5, the composition of individual congeners differs by 5-10 %. If a sample is collected on the French coast (therefore, contaminated with DP-5), and is quantified with DP-5 and Aroclor 1254, the difference observed in concentration could be in the order of 5-10 %. This shows the importance of choosing one common standard for the quantification of global industrial formulations or the importance of quantifying with individual congeners.

Percent contribution of individual chlorobiphenyls to Clophen A 50 and Aroclor 1254.

PCB N°	Clophen A50	Aroclor 1254	PCB N°	Clophen A50	Aroclor 1254
17	0	0.19	115	0.28	0.3
18	0	0.41	118	10.9	6.39
28	0.05	0.25	119	0.19	0.14
31	0.05	0.22	122	0.19	0.5
33	0.11	0.14	123	0.85	0.81
40	0.28	0.2	126	0.08	0
41	0.83	0.64	128	3.04	2.07
42	0.13	0.23	129	0.83	0.23
44	2.46	2.03	130	0.83	0.63
47	0.18	0.11	131	0.06	0.16
48	0.17	0.14	132	2.57	1.98
49	1.96	1.64	134	0.52	0.49
52	5.53	5.18	135	1.61	1.62
53	0.06	0.09	136	0.91	1.12
56	0.44	0.58	137	0.25	0.25
60	0.34	0.54	138	3.61	3.2
63	0.15	0.05	141	0.98	1.04
64	0.71	0.45	146	0.8	0.83
66	0.5	0.59	149	4.5	2.21
67	0.13	0.09	151	1.22	1.17
70	3.85	3.21	153	4.17	4.26
74	1.35	0.78	156	1.43	1.62
82	1.05	0.95	157	0.31	0
83	0.53	0.45	158	0.98	0.77
84	2.08	1.95	167	0.35	0.21
85	1.85	1.66	170	0.65	0.31
87	4.22	3.78	171	0.5	0.5
90	0.85	0.93	172	0.09	0.05
91	0.92	0.83	173	0.09	0.09
92	1.53	1.58	174	0.37	0.34
95	6	6.02	175	0.11	0.05
96	0.05	0.08	176	0.43	0.32
97	2.8	2.55	177	0.21	0.21
99	4.06	3.6	178	0.19	1.35
100	0.15	0.1	179	0.2	0.21
101	7.72	7.94	180	0.53	0.38
105	1.9	3.83	183	0.21	0.17
107	0.94	0.72	187	0.3	0.32
110	6.27	5.85	190	0.05	0.08
			201	0.6	0.68

11. QUALITY ASSURANCE / QUALITY CONTROL

Guidelines on the QA/QC requirements for analysis of sediments and marine organisms are detailed in Reference Method No 57, "Contaminant monitoring programs using marine organisms: Quality assurance and good laboratory practice". Brief descriptions of issues that must be addressed in the course of understanding the procedures described here are given below.

11.1. Precision

The precision of the method should be established by replicate analysis of samples of the appropriate matrix. Estimate the precision of the entire analytical procedure by extracting five sub-samples from the same sample after homogenisation. Alternatively, perform replicate analysis of an appropriate certified reference material (RM; see below) containing the analytes of interest. The principal advantage of using a RM is that the material permits the simultaneous evaluation of accuracy while offering a well homogenised sample. Precision should be evaluated as a matter of course during the initial implementation procedure just before initiation of sample analysis.

11.2. Accuracy

The accuracy of the methods described here must be confirmed by analysis of a suitable RM (i.e. appropriate matrix, analytes) prior to initiation of sample analysis. Agreement between measured and certified concentrations for any individual analyte should be within 35 % and on average within 25%. It is advisable to introduce RMs on a regular basis (e.g. every 10-20 samples) as a method of checking the procedure. Further description of the preparation of control charts and criteria for data acceptance are discussed in Reference Method N° 57.

11.3. Blanks

Blanks represent an opportunity to evaluate and monitor the potential introduction of contaminants into samples during processing. Contributions to the analyte signal can arise from contaminants in the reagents, those arising from passive contact between the sample and the environment (e.g. the atmosphere) and those introduced during sample handling by hands, implements or glassware. It is essential to establish a consistently low (i.e. with respect to analytes) blank prior to initiating analysis or even the determination of the method detection limit. In addition, it is necessary to perform blank determinations on a regular basis (e.g. every batch of samples).

11.4. Recovery

Recovery reflects the ability of the analyst to fully recover surrogate compounds introduced to the sample matrix or blank at the beginning of the procedure. The primary criteria for selection of compounds to be used for testing recovery are that they: 1) have physical (i.e. chromatographic/partitioning) properties similar to and if necessary spanning those of the analytes of interest, 2) do not suffer from interferences during gas chromatographic analysis, 3) are baseline resolved from the analytes of interest.

Recovery should be tested on all samples and blanks as a routine matter of course. Recoveries below 70% are to be considered unacceptable. Recoveries in excess of 100 % may indicate the presence of interference.

11.5. Archiving and reporting of results

Every sample should have an associated worksheet which follows the samples and the extracts through the various stages of the procedure and upon which the analyst notes all relevant details. An example of such a worksheet is given below. Each laboratory should construct and complete such a worksheet. Relevant chromatograms should be attached to the worksheet. Analyses should be grouped

and composite or summary analysis sheets archived with each group. Final disposal of the data will depend on the reasons for which it was collected but should follow the overall plan model.

All processed samples should be archived at all steps of the procedure:

- deep frozen (in the deep-freezer as it was received).
- freeze-dried (in sealed glass container kept in a dark place).
- extracted (after injection on the GC, sample extracts should be concentrated down to 1 ml and transferred into sealed glass vials, a Pasteur pipette sealed with a butane burner is adequate and cheap).

Sample: IAEA-357 : Marine Sediment

wet wt.
-------- = ..., % water in freeze dried sample determined by drying at 105°C : .....
dry wt.

.......g freeze-dried wt. extracted with hexane in Soxhlet extractor for 8 hours.

.......pg PCB Nº29, ...pg PCB Nº198, ...pg ε HCH and ….. pg Endosulfan Id_4 were added as internal standard.

The ....ml extract was reduced by rotary evaporator to approximately ......ml.

This was treated with sodium sulfate to dry the extract. Then treated with mercury to remove sulphur. This was further reduced to ....ml for lipid determinations. Corrected dry wt. : ....g.

Lipid determinations:

.............ml total extract;

10 µl aliquots weighed on micro-balance: ....mg; ....mg; ....mg.

HEOM = ...mg/g dry weight.

...........mg lipid subjected to column chromatography fractionation on Florisil.

F1: ....ml hexane

F2: ....ml hexane/dichloromethane (70:30)

F3: ....ml dichloromethane

GC determinations:

PCB Nº29 : ....ng recovered in F1 : ....% Recovery.

PCB Nº198 : ....ng recovered in F1 : ....% Recovery.

ε HCH : ....ng recovered in F2 : ....% Recovery.

Endosulfan Id_4: ....ng recovered in F3 : ....% Recovery.

Attach tabulation of individual compounds quantified in sample.

Sample worksheet for analysis of chlorinated compounds in marine sediments.

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ANNEX

PREPARATION OF THE SOLUTION OF INTERNAL STANDARDS: PCB No 29, PCB No 198, ε HCH and Endosulfan I d4

Stock Solution of PCB No 29:

1 ml from the original vial (250ng/µl) should be transferred into a 100 ml volumetric flask, and then the volume is adjusted to 100 ml with hexane. This stock solution contains:

2.5 ng/µl of PCB No 29

Stock Solution of Endosulfan I d4:

1 ml from the original vial (250ng/µl) should be transferred into a 100 ml volumetric flask, and then the volume is adjusted to 100 ml with hexane. This stock solution contains:

2.5 ng/µl of Endosulfan I d_4

Working solution of internal standards:

0.5 ml from the stock solution of PCB No 29 (2.5 ng/µl) should be transferred into a 50 ml volumetric flask, then, 0.5 ml from the stock solution of Endosulfan I d4 (2.5 ng/µl) should be transferred into the volumetric flask, then 1 ml from the original vial (1ng/µl) of ε HCH should be transferred into that volumetric flask, then 0.5 ml from the concentrated solution (2ng/µl) of PCB No 198, and the volume adjusted to 50 ml with hexane. This working solution contains:

25 pg/µl of PCB No 29
20 pg/µl of PCB No 198
20 pg/µl of ε HCH
25 pg/µl of Endosulfan I d4

CAUTION: VIALS SHOULD BE COOLED AT 20°C PRIOR TO OPENING

Preparation of the Aroclor 1254 solution

Preparation of the stock solution:

1 ml from the original vial should be transferred into a 100 ml volumetric flask, then, the volume is adjusted to 100 ml with hexane. This stock solution contains:

6.5 ng/µl of Aroclor 1254

Preparation of the working solution:

1 ml from this stock solution should be transferred into a 50 ml volumetric flask and the volume adjusted to 50 ml with hexane. This working solution contains :

0.13 ng/µl of Aroclor 1254

CAUTION : VIAL SHOULD BE COOLED TO 20 °C PRIOR TO OPENING

Preparation of the Aroclor 1260 solution

Preparation of the stock solution:

1 ml from the original vial should be transferred into a 100 ml volumetric flask, then the volume is adjusted to 100 ml with hexane. This solution contains:

5.44 ng/µl of Aroclor 1260

Preparation of the working solution:

1 ml from the stock solution should be transferred into a 50 ml volumetric flask, then the volume is adjusted to 50 ml with hexane. This working solution contains

0.1088 ng/µl of Aroclor 1260

CAUTION: VIAL SHOULD BE COOLED TO 20 °C PRIOR TO OPENING

Preparation of the pp' DDE, pp' DDD and pp' DDT solution

pp' DDE:

Stock solution: 1 ml from the original vial should be transferred into a 100 ml volumetric flask, then the volume is adjusted to 100 ml with hexane. This stock solution contains:

5 ng/µl of pp' DDE

pp' DDD:

Stock solution: 1 ml from the original vial should be transferred into a 100 ml volumetric flask, then the volume is adjusted to 100 ml with hexane. This solution contains:

5 ng/µl of pp' DDD

pp' DDT:

Stock solution: 1 ml of the original vial should be transferred into a 100 ml volumetric flask, then the volume is adjusted to 100 ml with hexane. This stock solution contains:

5 ng/µl of pp' DDT

Working solution: pp' DDE, pp' DDD and pp' DDT together.

1 ml from the stock solution of pp' DDE, 2 ml of the stock solution of pp' DDD and 3 ml of the stock solution of pp' DDT should be transferred into a 100 ml volumetric flask and the volume adjusted to 100 ml with hexane. This solution contains

- pp' DDE : 50 pg/µl
- pp' DDD : 100 pg/µl
- pp' DDT : 150 pg/µl

NOTE: Further dilution may be necessary depending on the sensitivity of the EC Detector.

CAUTION: VIAL SHOULD BE COOLED TO 20 °C PRIOR TO OPENING

Preparation of Aldrin, Diedrin and Endrin standard solutions:

Aldrin:

Stock solution: 1 ml from the original vial should be transferred into a 100 ml volumetric flask, then the volume is adjusted to 100 ml with hexane. This solution contains:

5 ng/µl of Aldrin

Dieldrin:

Stock solution: 1 ml from the original vial should be transferred into a 100 ml volumetric flask, then the volume is adjusted to 100 ml with hexane. This solution contains:

5 ng/µl of Dieldrin

Endrin:

Stock solution: 1 ml from the original vial should be transferred into a 100 ml volumetric flask, then the volume is adjusted to 100 ml with hexane. This solution contains:

5 ng/µl of Endrin

Working solution: Aldrin, Dieldrin and Endrin together.

1 ml from the stock solution of Aldrin, 1 ml from the stock solution of Dieldrin and 1 ml from the stock solution of Endrin are transferred into a 100 ml volumetric flask and the volume is adjusted to 100 ml with hexane. This working solution contains:

Aldrin : 50 pg/µl
Dieldrin : 50 pg/µl
Endrin : 50 pg/µl

NOTE: Further dilution may be necessary depending on the sensitivity of the detector.

CAUTION: VIALS SHOULD BE COOLED TO 20 °C PRIOR TO OPENING

Preparation of the HCB and Lindane standard solutions:

HCB:

Stock solution: 1 ml from the original vial should be transferred into a 100 ml volumetric flask, then the volume is adjusted to 100 ml with hexane. This solution contains:

5 ng/µl of HCB

Lindane:

Stock solution: 1 ml from the original vial should be transferred into a 100 ml volumetric flask, then the volume is adjusted to 100 ml with hexane. This solution contains:

5 ng/µl of lindane

Working solution:

1 ml from the stock solution of HCB and 1 ml from the stock solution of Lindane are transferred into a 100 ml volumetric flask, then the volume is adjusted to 100 ml with hexane. This solution contains:

HCB	:	50 pg/µl
Lindane	:	50 pg/µl

NOTE: further dilution may be necessary depending on the sensitivity of the EC Detector.

CAUTION: VIALS SHOULD BE COOLED TO 20 °C PRIOR TO OPENING

Preparation of the PCB congeners solution

In a 100 ml volumetric flask, transfer 1 ml from the original vial. Adjust to 100 ml with hexane in order to obtain the working solution with the following concentrations:

CB Nº:	Compounds:	Concentrations (pg/µl)
8	2,4’	17.50
18	2,2’,5	12
31	2,4’,5	10.6
28	2,4,4’	4.6
52	2,2’,5,5’	8.6
49	2,2’,4,5’	12.1
44	2,2’,3,5’	10.7
66	2,3’,4,4’	5.5
95	2,2’,3,5’,6	5.7
101	2,2’,4,5,5’	9.3
110	2,3,3’,4’,6	11.1
149	2,2’,3,4’,5’,6	12.1
118	2,3’,4,4’,5	8.5
153	2,2’,4,4’,5,5’	8.4
138	2,2’,3,4,4’,5’	13.8
183	2,2’,3,4,4’,5’,6	10.3
174	2,2’,3,3’,4’,5,6’	9.4
177	2,2’,3,3’,4’,5,6	9.5
180	2,2’,3,4,4’,5,5’	16.3
170	2,2’,3,3’,4,4’,5	13.4
199	2,2’,3,3’,4,5,5’,6’	9.3
194	2,2’,3,3’,4,4’,5,5’	12.6

Separate into 10 volumetric flasks of 10 ml, seal with Teflon tape and keep in refrigerated place in order not to evaporate them.

<u>CAUTION: VIAL SHOULD BE COOLED TO 20 °C PRIOR TO OPENING</u>

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Annex V:

HELCOM (2012). COMBINE - Guideline for the determination of heavy metals in biota. Annex B-12, Appendix 4: Technical note on the determination of trace metallic elements in biota (5.2.1)

HELCOM Manual for marine monitoring in the COMBINE programme

ANNEX B-12, APPENDIX 4: TECHNICAL NOTE ON THE DETERMINATION OF TRACE METALLIC ELEMENTS IN BIOTA

1. INTRODUCTION

Metallic elements appear in different marine biological matrices in trace concentrations, ranging from the mg/kg through the ƒÊg/kg to the ng/kg level. Stoeppler (1991) provided a comprehensive review of the most frequently used techniques for quantitative analysis of metallic trace elements, such as optical atomic absorption, fluorescence or emission spectrometry, anodic, cathodic or adsorptive stripping voltammetry, isotope dilution mass spectrometry and total reflection X-ray fluorescence, respectively. In spite of the powerful instrumental techniques presently in use, various analytical error sources have to be taken into consideration that may significantly influence the accuracy of the analytical data.

2. WORKING CONDITIONS

For each step of the analytical procedure, contamination of the sample may occur from the environment (laboratory air dust particles and the analyst), from sample containers or packing materials, from instruments used during sample pre-treatment and sample preparation, and from the chemical reagents used for analysis. The predominant purpose of the analytical clean laboratory is to eliminate contamination, which may be airborne or laboratory-induced, as far as possible and to control the total analytical blank.
Contamination by particles from the laboratory air may be controlled by a high-efficiency particulate filter. (A clean room is designed to maintain air with 100 particles per ft3 or 3.6.103 per m3 of 0.5 ƒÊm particles (class 100 of U.S. Federal Standards 209), or better, preferably with a minimum of activity in the room.) U.S. Federal Standards 209 describes designs for complete laminar flow rooms, clean benches, and fume hoods, and contains information on design, testing, and maintenance of clean rooms, and should be considered an essential reference for those interested in a clean laboratory.
To control the analytical blank for analysis of metallic trace elements, one must not only maintain good laboratory air quality, but also select the appropriate composition and type of construction materials used to build the laboratory. Principally, contaminants must be effectively removed at the source to minimize their uncontrolled distribution in the analytical clean laboratory. Accordingly, the laboratory's walls should be cleaned easily and therefore painted with special metal-free wipe-resistant paints. Surfaces of working areas should be protected with, for example, disposable plastic (polyethylene, PTFE) foils. The floors should, for example, be covered with adhesive plastic mats. Details of the design that are essential for obtaining a working laboratory with low trace element blanks are described by Moody (1982), Mitchell (1982a), Boutron (1990), and Schmidt and Gerwinski (1994).

3. PREATREATMENT OF LABORATORY WARE AND REAGENTS, CONTAMINATION CONTROL

Chemically resistant materials, used in the production of high-quality laboratory ware appropriate for metallic trace element analysis, include low- and high-density polyethylene (LDPE and HDPE), polypropylene (PP), polytetrafluorethylene (PTFE), perfluoralkoxy (PFA), ethylenetetrafluorethylene (ETFE), tetrafluorethyleneper- fluorpropylene (FEP), borosilicate and quartz glass, respectively. With appropriate pretreatment and handling, these materials meet the requirements of purity necessary for the required analytical investigations. Cleaning procedures for plastic and glass laboratory ware were comprehensively dealt with by Moody and Lindstrom (1977), Tschopel et al. (1980), Kosta (1982) and Boutron (1990). Generally, immersion in diluted (10-25 % v/v) high-purity nitric acid at room temperature for a period of one to three days, followed by repeated rinsing with high-purity water, is recommended. Steaming in high-purity acids (predominantly nitric acid) is also very effective to remove impurities from container surfaces and condition them for subsequent analysis.

The materials mentioned above for the production of laboratory ware exhibit some adsorptive or exchange properties. Boundary-surface interactions can be important, particularly when very dilute analytical solutions are being handled, since uncontrollable losses through sorption of element ions can occur (Tschopel et al., 1980; Harms, 1985). Based on this information, it is imperative that volumetric flasks, reagent vessels, pipette tips, etc., for handling samples, sample solutions and low-level reference or analyte solutions must never be used for transferring or processing stock calibration solutions, analytes solutions or concentrated reagents. Considerable quantities of analytes may be adsorbed from such solutions by the respective container surfaces, residuals of which may be leached later when dilute sample or analyte solutions are handled.
The availability of high-purity reagents is a key condition for reliable investigations of metallic trace element concentrations. For many analytical problems, the level of a specific contaminant can adequately be controlled only by applying specific purification methods. The first order of priority in regard to high-purity reagents is a sufficient supply of high-purity water. Ion-exchange units are universally accepted as an effective means of removing dissolved ionic species from water. Since high-purity water is frequently used in metallic trace element analysis, equipment for sustainable production of high-purity water by high-purity mixed-bed ion exchange resins should be available. The next most important group of reagents are mineral acids. Contamination of the sample by residual concentrations of metallic trace elements in the acids used for dissolution or decomposition represents a major problem. Purification of the acids is essential to ensure acceptable blanks.
Isothermal (isopiestic) distillation can produce volatile acids (and ammonia) of medium concentration in high-purity form. For example, pure hydrochloric acid (and ammonia) can be generated by placing an open container of concentrated reagent-grade acid adjacent to a

container of high-purity water, within a closed system (such as a desiccator) at room temperature. Acid vapours are continuously transferred into the water until equilibrium is obtained. Purification by sub-boiling distillation is based on motionless evaporation of the liquid by infrared heating at the surface to prevent violent boiling. Different purification systems are described in detail by Matthinson (1972), Kuehner et al. (1972), Dabeka et al. (1976), Tschopel et al. (1980), Mitchell (1982b), Moody and Beary (1982), Moody et al. (1989), and Paulsen et al. (1989). Acids of extremely high purity are produced by multiple batchwise distillation of reagent-grade acids in a silica apparatus, which is placed in a laminar-flow hood.

4. SAMPLE PRETREATMENT

If the determinands are heterogeneously distributed in the sample material, it may be preferable to homogenize prior to taking subsamples for analysis. However, this procedural step is problematic, since uncontrollable contamination through the homogenizing tool may occur. Cryogenic homogenization at liquid nitrogen temperature and application of high-purity material such as quartz, PTFE, titanium or stainless steel for the construction of homogenizing devices may help to minimize contamination (Iyengar, 1976; Iyengar and Kasperek, 1977; Klussmann et al., 1985).

5. SAMPLE DECOMPOSITION

For accurate direct measurements of metallic trace element contents in biological matrices, appropriate calibration (reference) standards are lacking in most instances. Therefore, multi-stage, easy to calibrate methods are still necessary, which include decomposition procedures and transformation of biological material into solution.
As a general rule wet sample is to be subject to decomposition procedures to avoid contamination or loss of determinands. A general sample decomposition procedure cannot be recommended due to the diverse composition of materials to be analysed, as well as to the different elements to be determined, and also because of the variety of possible analytical methods applied. However, the following minimum requirements should be met:

- complete destruction of all organic material of the sample,
- avoidance of determinand losses,
- avoidance of contamination.

Complete decomposition of the organic matrix is a prerequisite for a variety of the subsequently used instrumental determination techniques. Residual dissolved organic carbon from biological materials incompletely disintegrated after decomposition with nitric acid causes problems particularly in voltammetric and polarographic determinations. Both are sensitive to interference from chelating and electroactive organic components coexisting in incompletely decomposed samples during analysis (Pratt et al., 1988; Wurfels

et al., 1987, 1989). Residual dissolved organic carbon compounds even of low molecular weight can change the equilibria in the spray chambers for sample introduction in atomic emission spectrometry (AES), optical emission spectrometry (OES), and atomic absorption spectrophotometry (AAS) by changing the viscosity of the sample solution. In such cases, comparison with pure aquatic calibration standard solutions can lead to erroneous results. In graphite furnace atomic absorption spectrophotometry (GFAAS), residual organic carbon may undergo complicated secondary reactions with the analyte prior to or during the atomization process. Such 'matrix interferences' alter the rate at which atoms enter the optical path relative to that obtained for an undisturbed element standard (Harms, 1985; and other references cited there).

The comparatively simple dry ashing method using a muffle furnace is problematic, since both uncontrollable losses of the determinands and contamination through contact with the furnace material may occur.

Both, application of a carefully developed and controlled temperature programme and modifying the matrix prior to the ashing procedure (addition of ashing aids agents) may be suitable to prevent losses of volatile elements (special analytical problems concerning mercury determination are described in Attachment 1). The use of special materials (quartz, titanium, stainless steel) for the construction of sample containers may be helpful to minimise contamination.

In the widely applied wet ashing procedure in open systems, the sample is treated with acids, mainly nitric, sulphuric and perchloric acids, in different ratios and under different conditions. Usually large quantities of reagents and voluminous apparatus with large surfaces are needed for complete destruction of the organic material. Serious contamination problems (too high blank values) may arise, if insufficiently purified acids are used.

The rate of reaction and efficiency of acid decomposition increase substantially with elevated temperatures. Accordingly, closed-vessel techniques, using conventional heating or microwave energy, have an advantage over open systems. As a result of the closed systems with vessels manufactured of dense and very pure material (PTFE, PFA, quartz), loss of elements through volatilisation and contamination by desorption of impurities from the vessel surface are significantly reduced. In addition, since only small quantities of high-purity acid (usually nitric acid) need to be used, extremely low analytical blanks can be obtained. Kingston and Jassie (1986, 1988) comprehensively considered the fundamental parameters governing closed vessel acid decomposition at elevated temperatures using a microwave radiation field. Microwave systems enable a very fast energy transfer to the sample and a very rapid build up of high internal vessel temperature and pressure, with the advantage of an enormous reduction in digestion time occurs. Furthermore, a reduction of acid volume

(McCarthy and Ellis, 1991) and contamination reduction during the decomposition process were found (Dunemann, 1994; Sheppard et al., 1994).

The application of microwave energy must be carefully controlled to avoid explosions; a pressure-relief system is recommended for safe operation (Gilman and Grooms, 1988). At this stage of development, it can be concluded that advances in pressure and temperature feedback control features have contributed to the acceptance of microwave sample decomposition in analytical chemistry.

6. CALIBRATION

For calibration purposes, single element standard stock solutions at a concentration of 1000 mg/l, purchased from a qualified manufacturer, should be available. The actual concentration of the named element should be stated on the label together with the date of the preparation of the standard solution.

Fresh stock standard solutions should be compared with the old standard solutions. Traceability can be ensured by the use of CRM(s) or participation in intercomparison exercises (EURACHEM, 2003).

Single or mixed element working standard solutions for calibration purposes are prepared by dilution of the standard stock solutions using dilute acid, as required.
Both stock standard and working standard solutions are stored in polyethylene, borosilicate or quartz volumetric flasks. Working standard solutions at concentrations less than 100 ƒÊg/l should be freshly prepared for every batch of samples and kept no longer than two weeks. The calibration procedure must meet some basic criteria in order to give the best estimate of the true (but unknown) element concentration of the sample analysed. These criteria are as follows:

• The amounts or concentrations of standards for the establishment of the calibration function must cover the range as related to practical conditions. The mean of the range should be roughly equal to the expected analyte concentration in the sample.
• The required analytical precision must be achievable and known throughout the entire range.
• The measured value (response) at the lower end of the range must be significantly different from the procedural analytical blank.
• The chemical and physical properties of the calibration standards must closely resemble those of the sample under investigation.
• The calibration standards must be processed through the entire analytical procedure in the same manner as the sample.

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

7. DETERMINATION

In an analytical series, especially with the number of samples >10, the control of calibration settings should be carried out with 2-3 calibration solution between environmental 10 samples. The analytical series should contain also a control sample of LRM or CRM.

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Annex VI:

IAEA (2013). Recommended methods for the determination of petroleum hydrocarbons in biological samples (5.2.2)

REPORT

RECOMMENDED METHOD FOR THE DETERMINATION OF PETROLEUM HYDROCARBONS IN BIOLOGICAL SAMPLES

IAEA/NAEL Marine Environmental Studies Laboratory in co-operation with UNEP/MAP MED POL

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RECOMMENDED METHOD FOR THE DETERMINATION OF PETROLEUM HYDROCARBONS IN BIOLOGICAL SAMPLES

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NOTE: This method is not intended to be an analytical training manual. Therefore the method is written with the assumption that it will be used by formally trained analytical chemists. Several stages of this procedure are potentially hazardous; user should be familiar with the necessary safety precautions.

In addition, the IAEA recommended methods are intended to be guidance methods that can be used by laboratories as a starting point for generating their own standard operating procedure. If performance data are included in the method they must not be used as absolute QC acceptance criteria.

1. Sampling

Detailed guidelines for collecting biological samples are available in Reference Method No 12 Rev.2, UNEP/FAO/IAEA/IOC.

2. General discussion

Following collection of biological samples using appropriate techniques, samples are stored in non-contaminating jars at -20 °C until analysis. For analysis, the samples are defrosted and prepared for solvent extraction. To achieve a satisfactory recovery of the petroleum hydrocarbons, samples are freeze-dried. Samples are then Soxhlet extracted using methanol. Following initial clean-up treatments (partial removal of lipids by saponification), extracts are fractionated using column chromatography with silica and alumina. Quantification is done by GC-FID and GC-MS. Complementary guidelines for the analytical procedures are available in the Reference Method No 20.

3. Apparatus

- Glass jars and aluminium foil, stainless steel knives, scoops, forceps, labels, marking pens, log book.
- Insulated plastic boxes for transporting samples. Ice or dry ice.

- Deep freezer (-18 to -20 °C) for sample preservation (frost free type freezers heat to above zero during frost removal cycles and they cannot be used for long term storage).
- Rotary evaporator.
- Soxhlet extraction apparatus and heaters or Microwave oven
- Glassware including boiling flasks, ground glass stoppers, beakers, Erlenmeyer flasks, separatory funnels, centrifuge tubes, weighing bottles, pipettes, tissue grinders.
- Drying oven (temperature range up to at least 300 °C) for determining sample dry weights, baking of contaminant residues from glassware and reagents.
- Centrifuge and tubes.
- Freeze-dryer and porcelain mortar and pestle.
- Analytical balance with a precision of 0.1 mg and an electro-balance with a precision of at least 1 µg.
- Stainless steel tweezers and spatulas.
- Dessicator - completely cleaned and with no grease applied to sealing edges.
- Supply of clean dry nitrogen.
- Columns for the silica/alumina chromatography.
- Mechanical blender (food mixer).
- Vacuum pump (water-jet air pump).
- Ultrasonic bath.

4. Reagents

4.1. List of reagents

- Demineralized distilled water produced by distillation over potassium permanganate (0.1 g $KMnO_4$ per liter) or equivalent quality, demonstrated free from interfering substances.
- Detergent.
- Sulfochromic cleaning solution made from concentrated sulfuric acid and potassium dichromate.

- Concentrated H_2SO_4 (d 20°C: 1.84 g/ml).
- H_2SO_4 1M
- KOH
- Potassium dichromate.
- Hexane," distilled in glass" quality.
- Dichloromethane, "distilled in glass" quality.
- Methanol, "distilled in glass" quality.
- Acetone, "distilled in glass" quality.
- Anhydrous sodium sulfate.
- Carborundum.
- Glass wool.
- pH Paper.
- Silica gel Merck Kieselgel 60 (0.04-0.063 mm, 230-400 mesh).
- Aluminium oxide neutral Merck 90 Active (0.063-0.200 mm, 70-230 mesh).
- n-C_{24}-d_{50}, Friedeline, Hexamethylbenzene , Naphthalene-d_8, Acenaphtene-d_{10}, Phenanthrene-d_{10}, Chrysene-d_{10}, Perylene-d_{12}, Fluorene-d_{10}, Benzo(a)pyrene-d_{12}.
- Standard solutions of aliphatic and aromatic hydrocarbons.

Working solutions from the stock reference solutions are prepared on a regular basis and stored in clean glass volumetric flasks tightly capped with non-contaminating materials such as Teflon or glass. Extreme care must be taken to ensure that standards have not changed their concentrations through solvent evaporation.

4.2. Cleaning of reagents and adsorbents

4.2.1. Cleaning of glassware

Scrub all glassware vigorously with brushes in hot water and detergent. Rinse with tap water and with distilled water. Rinse with acetone followed by hexane or alternatively bake overnight in an oven at 450 °C. All glassware should be stored in dust free cabinets and tightly sealed with pre-cleaned aluminum foil when not in use. Ideally glassware should be rinsed with the same solvent just before use.

For more detailed information, consult Reference Method No 65: UNEP/IOC/IAEA: Reagent and laboratory ware clean-up procedures for low level contaminant monitoring.

4.2.2. Cleaning of reagents

Powdered or crystalline reagents, such as anhydrous sodium sulfate (Na_2SO_4)*, glass wool* and carborundum boiling chips*, are thoroughly cleaned before use. They are extracted with hexane in a Soxhlet apparatus for 8 hours and then with methanol or dichloromethane for another 8 hours. For those indicated by an *, this will require pre-combustion in a muffle furnace at approximately 400 °C.

4.2.3. Cleaning of adsorbents

Preparation of silica and alumina: silica gel and alumina are pre-cleaned by Soxhlet extraction, first for 8 hours with methanol and then for 8 hours with hexane. They are dried at 50 °C to remove the solvent, then at 200 °C for 8 hours and then stored in amber bottle.

Before use, they are activated at 200 °C for 4 hours and partially deactivated with 5 % water.

The deactivation procedure is carried out by adding the water to the sorbent, and mixing by gentle shaking for a few minutes. The equilibration is reached overnight.

4.2.4. Cleaning of extraction thimbles

Paper extraction thimbles should be cleaned prior to sample extraction. For use in the extraction of biological samples, the extraction can be performed in the Soxhlet apparatus with 250 ml of a mixture methanol / dichloromethane (50:50) for 8 hours cycling the solvent through at a rate of 4 to 5 cycles per hour. Add into the solvent a few carborundum boiling chips to get a regular ebullition.

The use of disposable paper thimbles for the extraction procedure rather than re-usable glass fiber thimbles is recommended due to the difficulties encountered in cleaning the latter.

5. Procedure

5.1. Extraction of freeze-dried samples

A 50 to 100 g fresh weight sub-sample is selected from the sample. This sub-sample is weighed and freeze-dried. When the sub-sample appears to be dry, it is weighed again and the dry to wet ratio is calculated.

Just before starting analysis, as results have to be reported on a dry weight basis, the percent moisture or water content in a sample can be determined by weighing an aliquot, not used for analysis, of the sample before and after drying. The drying is done by heating a few grams (1-2 g) of the sample in an oven at 105°C for 24 hours to a constant weight.
The freeze-dried sub-sample is carefully pulverized in a cleaned pestle and mortar.

5 to 10 grams of freeze-dried sample are extracted with a Soxhlet extractor with 200 ml of methanol. Internal standards are added to the sample in the extraction thimble before extraction starts:

- *n*-C_{24}-d_{50} for the aliphatic hydrocarbon fraction (Friedeline as GC internal standard, spiked right before GC injection)
- Hexamethylbenzene for the unresolved compounds from the aromatic hydrocarbon fraction (Friedeline as GC internal standard, spiked right before GC injection)
- Naphthalene-d_8, Acenaphtene-d_{10}, Phenanthrene-d_{10}, Chrysene-d_{12}, Perylene-d_{12} for the aromatic hydrocarbon fraction (Fluorene-d_{10}, Benzo(a)pyrene-d_{12} as GC internal standard spiked right before GC injection)

After the extraction is completed, 20 ml of 2 M KOH are added to the flask and the extraction is continued for 2 more hours in order to saponify the lipids.

The content of the extraction flask is then transferred into a separatory funnel with 30 ml of water (distilled and extracted with hexane) and extracted with 90 ml of

hexane and re-extracted again twice with 50 ml of hexane. Then all hexane extracts are combined, filtered through glass wool and dried with anhydrous sodium sulfate.

The aqueous phase is not discarded as it is used for total lipid weight determination, it's acidified with 1 M sulfuric acid and extracted 3 times in a separatory funnel with 30 ml of hexane. Then all hexane extracts are combined, filtered through glass wool and dried with anhydrous sodium sulfate.

Alternative method:

Using the microwave oven: 3-5 g of biota is placed in a glass tube with 30 ml of methanol, the oven is set at 1200 Watts, the temperature is programmed to reach 115°C in 10 min. and then isothermal at 115°C for 20 min. (internal standards are added before extraction starts).

After cooling 5 ml of 2 M KOH are added to the glass tube and the oven is set at 1200 Watts, the temperature is programmed to reach 90°C in 5 min. and then isothermal at 90°C for 10 min. in order to saponify lipids.

The content of the glass tube is filtered through glass wool and transferred into a separatory funnel with 5 ml of water (distilled and extracted with hexane) and extracted with 20 ml of hexane and re-extracted again twice with 15 ml of hexane. Then all hexane extracts are combined, filtered through glass wool and dried with anhydrous sodium sulfate.

The aqueous phase is not discarded as it is used for total lipid weight determination, it's acidified with 2 ml of 1 M sulfuric acid and extracted 3 times in a separatory funnel with 10 ml of hexane. Then all hexane extracts are combined, filtered through glass wool and dried with anhydrous sodium sulfate.

5.2. Concentration of the extract

The hexane fraction (50 ml), containing the non-saponifiable lipids and consequently the petroleum hydrocarbons is concentrated with a rotary evaporator down to about 15 ml (maximum temperature: 30°C). Then transferred in a graduated tube and concentrated with nitrogen down to a volume corresponding to 1 ml/ gram of freeze-dried sample extracted (this will avoid the precipitation of the lipids in the tube).

The hexane fraction (30 ml) containing the saponifiable lipids is concentrated with a rotary evaporator down to about 15 ml and then transferred in a graduated tube and concentrated with nitrogen.
The lipids are weighed with the electro-balance. The total lipid content is the sum of the lipid found in the first hexane fraction and this one. Then this fraction is discarded.

5.3. Extractable organic matter (EOM)

Solvent extractable organic matter (E.O.M.) is determined in the following manner. On the weighing pan of an electrobalance, a known volume of the extract (up to 100 µl) is evaporated and the residue weighed to about ± 1 µg. If the residue is less than 2 µg, pre-concentration of the original extract is required.

The quantity of E.O.M. is

$$\text{E.O.M. } (\mu g/g) = \frac{\text{Weight of residue } (\mu g) \times \text{Volume of extract (ml)} \times 1000}{\text{Volume evaporated } (\mu l) \times \text{Quantity of sample extracted (g)}}$$

The total E.O.M. is the sum of both non-saponified and saponified lipids.

5.4. Clean-up procedure and fractionation

Especially in the case of biota samples, it is necessary to clean-up the extract before proceeding with the analysis. The clean-up should remove non-petroleum hydrocarbons material that fluoresces under certain conditions. Furthermore, materials that may cause quenching will be removed simultaneously.

5.4.1. Fractionation

The clean-up and separation are achieved by a simple column chromatographic partition as follows:

A chromatography column is prepared using 50 ml burette in which a piece of glass wool is added near the stopcock to maintain the packing material. Then, 5 g of silica are transferred into the column, then 10 g of alumina and on top 1 g of sodium sulfate is added in order to avoid the disturbance of the first layer when solvents are poured into the column.

Separation of compounds:

The sample (maximum 300 mg of non-saponified lipids) is applied on top of the column. A first fraction is obtained by eluting the sample with 20 ml of hexane (F1), this fraction will contain the saturated aliphatics. The second fraction (F2) is obtained by eluting with 30 ml of a mixture of hexane and dichloromethane (90:10), this fraction will contain the unsatured and aromatic hydrocarbons.

6. Gas Chromatography Conditions

6.1. Quantification of petroleum hydrocarbons

Gas Chromatograph	Agilent 7890
Detector	Flame Ionization Detector (FID)
Injection mode	Splitless
Carrier gas	Helium 1.2 ml min-1
Column	HP-5 (crosslinked 5% Ph Me Silicone) 30 m x 0.25 mm i.d. x 0.25 µm film thickness
Injector temperature	270°C
Detector temperature	300°C
Oven temperature program	60°C initial for 1 min., 60°C to 290°C at 4°C min^{-1}, 290°C for 40 min.

6.2. Quantification of PAHs

Gas Chromatograph	Agilent 6890 N
Detector	MSD 5975
Injection mode	Splitless
Carrier gas	Helium 1.5 ml min^{-1}
Column	DB-XLBMSD 30 m x 0.25 mm i.d. x 0.25 µm film thickness
Injection specifications	inj. press.: 13 psi, Constant flow on 13 psi at 60°C, Temp. injector 270°C
Transfer line	280°C
Ion source	240°C
Analyzer	100°C
Oven temperature program	60°C initial, 60°C to 100°C at 10°C min^{-1}, 100°C to 285°C at 4°C min^{-1}, 285°C for 20 min.

6.3. Target ions to use for quantification and confirmation ions and their relative abundance for GC/MS analyses of PAHs

Compound	Target	Confirming	% Abundance
Benzene	78		
C_1 - benzene	92		
C_2 - benzene	106		
C_3 - benzene	120		
C_4 - benzene	134		
d_8 - Naphthalene	136	134	8
Naphthalene	128	127	10
C_1 - naphthalene	142	141	80
C_2 - naphthalene	156	141	47 - 95
C_3 - naphthalene	170	155	61 - 300
C_4 - naphthalene	184	169	189
Acenaphthylene	152	151	20
d_{10} - Acenaphthene	164	162	97
Acenaphthene	154	153	86
d_{10} - Fluorene	176	174	93
Fluorene	166	165	80
C_1 - fluorene	180	165	95 - 144
C_2 - fluorene	194	179	25
C_3 - fluorene	208	193	
d_{10} - phenanthrene	188	187	22
Phenanthrene	178	179	16
Anthracene	178	176	20
C_1 - phenanthrene/anthracene	192	191	39 - 66
C_2 - phenanthrene/anthracene	206	191	16 - 150
C_3 - phenanthrene/anthracene	220	205	
C_4 - phenanthrene/anthracene	234	219, 191	73 - 297
Dibenzothiophene	184	185	14
C_1 - dibenzothiophene	198	197	53
C_2 - dibenzothiophene	212	211	

C_3 - dibenzothiophene	226	211	
C_4 - dibenzothiophene	240	211	
Fluoranthene	202	200	17
Pyrene	202	200	21
C_1 - fluoranthene/pyrene	216	215	36 - 64
Benz[a]anthracene	228	226	19
d_{12} - Chrysene	240	236	26
Chrysene	228	226	21
C_1 - benzanthracene/chrysene	242	243	20
C_2 - benzanthracene/chrysene	256	241	75 - 131
C_3 - benzanthracene/chrysene	270	255	
C_4 - benzanthracene/chrysene	284	269, 241	
d_{12} - perylene	264	260	24
Perylene	252	253	22
Benzo[b or k]fluoranthene	252	253	23
d_{12} - Benzo[a]pyrene	264	260	20
Benzo[a or e]pyrene	252	253	22
Indeno[1,2,3-c,d]pyrene	276	138	50
Dibenz[a,h]anthracene	278	279	24
Benzo[g,h,i]perylene	276	138	37

7. Quantification

The most widely used information for identification of a peak is its retention time, or its relative retention time (i.e., the adjusted retention time relative to that of a selected reference compound). Retention behavior is temperature dependent and comparison of retention times obtained at two or more temperatures may aid in determining a peak's identity. However, retention times are not specific and despite the high resolution offered by capillary columns, two compounds of interest in the same sample may have identical retention times.

In gas chromatography, results are usually quantified by either external calibration or internal calibration. Compounds identification is confirmed by GC-MS.

7.1. External Calibration

An external calibration is performed by injecting standard samples containing varying concentrations of the compound to be analyzed and creating a calibration curve (area vs. concentration). A response factor (RF) is calculated, for each target compounds, using the following equation:

$$RF = \frac{\text{Peak Area}}{\text{Sample Amount}}$$

The unknown samples are injected and the amounts of target compounds are then calculated with the following equation:

$$Amount = \frac{\text{Peak Area}}{\text{Response Factor}}$$

The method based on the external calibration doesn't take into account any variance in gas chromatograph performance and it requires the final volume of sample injected and the final volume of the extract.

7.2. Internal Calibration

This method is based on the use of an ***internal standard*** which is defined as a non-interfering compound added to a sample in known concentration in order to eliminate the need to measure the sample size in quantitative analysis and for correction of instrumental variation.

In this method, the internal standard is added to each sample and standard solution.

In a multiple point internal calibration each analyses contains the internal standard whose total amount is kept constant and the analyte of interest whose amount covers the range of concentrations expected. The ratio of the areas of the internal standard and analyte are then used to construct the calibration curve.

A multiple points relative response factor (RRF) calibration curve is established for analytes of interest for each working batch. A RRF is determined, for each analyte, for each calibration level using the following equation:

Where:

$$RRF(X) = \frac{\text{Area (X)}}{\text{Area (IS)}} \text{ x } \frac{\text{Qty (IS)}}{\text{Qty (X)}}$$

Area (X) = the area of the analyte to be measured (target compound)

Area (IS) = the area of the specific internal standard

Qty (X) = the known quantity of the analyte in the calibration solution

Qty (IS) = the known quantity of the internal standard in the calibration solution

The relative response factors determined for each calibration level are averaged to produce a mean relative response factor (mRRF) for each analyte. The percent relative standard deviation (%RSD) for the 3 response factor must be less than or equal to 15%, for each analyte.

$$\%RSD = \frac{\text{Standard deviation of the RRFs}}{\text{Average of the RFs}} \text{ x } 100$$

Sample analyte concentrations are calculated based on the quantity and response of the internal standard.

The following equation gives the amount of analyte in the solution analysed.

$$Qty\text{ (X)} = \text{Qty (IS) x } \frac{\text{Area (X)}}{\text{Area (IS)}} \text{ x } \frac{1}{\text{mRR}F(X)}$$

Where:

Qty (X) = the unknown quantity of the analyte in the sample

Qty (IS) = the known quantity of the internal standard added to the sample

Area (X) = the area of the analyte

Area (IS) = the area of the internal standard

mRRF (X) = the average response factor of the analyte

Sample analyte concentrations are then calculated by dividing the amount found (Qty) by the grams of samples extracted.

8. Quality assurance/quality control

Guidelines on the QA/QC requirements for analysis of sediments and marine organisms are detailed in Reference Method No 57, "Contaminant monitoring programs using marine organisms: Quality assurance and good laboratory practice".

The precision of the method is established by the replicate analysis of samples of the appropriate matrix. The precision of the entire analytical procedure is estimated by extracting five sub-samples from the same sample after homogenization. Precision is evaluated as a matter of course during the initial implementation procedure just before initiation of sample analysis.

8.1. Accuracy

The accuracy of the methods is confirmed by analysis of a suitable RM (i.e. appropriate matrix, analytes) prior to initiation of sample analysis. Agreement between measured and certified concentrations for any individual analyte should be within 35 % and on average within 25 %. Reference Materials are introduced on a regular basis (e.g. every 10-15 samples) as a method of checking the procedure. Further description of the preparation of control charts and criteria for data acceptance is elaborated in Reference Method No 57.

8.2. Blanks

Blanks represent an opportunity to evaluate and monitor the potential introduction of contaminants into samples during processing. Contributions to the analyte signal can arise from contaminants in the reagents, those arising from passive contact between the sample and the environment (e.g. the atmosphere) and those introduced during sample handling by hands, implements or glassware. It is essential to establish a consistently low (i.e. with respect to analytes) blank prior to initiating analysis or even the

determination of the method detection limit. In addition, it is necessary to perform blank determinations on a regular basis (e.g. every batch of samples).

8.3. Recovery

Recovery reflects the ability of the analyst to fully recover surrogate compounds introduced to the sample matrix or blank at the beginning of the procedure. The primary criteria for selection of compounds to be used for testing recovery are that they: 1) have physical (i.e. chromatographic/partitioning) properties similar to and if necessary spanning those of the analytes of interest, 2) do not suffer from interferences during gas chromatographic analysis, 3) are baseline resolved from the analytes of interest.

Recovery should be tested on all samples and blanks as a routine matter. Recoveries should be within 60% - 125%. However lower recoveries might be expected for low molecular weight PAHs (d_8-Naphtalene for example) due to their higher volatility. Recoveries higher than 100 % may indicate the presence of interferences.

8.4. Archiving and reporting of results

Every sample should have an associated worksheet which follows the samples and the extracts through the various stages of the procedure and upon which the analyst notes all relevant details. An example of such a worksheet is given below. Each laboratory should construct and complete such a worksheet. Relevant chromatograms should be attached to the worksheet. Analyses should be grouped and composite or summary analysis sheets archived with each group. Final disposal of the data will depend on the reasons for which it was collected but should follow the overall plan model.

All processed samples should be archived at all steps of the procedure:

- deep frozen (in the deep-freezer as it was received).
- freeze-dried (in sealed glass container kept in a dark place).

- extracted (after injection on the GC, sample extracts should be concentrated down to 1 ml and transferred into sealed glass vials, a Pasteur pipette sealed with a butane burner is adequate and cheap).

9. Bibliography

- Reference Method No 20, UNEP/IOC/IAEA: Determination of petroleum hydrocarbons in sediments. UNEP, 1992.
- Reference Method No 65, UNEP/IOC/IAEA: Reagent and laboratory ware clean-up procedures for low-level contaminants monitoring. UNEP, 1995.
- Reference Method No 57, UNEP/IOC/IAEA/FAO: Contaminant monitoring programs using marine organisms: Quality assurance and good laboratory practice. UNEP, 1990.
- Reference Method No 6, UNEP/FAO/IOC/IAEA: Guidelines for monitoring chemical contaminants in the sea using marine organisms. UNEP, 1993.
- Reference Method No 12 Rev.2, UNEP/FAO/IAEA/IOC: Sampling of selected marine organisms and sample preparation for the analysis of chlorinated hydrocarbons. UNEP, 1991.

Annex VII:

OSPAR (2008). JAMP Guidelines for monitoring contaminants in biota and sediments (5.2.4)

1.5.5.16 JAMP Guidelines for monitoring contaminants in biota and sediments

Request

ICES has received the following request from OSPAR:

Development of JAMP monitoring guidelines *(OSPAR no. 2-2007)*

To carry out the following development work with regard to the JAMP Guidelines for monitoring Contaminants in Sediments (OSPAR agreement 2002-16) and JAMP Guidelines for monitoring Contaminants in Biota (OSPAR agreement 1999-2) to ensure that monitoring guidance is in place to support a revised Co-ordinated Environmental Monitoring Programme.

- *a. develop draft technical annexes on monitoring of polybrominated diphenyl ethers and hexabromocycladodecane in sediments and biota following the structure of the existing technical annexes. SIME 2007 will be invited to clarify the congeners and compartments that are relevant for the development of monitoring guidance for brominated flame retardants.*
- *b. review the existing technical annexes on PAHs to see whether they are adequate for monitoring of the alkylated PAHs and, as appropriate, prepare advice on any revisions that are necessary.*
- *c. to develop a draft technical annex on monitoring of TBT and its breakdown products in biota"*

Advice on point (a) has been provided previously.

Summary

Alkyl PAH in sediment and biota

The current OSPAR technical annex for PAH analyses required updating as clear guidance was required to ensure quantification of alkyl homologues of PAHs and alkyl substituted sulphur-heterocyclic PAHs in biota and sediment. The proposed updated technical guidelines are presented in annexes 1 and 2 and are recommended to OSPAR for adoption as part of their JAMP guidelines for monitoring contaminants.

The technical annex for the analyses of parent and alkylated PAHs in biota contains information for the selection of species, sampling techniques, sample transport, conservation, and sample treatment (including extractions, clean-up, and pre-concentration).

The analytical protocol follows the same technical principles as for the analysis of unsubstituted, parent PAHs. However, HPLC with fluorescence detection (HPLC-UVF) cannot be used for the detailed analysis of individual alkylated PAHs. Gas chromatography with mass spectrometry (GC-MS) is presently the preferred analytical technique for the analysis of both parent and alkylated PAHs.

Organotins in biota

The technical annex for organotins in biota, as appended at annex 3 is proposed for adoption by OSPAR as part of the Joint Monitoring and Assessment Programme (JAMP) Guidelines for Monitoring Contaminants in Biota.

Explanation

The full text of the response is found in the attached technical annexes.

Sources of information

ICES. 2008a. Report of the Working Group on Marine Sediments (WGMS 2008). ICES CM 2008/MHC:03.
ICES. 2008b. Report of the Marine Chemistry Working Group (MCWG 2008).

ANNEX 1: Polyaromatic hydrocarbons in biota

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
C_1-Naphthalenes	142	C_1-234	248
C_2-Naphthalenes	156	C_2-Fluoranthenes/Pyrenes	230
C_3-Naphthalenes	170	Benz[*a*]anthracene	228
C_4-Naphthalenes	184	Chrysene	228
Acenaphthylene	152	2,3-Benzanthracene	228
Acenaphthene	154	C_1- Benz[*a*]anthracene/ Chrysene	242
Biphenyl	154	C_2- Benz[*a*]anthracene/ Chrysene	256
Fluorene	166	C_3- Benz[*a*]anthracene/ Chrysene	270
C_1-Fluorenes	180	Benzo[*a*]fluoranthene	252
C_2-Fluorenes	*194*	Benzo[*b*]fluoranthene	252
C_3-Fluorenes	*208*	Benzo[*j*]fluoranthene	252
Dibenzothiophene	184	Benzo[*k*]fluoranthene	252
C_1-Dibenzothiophenes	198	Benzo[*e*]pyrene	252
C_2-Dibenzothiophenes	212	Benzo[*a*]pyrene	252
C_3-Dibenzothiophenes	226	Perylene	252
Phenanthrene	178	Indeno[1,2,3-*cd*]pyrene	276
Anthracene	178	Benzo[*ghi*]perylene	276
C_1-Phenanthrenes/Anthracenes	192	Dibenz[*a,h*]anthracene	278
C_2-Phenanthrenes/Anthracenes	206	Benzo[*k*]fluoranthene	252
C_3-Phenanthrenes/Anthracenes	220	Cyclopenta[*cd*]pyrene	226
Fluoranthene	202	Naphtho[2,1-*a*]pyrene	302
Pyrene	202	Dibenz[*a,e*]pyrene	302
C_1-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 $\mu g\ kg^{-1}$ 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 d_8-naphthalene to d_{14}-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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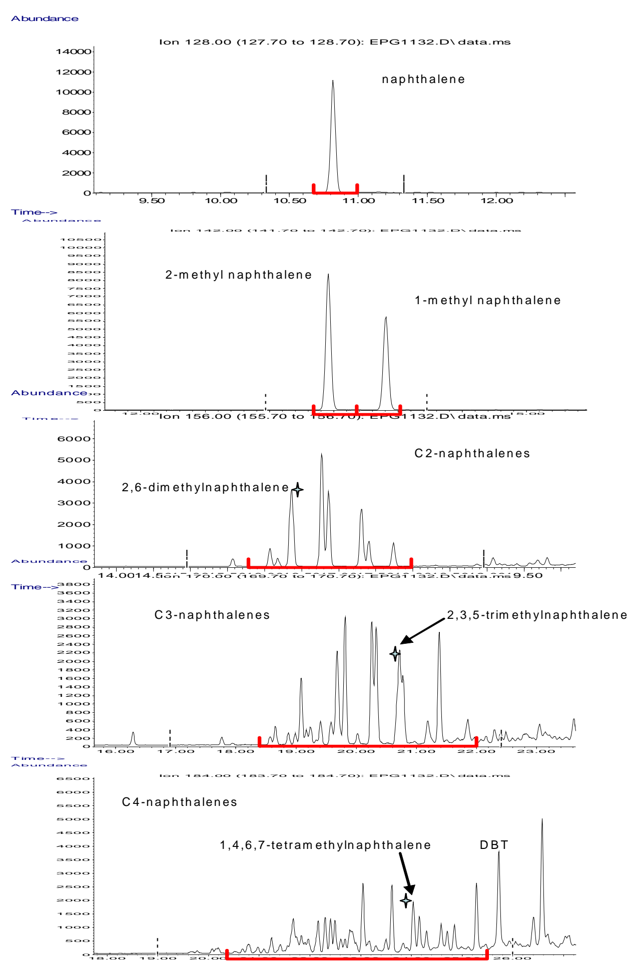
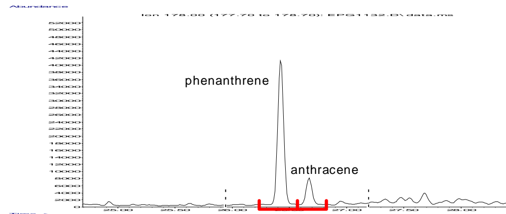
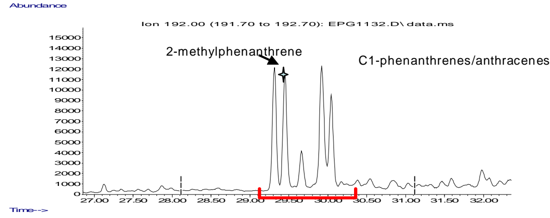
ANNEX 1 – APPENDIX 1

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




phenanthrene
anthracene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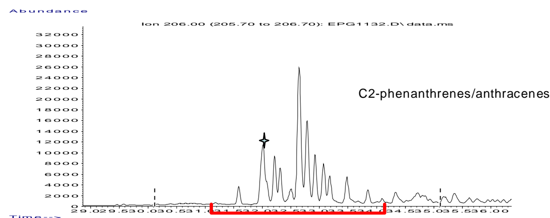
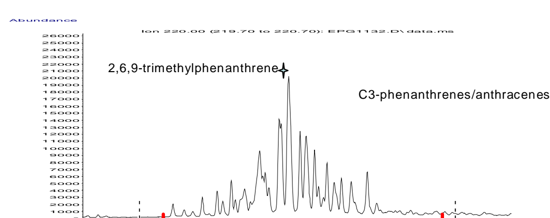
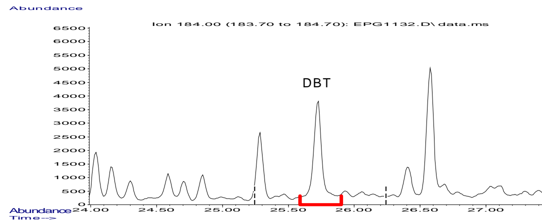
Ion 192.00 (191.70 to 192.70): EPG1132.D\ data.ms
2-methylphenanthrene
C1-phenanthrenes/anthracenes
Time-->


C2-phenanthrenes/anthracenes


2,6,9-trimethylphenanthrene
C3-phenanthrenes/anthracenes


Abundance
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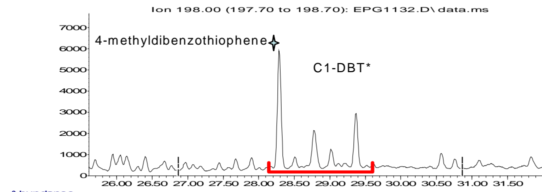
DBT
Time-->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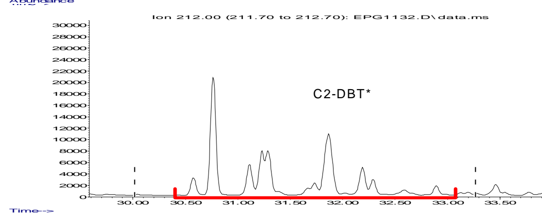
C1-DBT*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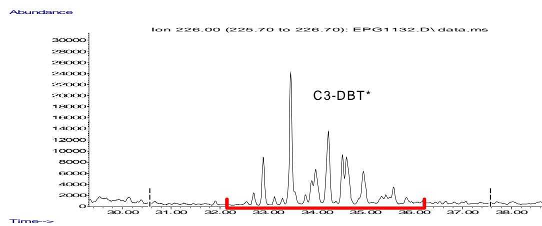
C2-DBT*
Time-->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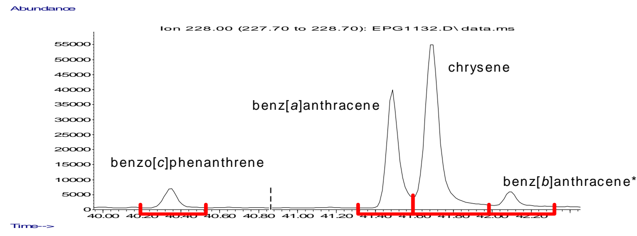
C3-DBT*
Time-->


Abundance
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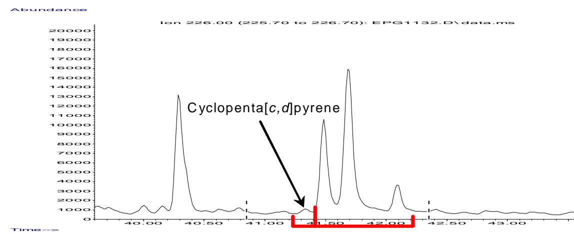
chrysene
benz[a]anthracene
benzo[c]phenanthrene
benz[b]anthracene*
Time-->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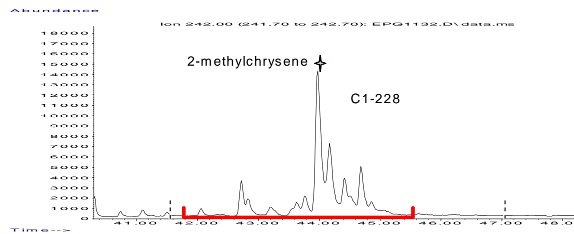
Cyclopenta[c,d]pyrene
Time-->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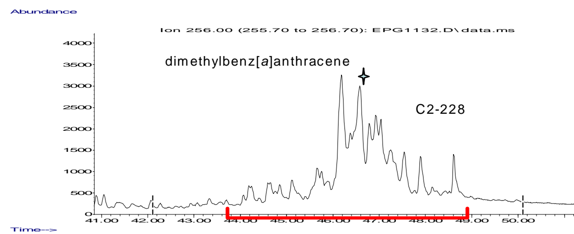
C1-228
Time-->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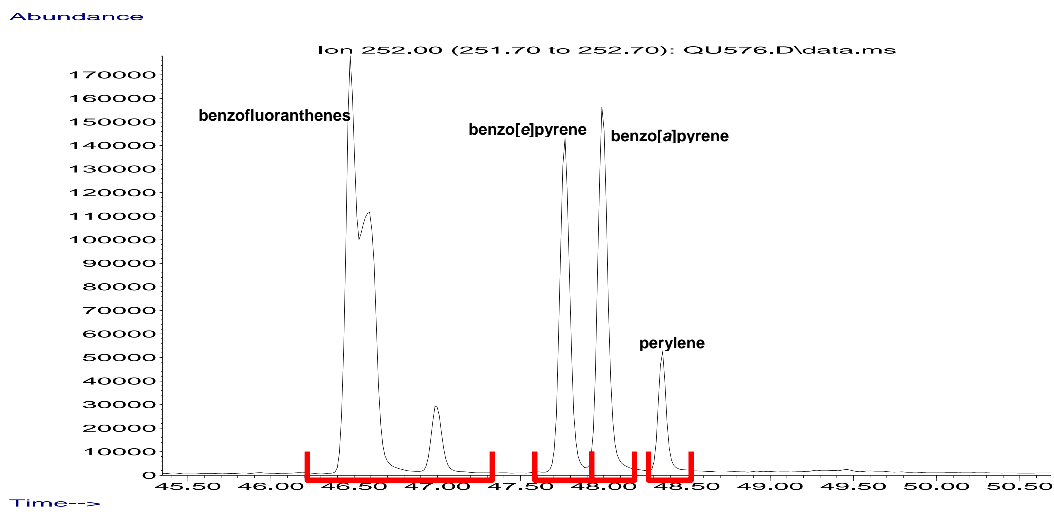
dimethylbenz[a]anthracene
C2-228
Time-->


Abundance
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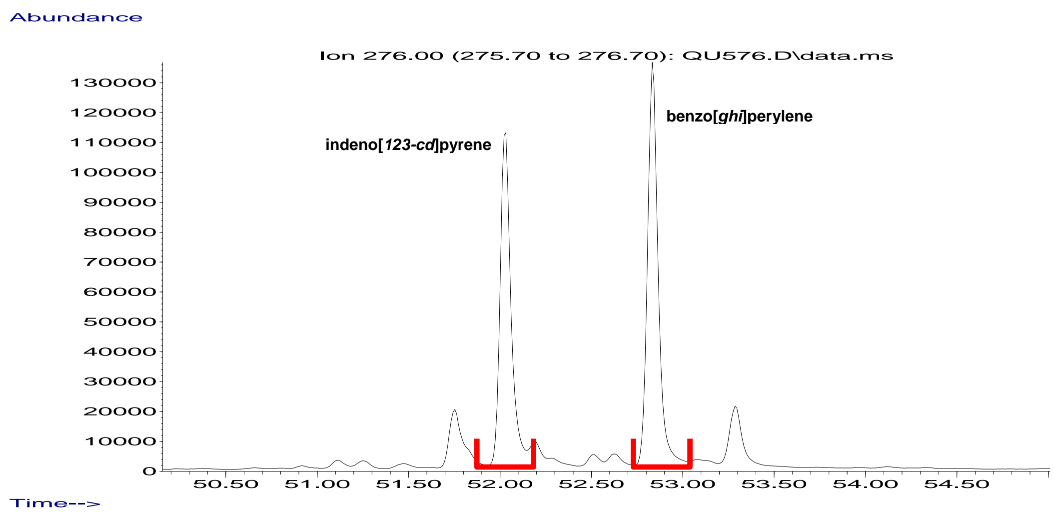
benzofluoranthenes
benzo[e]pyrene
benzo[a]pyrene
perylene
Time-->


Abundance
Ion 276.00 (275.70 to 276.70): QU576.D\data.ms
indeno[123-cd]pyrene
benzo[ghi]perylene
Time-->

ANNEX 2

Technical annex: Polyaromatic hydrocarbons in sediments

Determination of parent and alkylated PAHs in sediments

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) consist of a variable number of fused aromatic 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) PAHs. 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).

This Technical Annex provides advice on the analysis of parent and alkylated polycyclic aromatic hydrocarbons (PAH) in total sediment, sieved fractions, and suspended particulate matter. The analysis of in sediments generally includes extraction with organic solvents, clean-up, high performance liquid chromatography (HPLC) with ultraviolet or fluorescence detection or gas chromatographic (GC) separation with flame ionisation (FID) or mass spectrometric (MS) detection (e.g., Fetzer and Vo-Dinh, 1989; Wise *et al*., 1995). All steps in the procedure are susceptible to insufficient recovery and/or contamination. Quality control procedures are recommended in order to check the performance of the method. These guidelines are intended to encourage and assist analytical chemists to critically reconsider their methods and to improve their procedures and/or the associated quality control measures, where necessary.

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

2. Pre-treatment and Storage

2.1 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.) that could affect the sampling process. It is 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 sediment 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, more volatile PAHs through evaporation (Law *et al*., 1994).

Plastic materials must not be used for sampling and storage owing to possible adsorption of the PAHs onto the container material. Samples should be transported in closed containers; a temperature of 25°C should not be exceeded. If the samples are not analysed within 48 hours after sampling, they must be stored at 4°C (short-term storage). Storage over several months is only possible for frozen, (i.e., below −20°C) and/or dried samples (Law and de Boer, 1995).

As PAHs are sensitive to photo-degradation, exposure to direct sunlight or other strong light must be avoided during storage of the samples as well as during all steps of sample preparation, including extraction and storage of the extracts (Law and Biscaya, 1994). The use of amber glassware is strongly recommended.

2.3 Blanks

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

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

- all solvents should be checked for impurities by concentrating the amount normally used to 10% of the normal end volume. This concentrate can then be analysed by GC and should not contain significant amounts of PAHs or other interfering compounds;

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

- Glassfibre filters used for the PLE (pressurised liquid extraction) method should be heated at 450°C overnight.

3. Pre-treatment

Before taking a subsample for analysis, the samples should be sufficiently homogenised. The intake mass is dependent on the expected concentrations. For the marine environment, as a rule of thumb, the mass of sample taken for analysis can be equal to an amount representing 50–100 mg organic carbon. PAHs can be extracted from wet or dried samples. However, storage, homogenisation and extraction are much easier when the samples are dry. Care must be taken if freeze-drying samples for the reasons described in 2.1. Possible losses and contamination have to be checked. Contamination can be checked by exposing 1–2 g C18-bonded silica to drying conditions and analysing it as a sample (clean-up can be omitted) (Smedes and de Boer, 1997). Contamination during freeze-drying can be reduced by placing a lid, with a hole about 3 mm in diameter, on the sample container, while evaporation of the water is not hindered.

4. Extraction and clean-up

Exposure to light must be kept to a minimum during extraction and further handling of the extracts (Law and Biscaya, 1994). Since photo-degradation occurs more rapidly in the absence of a sample matrix, first of all the standard solution used for checking the recovery of the procedure will be affected, allowing a proper detection of the influence of light. The most photo-sensitive PAH is benzo[*a*]pyrene, followed by anthracene.

4.1 Wet sediments

Wet sediments should be extracted using a stepwise procedure by mixing with organic solvents. Extraction is enhanced by shaking, Ultra Turrax mixing, ball mill tumbling or ultrasonic treatment. Water-miscible solvents, such as acetone, methanol, or acetonitrile, are used in the first step. The extraction efficiency of the first step will be low as there is a considerable amount of water in the liquid phase. For sufficient extraction, at least three subsequent extractions are needed. The contact time with the solvent should be sufficient to complete the desorption of the PAHs out of the sediment pores. Heating by microwave or refluxing will accelerate this process.

When utilising a Soxhlet, the extraction of wet sediments should be conducted in two steps. First, a polar solvent, such as acetone, is used to extract the water from the sediment, then the flask is replaced and the extraction continued with a less polar solvent or solvent mixture (e.g., acetone/hexane). Thereafter, the extracts must be combined. For both batch and Soxhlet extraction, water must be added to the combined extracts and the PAHs must be extracted to a non-polar solvent.

Extraction of wet sediments by pressurised liquid extraction (PLE) is a more recent method, requiring less solvent and time for the extraction process. Wet sediment is dried by mixing with sufficient anhydrous sodium sulphate to form a free flowing mixture and is packed into stainless steel tubes for extraction. Extractions are performed at elevated temperatures and pressures. Various extracting solvents (DCM, acetone, methanol, acetonitrile, hexane, DCM: acetone [1:1], hexane:acetone [1:1] were investigated by Saim *et al.* (1998) and as long as the solvent polarity was >1.89 (*i.e.* all

except hexane) no significant differences were noted. Extraction temperatures can be manipulated to suit the analytical requirements.

4.2 Dry sediments

Although all the methods mentioned above can also be used for dried sediments, Soxhlet extraction is the most frequently applied technique to extract PAHs from dried sediments. Medium-polar solvents such as dichloromethane or toluene, or mixtures of polar and non-polar solvents can be used. When using dichloromethane, losses of PAHs have occasionally been observed (Baker, 1993). Although toluene is not favoured because of its high boiling point, it should be chosen as solvent when it is expected that sediment samples contain soot particles. For routine marine samples, the use of a mixture of a polar and a non-polar solvent (e.g., acetone/hexane (1/3, v/v)) is recommended.

The extraction can be carried out with a regular or a hot Soxhlet (Smedes and de Boer, 1997). A sufficient number of extraction cycles must be performed (approximately 8 hours for the hot Soxhlet and 12 to 24 hours for normal Soxhlet). The extraction efficiency has to be checked for different types of sediments by a second extraction step. These extracts should be analysed separately.

PLE can also be used for the extraction of freeze-dried sediments. Instead of anhydrous sodium sulphate to dry the sediment the sample is mixed with a clean sand or diatomaceous earth to increase the surface area of the sediment. The same solvent mixtures detailed above for wet sediment extraction can be used for the dry sediments. Supercritical fluid extraction (SFE) has also been used for the extraction of organic compounds. The optimum conditions may vary for specific sediments (e.g., Dean *et al*., 1995; Reimer and Suarez, 1995).

4.3 Clean-up

The crude extract requires a clean-up to remove the many other compounds which are co-extracted (e.g., Wise *et al*., 1995). Due to chlorophyll-like compounds extracted from the sediment, the raw extract will be coloured and also contain sulphur and sulphur-containing compounds, oil, and many other natural and anthropogenic compounds. Selection of the appropriate clean-up method depends on the subsequent instrumental method to be used for analysis. Prior to the clean-up, the sample must be concentrated and any polar solvents used in the extraction step should be removed. The recommended acetone/hexane mixture will end in hexane when evaporated because of the formation of an azeotrope. Evaporation can be done either using a rotary evaporator or parallel evaporating systems such as Syncore. Especially for the rotary evaporator, care should be taken to stop the evaporation in time at about 5 ml. For further reducing the volume, a gentle stream of nitrogen should be applied. The extract should never be evaporated to dryness. The drawback of the rotary evaporator is that more volatile components may be lost during the nitrogen drying stage whilst the heavier components stick to the glassware. The Buchi Syncore Analyst also uses glass tubes but the system is sealed, avoiding contamination from the lab 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.

For removing more polar interferences from the extract, deactivated aluminium oxide (10 % water), eluted with hexane, as well as silica or modified silica columns, e.g., aminopropylsilane, eluted with toluene or a semipolar solvent mixture such as hexane/acetone (95/5, v/v) or hexane/dichloromethane (98/2, v/v), can be used. Gel permeation chromatography (GPC) can be used to remove high molecular weight material and sulphur from the extracts.

For GC-MS analysis, sulphur should be removed from the extracts, in order to protect the detector. This can be achieved by the addition of copper powder, wire or gauze during or after Soxhlet extraction. Copper can also be added to the PLE cell, however, this is not always sufficient and further treatment with copper may be required following extraction. Ultrasonic treatment might improve the removal of sulphur. As an alternative to copper, other methods can be used (Smedes and de Boer, 1997).

Aliphatic hydrocarbons originating from mineral oil interfere with the flame ionisation detection. They can be removed from the extract by fractionation over columns filled with activated aluminium oxide or silica. The first fraction eluting with hexane is rejected. The PAHs elute in a second fraction with a more polar solvent, e.g., diethylether or acetone/hexane. When applying fractionation, the elution pattern has to be checked frequently. This should be carried out in the presence of sample matrix, as that can partially deactivate the clean-up column, resulting in earlier elution of the PAHs than in 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.

4.4 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. 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 that 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 A2.1, and 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 A2.1 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	Benzo[b]naphtho[2,3-*d*]thiophene	234
C_1-Naphthalenes	142	C_1-benzonaphthothiophenes	248
C_2-Naphthalenes	156	C_2-Fluoranthenes/Pyrenes	230
C_3-Naphthalenes	170	Benz[*a*]anthracene	228
C_4-Naphthalenes	184	Chrysene	228
Acenaphthylene	152	2,3-Benzanthracene	228
Acenaphthene	154	C_1- Benz[*a*]anthracene/ Chrysene	242
Biphenyl	154	C_2- Benz[*a*]anthracene/ Chrysene	256
Fluorene	166	C_3- Benz[*a*]anthracene/ Chrysene	270
C_1-Fluorenes	180	Benzo[*a*]fluoranthene	252
C_2-Fluorenes	*194*	Benzo[*b*]fluoranthene	252
C_3-Fluorenes	*208*	Benzo[*j*]fluoranthene	252
Dibenzothiophene	184	Benzo[*k*]fluoranthene	252
C_1-Dibenzothiophenes	198	Benzo[*e*]pyrene	252
C_2-Dibenzothiophenes	212	Benzo[*a*]pyrene	252
C_3-Dibenzothiophenes	226	Perylene	252
Phenanthrene	178	Indeno[1,2,3-*cd*]pyrene	276
Anthracene	178	Benzo[*ghi*]perylene	276
C_1-Phenanthrenes/Anthracenes	192	Dibenz[*a,h*]anthracene	278
C_2-Phenanthrenes/Anthracenes	206	Benzo[*k*]fluoranthene	252
C_3-Phenanthrenes/Anthracenes	220	Cyclopenta[*cd*]pyrene	226
Fluoranthene	202	Naphtho[2,1-*a*]pyrene	302
Pyrene	202	Dibenz[*a,e*]pyrene	302
C_1-Fluoranthenes/Pyrenes	216	Dibenz[*a,i*]pyrene	302
Benzo[*b*]naphtho[2,1-*d*]thiophene	234	Dibenz[*a,l*]pyrene	302
Benzo[*b*]naphtho[1,2-*d*]thiophene	234	Dibenz[*a,h*]pyrene	302

6. 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 $\mu g\,kg^{-1}$ dry weight for individual PAH compounds should be achievable by GC-MS.

6.1 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 A2.2. 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 an 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 A2.2.

Table A.2.2 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, however, lead 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.

7. Calibration and quantification

7.1 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 A2.1. 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 d_8-naphthalene to d_{14}-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.

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.

7.3 Recovery

The recovery of analytes should be checked and reported. Given the wide boiling range of the PAHs to be determined, the recovery may vary with compound group, from the volatile PAHs of low molecular weight to the larger compounds. 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.

8. Analytical quality control

Planners of monitoring programmes must decide on the accuracy, precision, repeatability, and limits of detection and determination, which they consider acceptable. Achievable limits of determination for each individual component using GC-MS are 0.05 $\mu g\ kg^{-1}$ dry weight.

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., liver, muscle, mussel tissue) as the samples, and the determinand concentrations should be in the same range as those in the samples. Realistically, and given the wide range of PAH concentrations encountered, particularly in oil spill investigations, this is bound to involve some compromise. The data produced for the LRM in successive sample batches should be used to prepare control charts. It is also useful to analyse the LRM in duplicate from time to time to check within-batch analytical variability. The analysis of an LRM is primarily intended as a check that the analytical method is under control and yields acceptable precision, but a certified reference material (CRM) of a similar matrix should be analysed periodically in order to check the method bias. A marine sediment (NIST SRM 1941b)[1] is available, with certified values for 24 PAHs and a further 44 as reference (non-certified) values. 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.

9. Data reporting

The calculation of results and the reporting of data can represent major sources of error, as has been shown in intercomparison studies for PAHs. Control procedures should be established in order to ensure that data are correct and to obviate transcription errors. Data stored 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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[1] More info on https://srmors.nist.gov/view_detail.cfm?srm=1941B

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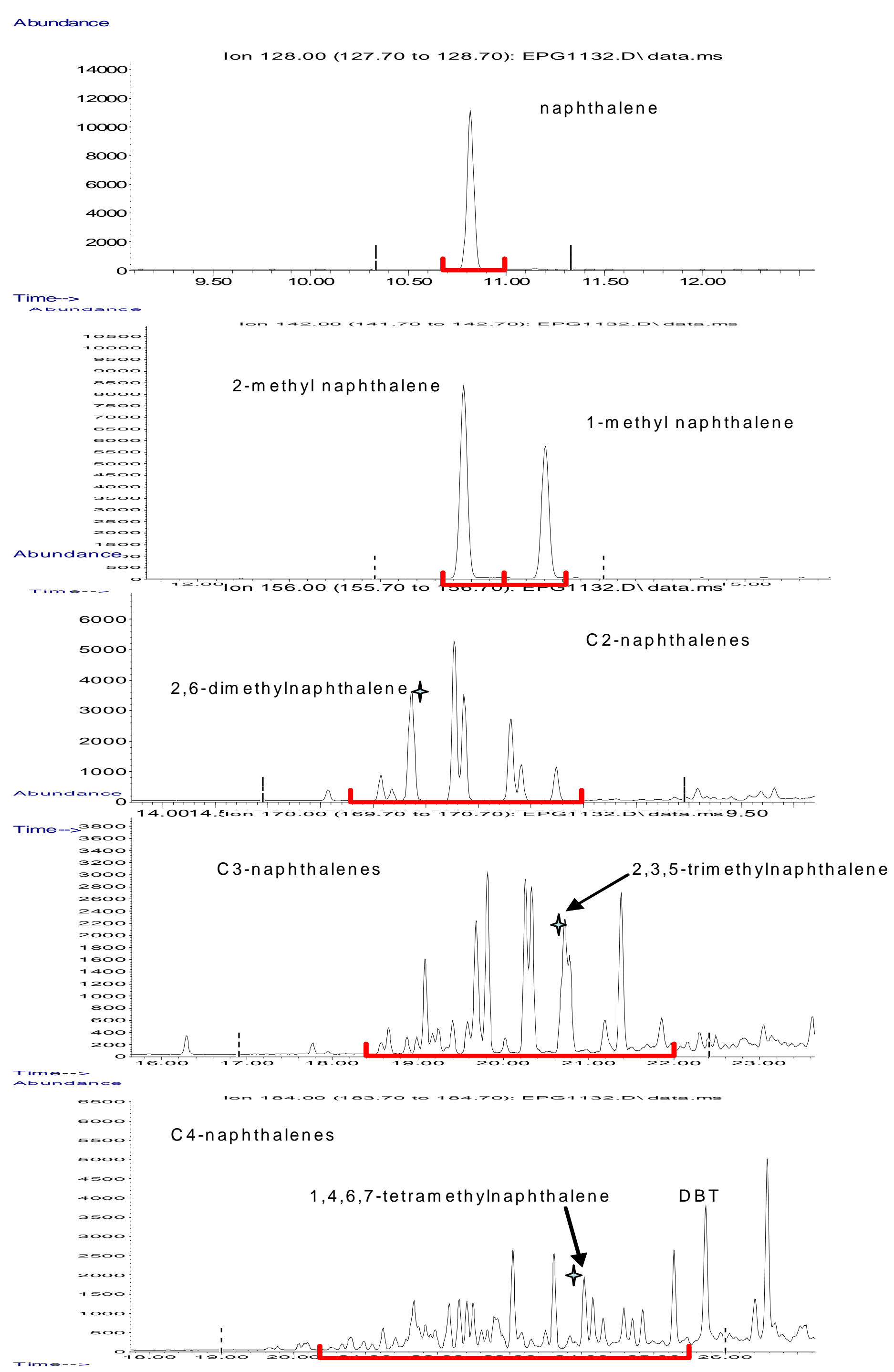
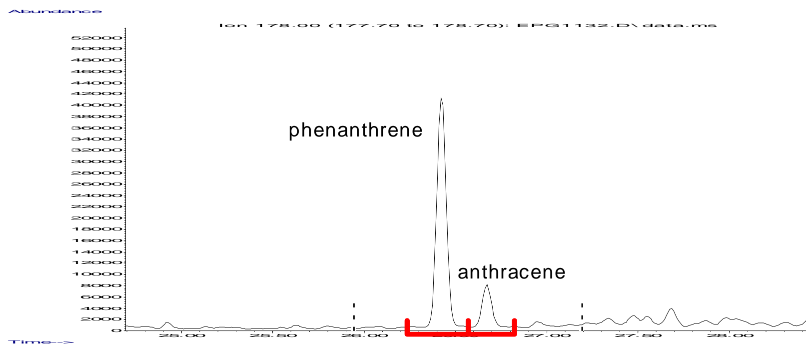
ANNEX 2 – APPENDIX 1

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

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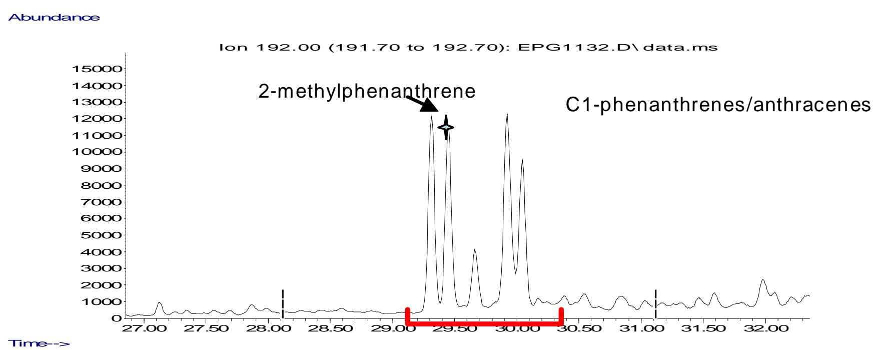
anthracene
Time-->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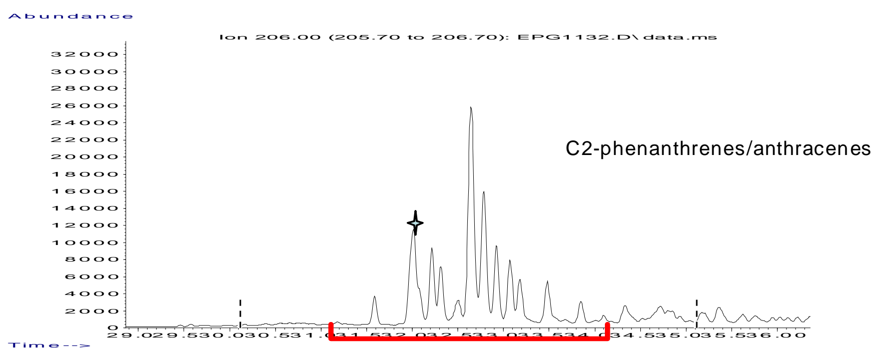
C1-phenanthrenes/anthracenes
Time-->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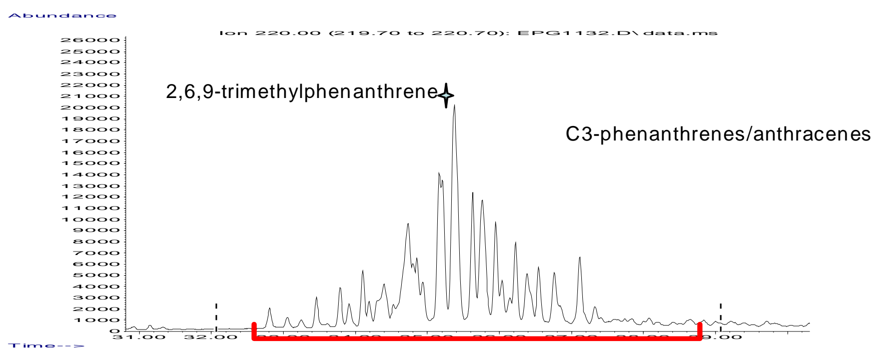
C2-phenanthrenes/anthracenes
Time-->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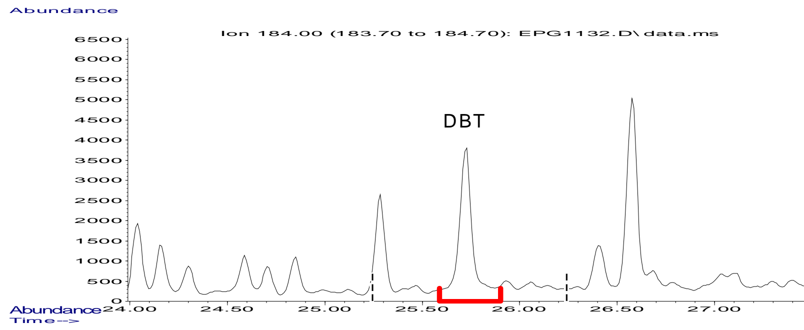
C3-phenanthrenes/anthracenes
Time-->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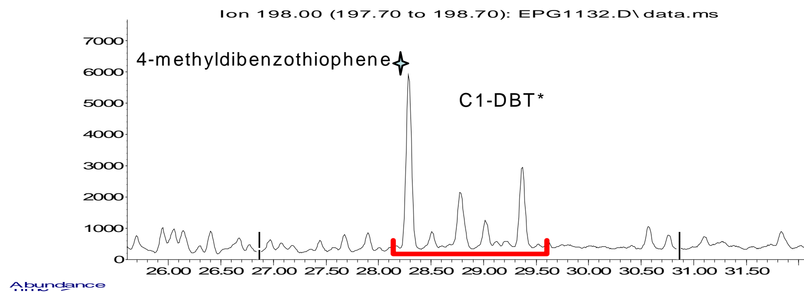
DBT
Time-->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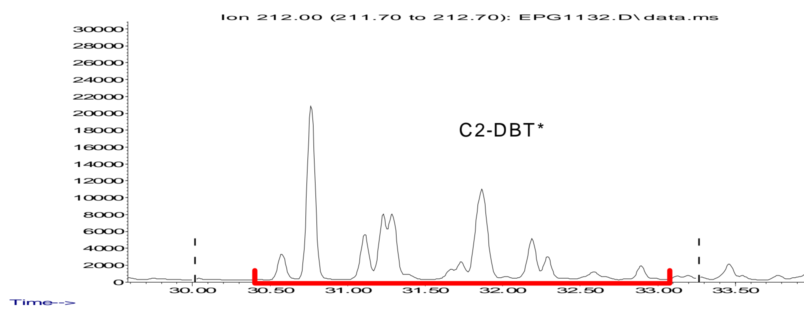
C1-DBT*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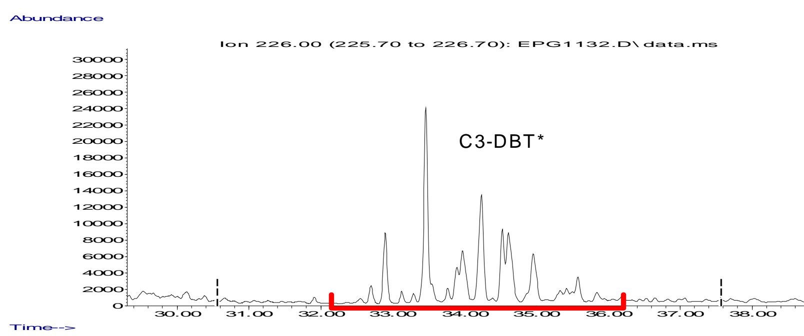
Time-->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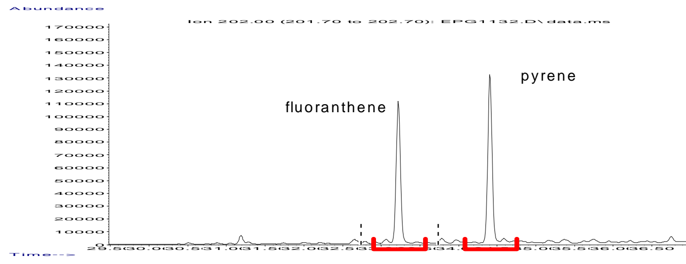
C3-DBT*
Time-->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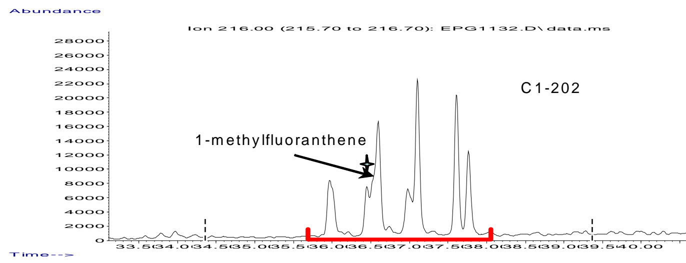
pyrene
fluoranthene
Time-->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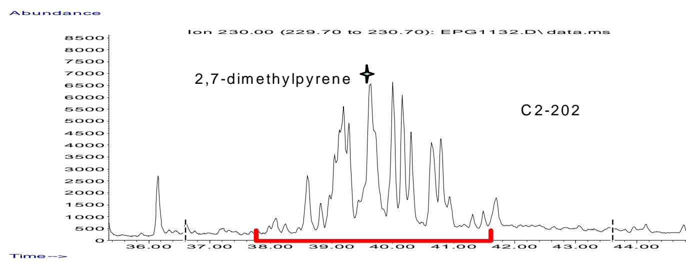
Time-->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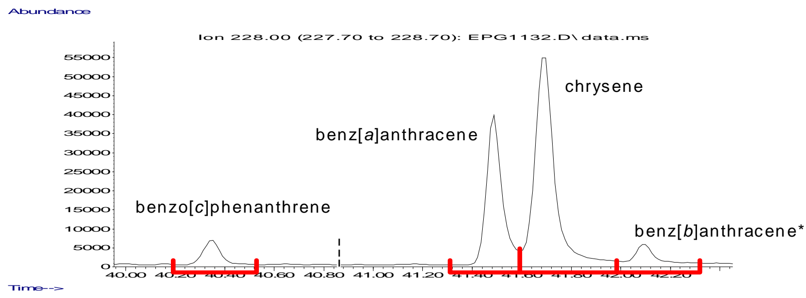
C2-202
Time-->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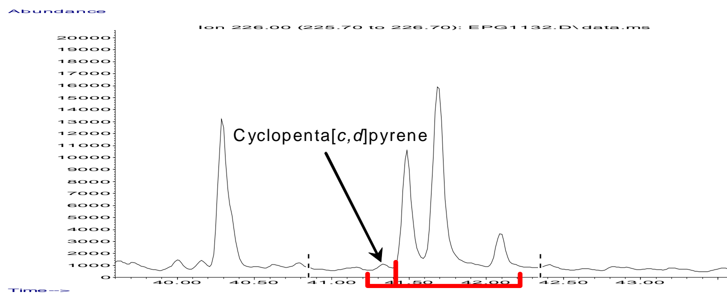
benzo[c]phenanthrene
benz[b]anthracene*
Time-->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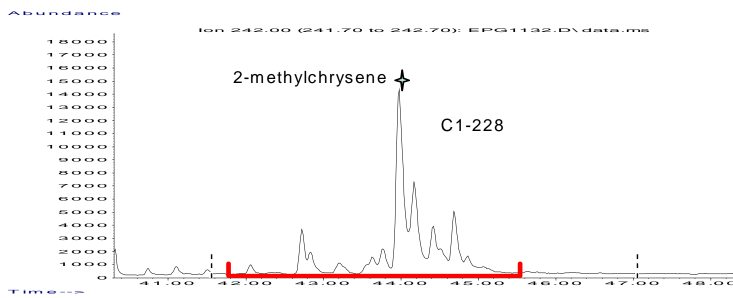
Time-->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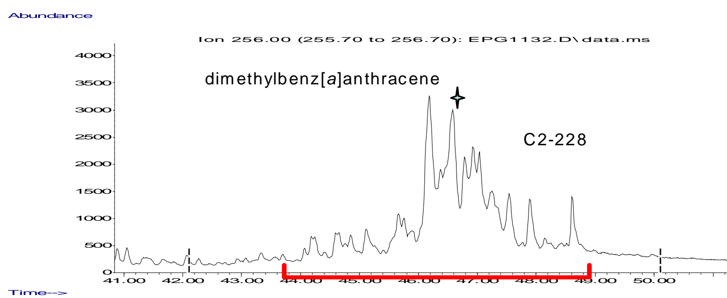
C1-228
Time-->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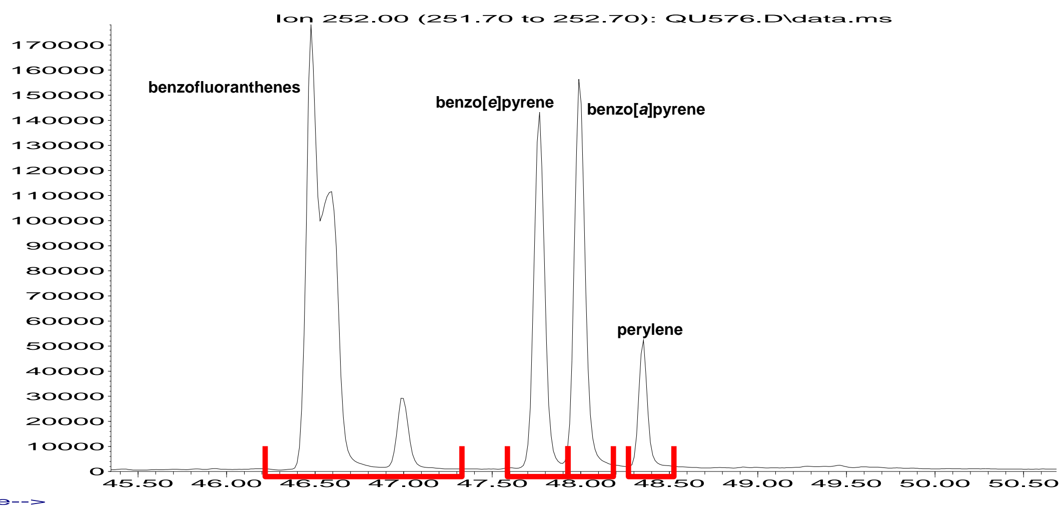
dimethylbenz[a]anthracene
C2-228
Time-->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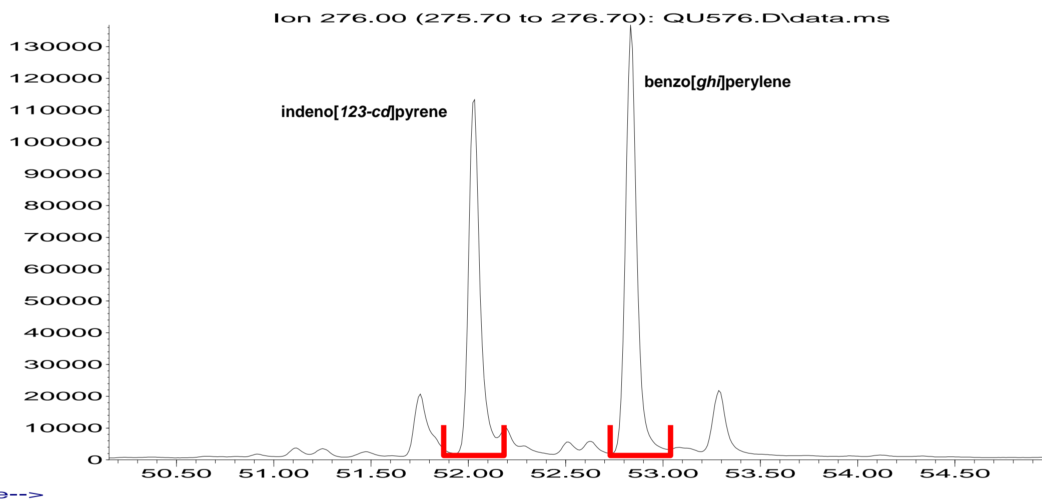
benzofluoranthenes
benzo[e]pyrene
benzo[a]pyrene
perylene
Time-->


Abundance
Ion 276.00 (275.70 to 276.70): QU576.D\data.ms
indeno[123-cd]pyrene
benzo[ghi]perylene
Time-->

ANNEX 3

Technical Annex: 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 JAMP 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 ($NaBEt_4$): Derivatisation with this complexing agent has been developed to minimise the analysis time. The $NaBEt_4$ procedure allows a simultaneous extraction-derivatisation in a buffered medium (optimum pH 4-5). $NaBEt_4$ derivatisation produces more thermally stable derivatives. However, $NaBEt_4$ 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 ($NaBH_4$). Hydride generation produces a large volume of hydrogen as a by-product, which facilitates the purging of butyltin hydrides from a large volume of sample.

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 then 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 coumpounds. 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
- location of sampling site (name, latitude, and longitude);
- date and time of sampling (GMT);
- sampling depth with respect to low tide (for sub-tidal sites only);
- irregularities and unusual conditions;
- name and institution of sampling personnel;
- number of pooled samples;
- number of individuals in pool;
- mean, minimum and maximum length and standard deviation;
- mean dry shell weight;
- mean soft tissue weight (wet weight);
- condition index.

7.2 Analytical and quality assurance parameters

- LRM and CRM results for a set of organotin compounds, reported on a wet weight basis;
- descriptions of the extraction, cleaning and instrumental determination methods;
- mean tissue lipid weight and method of extraction;
- the mean soft dry weight and method of determining water content if this differs from air drying to constant weight at 105°C (if sufficient material is available);
- the detection limit 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

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

Annex VIII:

References

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