



UNITED
NATIONS

EP

UNEP/MED WG.509/23



**Mediterranean
Action Plan**
Barcelona
Convention

30 August 2021
Original: English

Meeting of the MED POL Focal Points

Teleconference, 27-28 May and 6-7 October 2021

Agenda item 12: Harmonization and standardization of IMAP Pollution Cluster Monitoring

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

For environmental and cost-saving reasons, this document is printed in a limited number. Delegates are kindly requested to bring their copies to meetings and not to request additional copies.

Table of Contents

1	Introduction.....	1
2	Technical note for the sampling of marine biota for the analysis of heavy metals and organic contaminants.....	2
2.1	Protocol for the collection of fish for heavy metal and organic contaminants analysis	3
2.2	Protocol for the collection of bivalves for heavy metal and organic contaminants analysis....	4
2.3	Protocol for the dissection of fish to collect muscle and liver.....	5
2.4	Protocol for the dissection of bivalves	7
3	Technical note for the sample preservation of marine biota for the analysis of heavy metals and organic contaminants.....	8
3.1	Protocol for the treatment of biota samples prior to analysis of heavy metals.....	8
3.2	Protocol for the treatment of biota samples prior to analysis of organic contaminants.....	9

Annexes

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

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: ICES/OSPAR (2018). CEMP Guidelines for Monitoring Contaminants in Biota (5.2.2);

Annex V: References

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.

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.

In accordance with the Conclusions and Recommendations of the Integrated Meetings of the Ecosystem Approach Correspondence Groups on IMAP Implementation (CORMONs) (Videoconference, 1-3 Dec. 2020), and in particular paragraph 22, this Meeting requested the Secretariat to amend this Monitoring Guideline by addressing agreed technical proposals that were described in the Report of the Meeting in line with its agreement to proceed with submission of this document to the Meeting of MEDPOL Focal Points. Requested amendments included technical written suggestions that were provided by several Contracting Parties up to 10 days after the

Integrated Meeting of CORMONs. The amended document was shared by the Secretariat on 19 February 2021 for a period of 2 weeks for the non-objection by the Integrated Meetings of CORMONs on the introduced changes. Further to no objection from the Integrated Meeting of CORMONs, this Monitoring Guideline is submitted for consideration of present Meeting of MEDPOL Focal Points.

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, 2019a¹; UNEP (2019b²) 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 HELCOM (2012³) (Annex III) and ICES/OSPAR (2018⁴) (Annex IV) as well as the European Commission's guidance documents (EC 2010⁵ and 2014⁶). Given the suitability of any of these Guidelines in the context of IMAP, they could

¹ UNEP/MAP (2019a). 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

² UNEP (2019b). UNEP/MED WG.463/6. Monitoring Protocols for IMAP Common Indicators related to pollution

³ 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

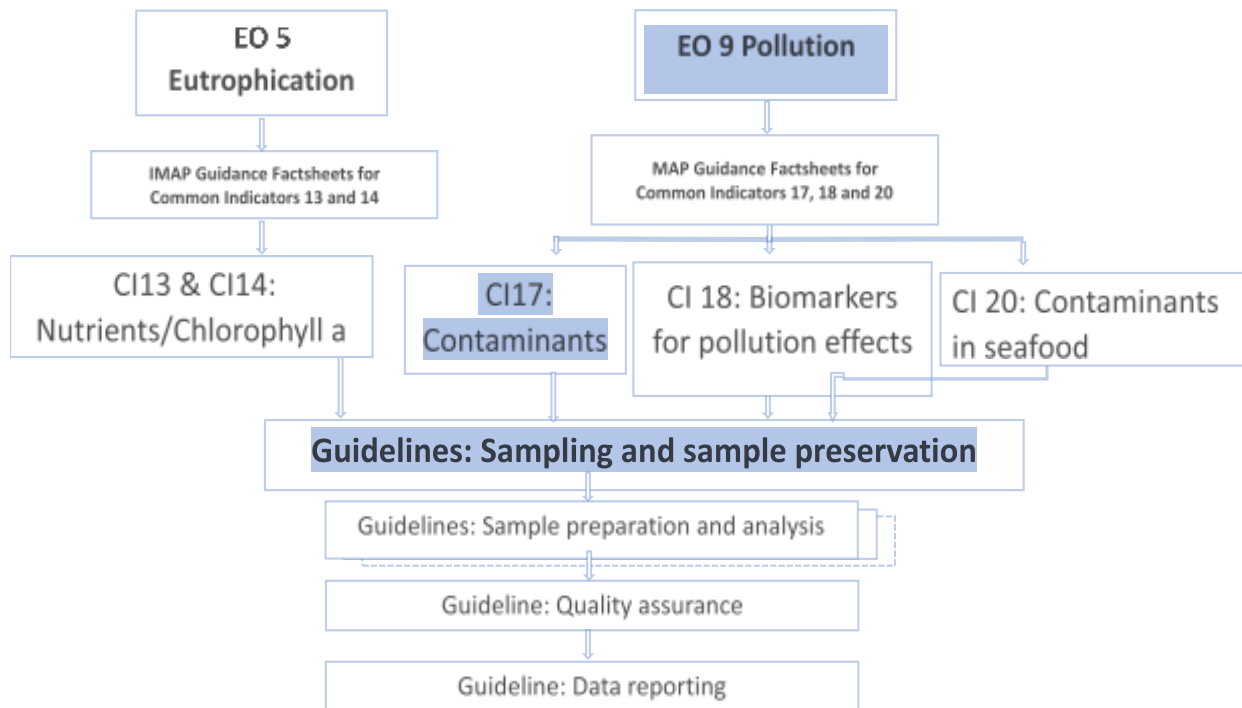
⁴ ICES/OSPAR (2018). CEMP Guidelines for Monitoring Contaminants in Biota

⁵ EC (2010). Guidance Document No: 25 Guidance on chemical monitoring of sediment and biota under the Water Framework Directive

⁶ EC (2014). Guidance Document No: 32 Guidance on biota monitoring under the Water Framework Directive

be further used by interested IMAP competent Mediterranean laboratories for developing their laboratory specific sampling and sample processing methodologies.

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, 2019b). 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, 2019c⁷).

9. For fish sampling, in line with the IMAP Monitoring Protocols for CI17 (UNEP, 2019b), 3-5 parallel composite samples (5-6 specimen for each fish sample) are collected from the same size class at each site. During the initial phase of the IMAP (identification of key sampling sites/stations) fish sampling should be done every 4 years and bivalves sampling yearly, while during the advanced phase (when it is a fully completed MED POL Phase IV implementation with the ongoing reporting of data sets) biota sampling should be done every 1 to 3 years, according the trends and levels assessed at the different stations/sites (UNEP, 2019a). EU requests Member States to determine the frequency of monitoring in sediment and/or biota so as to provide sufficient data for a reliable long-term trend analysis (2008/105/EC⁸). As a guideline, the Directive suggests a monitoring frequency of three years for sediment and biota, unless technical knowledge and expert judgment justify another interval.

10. 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 1967/2006⁹). 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 (2012) (Annex III.) and OSPAR (2018).

11. 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.

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

⁷ UNEP (2019c) UNEP/MED WG.467/8. Data Standards and Data Dictionaries for Common Indicators related to pollution and marine litter.

⁸ EC Directive 2008/105/EC (2008) on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Parliament and of the Council

⁹ EC Council Regulation No 1967/2006 concerning management measures for the sustainable exploitation of fisheries resources in the Mediterranean Sea

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 or aluminium clean working surface in the ship's laboratory. Before starting the handling of fish 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 °C) 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, 2019c).

16. In line with the IMAP Monitoring Protocols for CI17 (UNEP, 2019b), 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, 2012). 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, 2012).

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 HELCOM (2012) (Annex III) and OSPAR (2018) (Annex IV).

20. In places where no wild bivalves populations are found, caged bivalves can be used as an alternative option for monitoring (UNEP, 2019b). 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)¹⁰ and Galgani et al (2014)¹¹.

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 -20°C. 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

i) Dissection

22. Muscle tissues of fish has to be dissected while the organism is 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. 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 (2012) and OSPAR (2018) propose a similar procedure for fish dissection and removal of muscle for further analysis. 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. 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 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.

¹⁰ 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>.

¹¹ Galgani, F., Chiffolleau, J.F., Barrah, M., Drebiga, 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>.

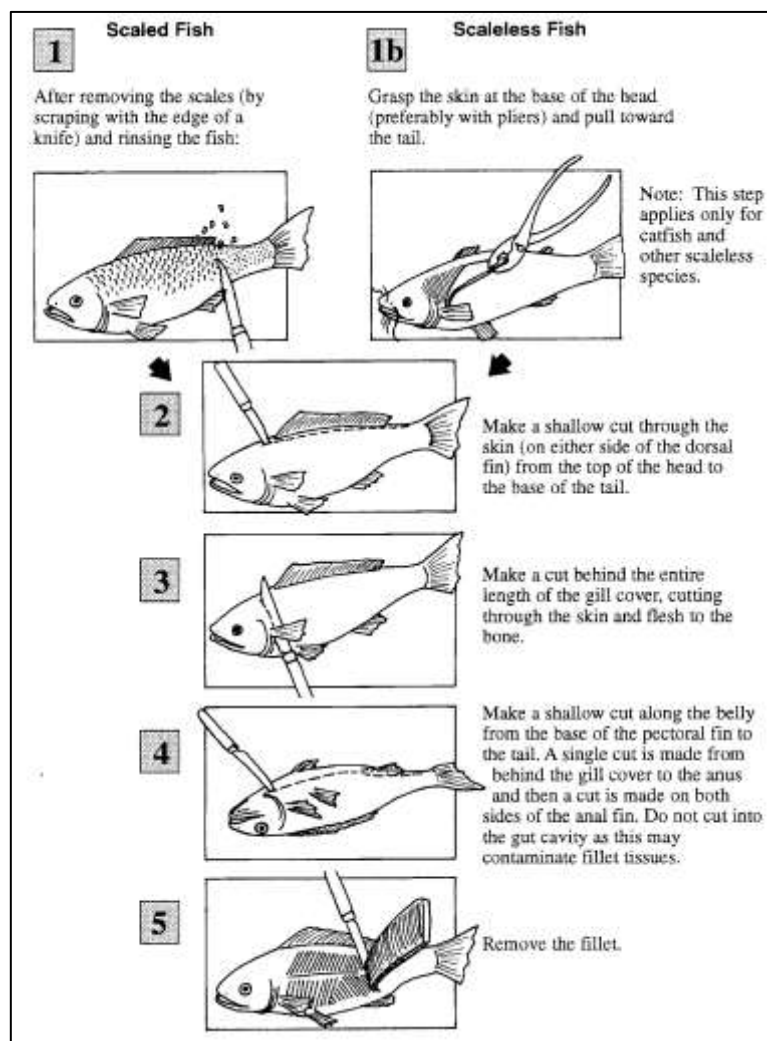


Figure 1. Fish filleting procedure (from US EPA, 2000¹²)

27. In case liver tissue is sampled for analysis (not a mandatory tissue in the framework of IMAP), 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 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.

¹²US EPA (2000). Guidance for Assessing Chemical Contaminant Data for Use in Fish Advisories Volume 1 Fish Sampling and Analysis. Third Edition.

31. HELCOM (2012) 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 HNO₃ 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, 2012). 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 (2019b) 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 (2012) (Annex III) and OSPAR (2018) (Annex IV).

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.

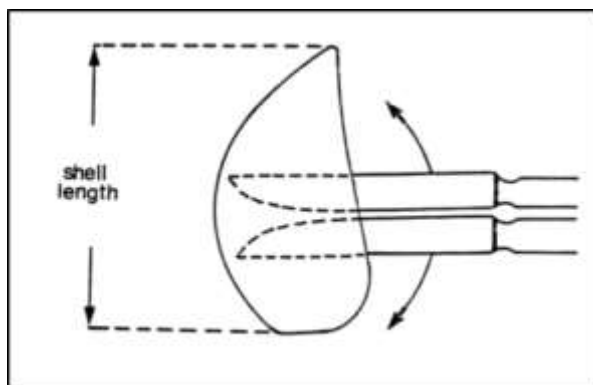


Figure 2. Cutting the abductor muscle

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 °C) 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. For metal (except volatile mercury) analysis, biota freeze-drying is the preferable procedure. Alternatively, the biota tissues may be dried at any temperature below 105°C until constant weight. For mercury analysis, to minimise losses due to evaporation, a biota tissue sub sample could be air dried at temperature <50°C (EC, 2010).

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, 2012). 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 are provided by OSPAR (2018) and HELCOM (2012).

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).

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 °C) 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. For organic contaminants analysis drying procedures depends on the compounds to be analysed. For chlorinated hydrocarbons biota can be freeze-dried taking care to avoid determinant loss through evaporation by keeping the temperature in the evaporation chamber below 0°C. (OSPAR, 2018). For PAH determination, freeze-drying of biota tissues may be a source of contamination due to the back-streaming of oil vapours from the rotary vacuum pumps. Furthermore, drying may result in losses of the lower molecular weight, more volatile PAHs through evaporation. A possible way to protect samples from contamination is to cover the sample containers with a filter paper perforated with a small hole (HELCOM, 2012). 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. 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%.

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

c) Storage of dried biota tissues

52. 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.

53. 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).

Annex I:

Guidelines for monitoring chemical contaminants in the sea using marine organisms



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

NOTE: This document has been prepared in co-operation between the United Nations Environment Programme (UNEP), The Food and Agriculture Organization of the United Nations (FAO), the International Atomic Energy Agency (IAEA) and the Intergovernmental Oceanographic Commission (IOC) under project FP/S102-88-03 (2849).

For bibliographic purposes this document may be cited as:

UNEP/FAO/IOC/IAEA: Guidelines for monitoring chemical contaminants in the sea using marine organisms. Reference Methods for Marine Pollution Studies No. 6, UNEP 1992.

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.

-
- | | | |
|-----|----------------|--|
| (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. |

- ii -

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.

CONTENTS

	<u>Page</u>
1. Scope and field of application	1
2. References	1
3. Introduction	2
4. Definitions	3
5. Aims of monitoring programmes	4
6. Pilot study	5
7. Designing a monitoring programme	6
8. Selection of contaminants	7
9. Selection of organisms	8
10. Location of sampling sites	10
11. Period and frequency of sampling	11
12. Size of sample	12
13. Selection of tissue	13
14. Appendix 1: Guidance on the planning of a pilot study	14
15. Appendix 2: Documentation of data	17
16. Appendix 3: Examples of chemical substances measured in marine organisms from monitoring purposes	18
17. Appendix 4: A) List of MED-POL species B) List of possible organisms for the assessment of contamination in the North Atlantic Region	19
18. Appendix 5: Detection of differences in levels of contaminants in marine organisms in relation to spatial; and trend monitoring programmes	21

1. SCOPE AND FIELD OF APPLICATION

This publication provides guidelines 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)

total mercury
organic mercury
cadmium
halogenated hydrocarbons

category II (optional)

total arsenic
radionuclides
polynuclear aromatic 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								
<u>Mytilus edulis</u> (common mussel)	+		?	+	+	+	+	+
<u>Littorina littorea</u> (gastropod)	+		+	?	+	+		
<u>Patella vulgata</u> (limpet, gastropod)	+		+		+	+		
Muddy substrate								
<u>Scrobicularia plana</u> (da Costa) (peppery furrow bivalve)	+	+	?	+	+	+		
<u>Macoma balthica</u> (bivalve)	+	+	?	+	+	+		
<u>Nereis diversicolor</u> (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 contaminant levels in the same part of the marine environment.

Annex II:

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



*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

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.

For bibliographic purposes this document may be cited as:

UNEP/FAO/IAEA/IOC: Sampling of selected marine organisms and sample preparation for trace metal analysis. Reference Methods for Marine Pollution Studies No. 7 Rev. 2, UNEP 1984.

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 ^{1/} includes ^{2/} ten regions and has over 120 coastal States participating in it.

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.

CONTENTS

	<u>Page</u>
1. Scope and field of application	1
2. References	1
3. Principles	1
4. Reagents	1
5. Apparatus	2
6. Sampling and transport	3
7. Sample preparation	5
8. Sampling and sample preparation protocol	10
Appendix A : Preparation of plastic tweezers	14
Appendix B : Specimen identification note	15

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 (FIRI/T158), pp. 124. FAU, 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 polyethylene 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.

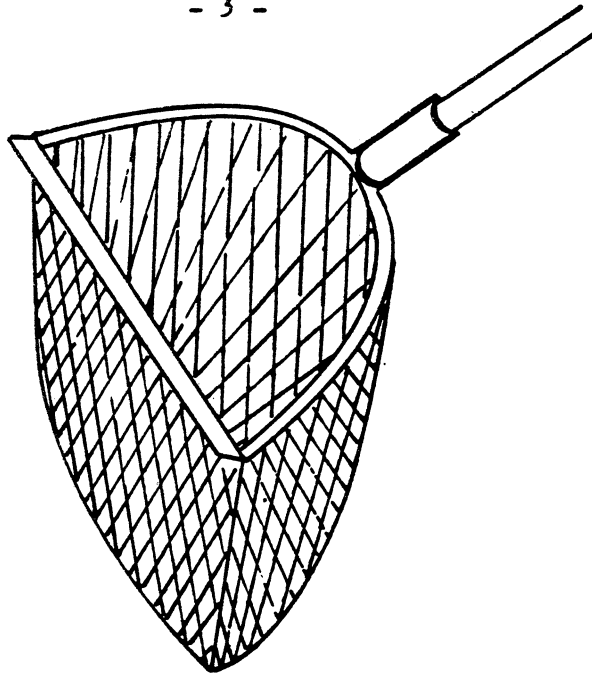


FIGURE 1 : SCRAPER FOR COLLECTING MUSSELS

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 plastic 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).

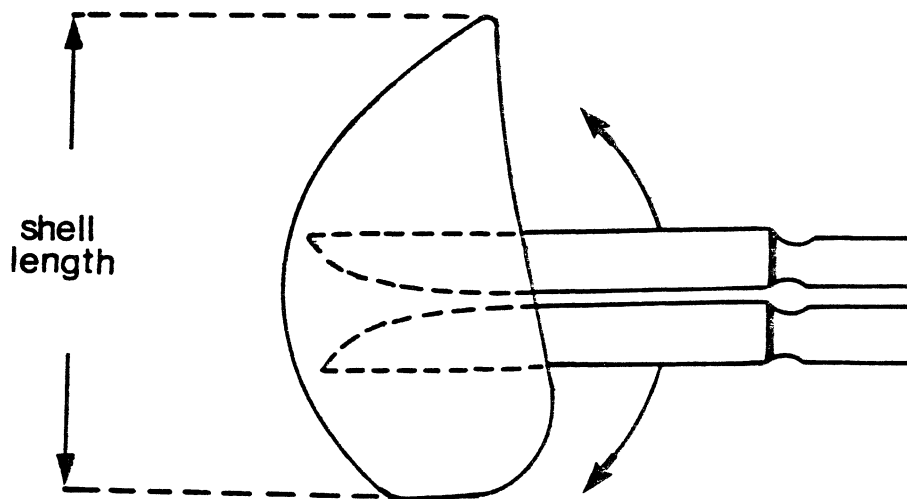


FIGURE 2 : CUTTING THE ADDUCTOR MUSCLE

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. Label 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.

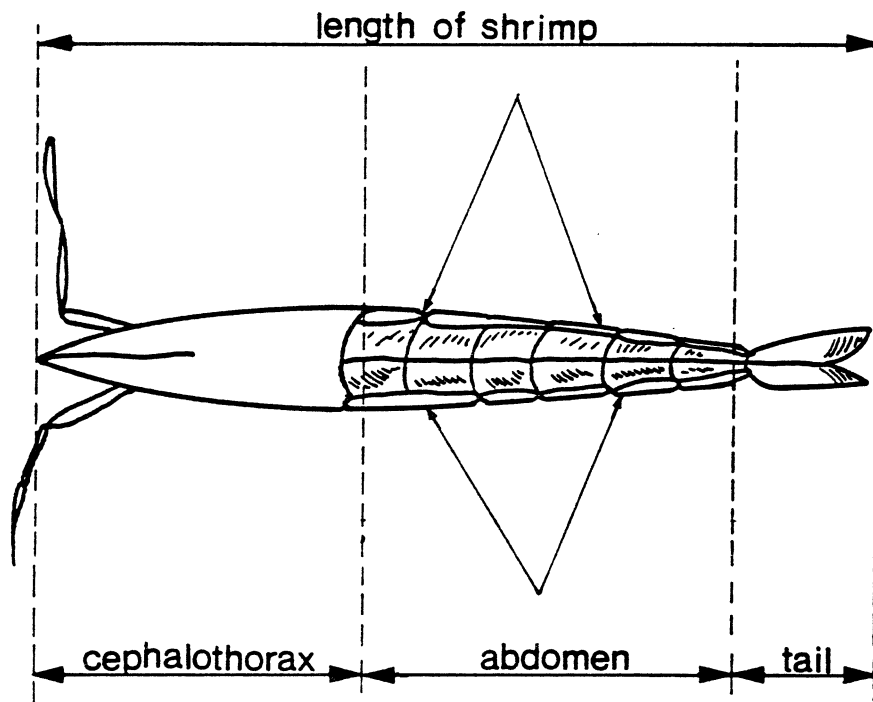


FIGURE 3 : SCHEMATIC DIAGRAM OF A SHRIMP
(arrows indicate where to cut after the legs have been removed)

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 sterinities (ventral exoskeleton); lift the sterinities 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.

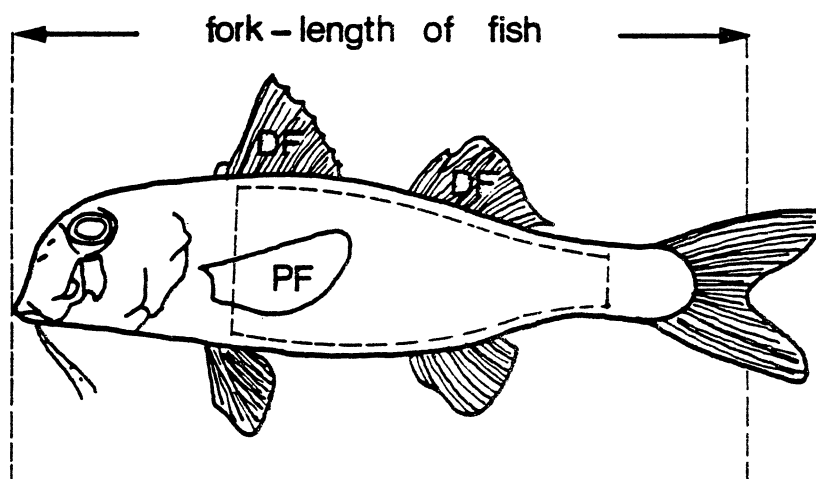


FIGURE 4 : SCHEMATIC DIAGRAM OF A FISH

(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 protocol 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 identification 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

Additional copies of this and other publications issued by
the Regional Seas Programme Activity Centre of UNEP can be
obtained from:

Regional Seas Programme Activity Centre
United Nations Environment Programme
Palais des Nations
GENEVA
Switzerland

Annex III:

HELCOM

Manual for marine monitoring in the COMBINE programme.

ANNEX B-12 Technical note on the determination of heavy metals and persistent organic compounds in biota.



ANNEX B-12 TECHNICAL NOTE ON THE DETERMINATION OF HEAVY METALS AND PERSISTENT ORGANIC COMPOUNDS IN BIOTA

- 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
- Appendix 2. Technical note on the determination of polycyclic aromatic hydrocarbons in biota
- Appendix 3. Technical note on the determination of chlorinated biphenyls and organochlorine pesticides in biota
- Appendix 4. Technical note on the determination of trace metallic elements in biota
- Appendix 5. Technical note on the determination of total mercury in marine biota

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 dissection 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 polynuclear 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 HNO₃ (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 (Oehlschläger, 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°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.

REFERENCES

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.

Last updated: 29.10.2012 (Annex number changed from Annex B 13 to Annex B 12)

Annex IV:

OSPAR COMMISSION

CEMP Guidelines for Monitoring Contaminants in Biota



CEMP Guidelines for Monitoring Contaminants in Biota

(OSPAR Agreement 1999-02)

1. Introduction

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

2. Purposes

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

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

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

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

policy (2008/105/EC), to assess whether certain regions or sub-regions, have reached or failed to reach Good Environmental Status.

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

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

- i. to identify sites where contaminant-specific biological effects programmes should be applied;
- ii. to investigate the chemical cause of observed biological effects;
- iii. How to improve and extend OSPAR's monitoring framework and better link it with the understanding of biological effects and ecological impacts of individual substances and the cumulative impacts of mixtures of substances.

3. Quantitative objectives

3.1 Temporal trends

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

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

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

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

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

3.2 Spatial distribution

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

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

or

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

or

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

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

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

3.3 Qualitative objectives

13. The quantitative results will be used to perform the assessment of the status of the marine environment with regard to hazardous substances. Further information on the assessment procedure,

the classification schemes and the threshold values used for distinguishing between the different classes from e.g. unacceptable to excellent or Good Environmental Status (WFD, MSFD) being achieved or not, can be found in the relevant OSPAR agreements and EC Directives and Decisions.

4. Sampling strategy

4.1 General

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

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

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

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

4.1.1 Species

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

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

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

- reflect changes in the concentration of contaminants in the surrounding environment;
- for a given species, have similar bioconcentration factors throughout the Maritime Area;

- accumulate the contaminant without being seriously affected by the concentrations encountered in the marine environment;
- be representative of the study area;
- be abundant throughout the study area;
- be of reasonable size, giving adequate amounts of tissue for chemical, biochemical and physiological analyses; restrictions to this may occur on different preconditions for performing the different tests and analytical methods;

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

- be easy to sample and hardy enough to survive in the laboratory, thus allowing:
 - defecation before analysis (if desired);
 - laboratory studies of contaminant uptake;
 - studies verifying biological field observations.

4.2 Sampling strategy for temporal trend monitoring

4.2.1 Species and sampling

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

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

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

Table 1: Sampling to minimise natural variability

Species	Number	Size ¹	Age	Sex ²	Tissue
Shellfish					
Mussel <i>Mytilus edulis</i> or <i>M. galloprovincialis</i>	3 pools of 20	Narrow length range	1-2 years	-	Whole soft body
Pacific oyster <i>Crassostrea gigas</i>		Narrow length range	2 years	-	Whole soft body
Flatfish					
Dab <i>Limanda limanda</i>	At least 12	Narrow length range	1-3 years	Single sex, females*	Muscle for Hg. Liver for all other determinands
Flounder <i>Platichthys flesus</i>	At least 12	Narrow length range	1-3 years	Single sex, females*	Muscle for Hg. Liver for all other determinands
Plaice <i>Pleuronectes platessa</i>	At least 12	Narrow length range	1-3 years	Single sex, females*	Muscle for Hg. Liver for all other determinands
Roundfish					
Cod <i>Gadus morhua</i>	At least 12	Narrow length range	Preferably 1-3years**		Muscle for Hg. Liver for all other determinands
Whiting <i>Merlangius merlangus</i>	At least 12	Narrow length range	2-3 years	Single sex, preferably females	Muscle for Hg. Liver for all other determinands
Hake <i>Merluccius merluccius</i>	At least 12	Narrow length range	2-3 years	Single sex, preferably females	Muscle for Hg. Liver for all other determinands
Herring <i>Clupea harengus</i>	At least 12	Narrow length range	1-2 years		Muscle for organic contaminants and Hg. Liver for other trace metals.
Eel pout <i>Zoarces viviparus</i>	At least 12	Narrow length range	2-3 years	Single sex, preferably females	Muscle for Hg. Liver for all other determinands
Seabird eggs					

¹ "Narrow length range" means that the length of the individuals collected should be constant from year to year at each site or should at least fall within a very narrow range. The length range could however vary between sites and hence is not specified in the table.

² The same sex should be sampled each year.

Species	Number	Size ¹	Age	Sex ²	Tissue
Common tern <i>Sterna hirunda</i>	10 ^{footnote 3}	-	1-5 days incubation	-	Whole egg content
Oyster catcher <i>Haematopus ostralegus</i>	10 ^{footnote 4}	-	1-5 days incubation	-	Whole egg content
Guillemot <i>Uria aalge</i>	10 ^{footnote 4}	-	1-5 days incubation	-	Whole egg content

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

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

Table 2: Length-stratified sampling

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

³ One egg taken randomly from each of 10 clutches.

⁴ The eggs should be collected as early as possible to avoid collecting replacement eggs.

4.2.2 Sampling area

Fish

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

Shellfish

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

Seabird eggs

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

4.2.3 Sampling frequency

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

4.2.4 Sampling period

Fish

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

Shellfish

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

Seabird eggs

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

4.3 Sampling strategy for spatial distribution monitoring

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

4.3.1 Species and sampling

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

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

Species	Spawning season	Reference
Shellfish		
Mussel		
<i>Mytilus edulis</i>	Spawning throughout the year but normally peaks in springtime and autumn	http://www.ukmarinesac.org.uk/communities/biogenic-reefs/br4_4.htm
<i>M. galloprovincialis</i>		
Pacific oyster	Summer months (above 20 °C)	http://www.fao.org/fishery/culturedspecies/Crassostrea_gigas/en
<i>Crassostrea gigas</i>		
Flatfish		
Dab		
<i>Limanda limanda</i>	January-August	http://www.fishbase.org/Reproduction/SpawningList.php?ID=695&GenusName=Limanda&SpeciesName=limanda&fc=440&StockCode=711
Plaice		
<i>Pleuronectes platessa</i>	January-June	http://www.fishbase.org/Reproduction/SpawningList.php?ID=1342&GenusName=Pleuronectes&SpeciesName=platessa&fc=440&StockCode=1360
Flounder		
<i>Platichthys flesus</i>	January-June	http://www.fishbase.org/Reproduction/SpawningList.php?ID=1341&GenusName=Platichthys&SpeciesName=flesus&fc=440&StockCode=1359
Roundfish		
Cod		
<i>Gadus morhua</i>	⁵	http://www.fishbase.org/Reproduction/SpawningList.php?ID=69&GenusName=Gadus&SpeciesName=morhua&fc=183&StockCode=79

⁵ Please use information on the actual timing of spawning in your sampling area. It is recommended to use Fishbase www.fishbase.org:

Whiting <i>Merlangius merlangus</i>	January-September	http://www.fishbase.org/Reproduction/SpawningList.php?ID=29&GenusName=Merlangius&SpeciesName=merlangus&fc=183&StockCode=39
Hake <i>Merluccius merluccius</i>	December-August	http://www.fishbase.org/Reproduction/SpawningList.php?ID=30&GenusName=Merluccius&SpeciesName=merluccius&fc=184&StockCode=40

Table 4: Spatial distribution sampling

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

Species	Recommended Number	Size (cm)	Tissue
Shellfish			
First choice Mussel <i>Mytilus edulis</i> or <i>M. galloprovincialis</i>	50 ^{footnote 4} ± 10%	3-6	Whole soft body
Second choice ¹ Pacific oyster <i>Crassostrea gigas</i>	10 ^{footnote 4} ± 10%	9-14 (2 years of age)	Whole soft body
Flatfish			
First choice Dab <i>Limanda limanda</i>	20 to 25 individuals, or 15 if justified, or 5 batches of 5 individuals	18-30	Muscle for Hg. Liver for all other determinands
Second choice ¹ Flounder <i>Platichthys flesus</i>	20 to 25 individuals, or 15 if justified, or 5 batches of 5 individuals	15-35	Muscle for Hg. Liver for all other determinands
Roundfish			
First choice Cod <i>Gadus morhua</i>	20 to 25 individuals, or 15 if justified, or 5 batches of 5 individuals	>20	Muscle for Hg. Liver for all other determinands
Second choice ¹ Whiting <i>Merlangius merlangus</i>	20 to 25 individuals, or 15 if justified, or 5 batches of 5 individuals	20-35	Muscle for Hg. Liver for all other determinands
Hake <i>Merluccius merluccius</i>	20 to 25 individuals, or 15 if justified, or 5 batches of 5 individuals	20-35	Muscle for Hg. Liver for all other determinands
Seabird eggs			
Common tern <i>Sterna hirunda</i>	10 ^{footnote 2}	-	Whole egg content

<http://www.fishbase.org/Reproduction/SpawningList.php?ID=69&GenusName=Gadus&SpeciesName=morhua&fc=183&StockCode=79>

Oyster catcher <i>Haematopus ostralegus</i>	10 ^{footnote 2}	-	Whole egg content
Guillemot <i>Uria aalge</i>	10 ^{footnote 3}	-	Whole egg content

¹ Where first choice species is not available.

² One egg taken randomly from each of 10 clutches.

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

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

4.3.2 Sampling area

Fish

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

Shellfish

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

Seabird eggs

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

4.3.3 Sampling period

Fish

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

Shellfish

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

Seabird eggs

36. Eggs should be sampled during the species-specific, year-specific and site-specific peak of the first laying cycle within the year. This generally occurs in May/June. Only fresh eggs should be taken

from full clutches. For each species, site and year, 10 eggs should be sampled with one egg taken randomly from each of 10 clutches from the first laying cycle within the year.

4.4 Sampling strategy for biological effects monitoring

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

5. Field sampling and sampling equipment

5.1 Fish

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

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

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

5.2 Shellfish

40. Only those individuals that are free of fouling and bored shells should be sampled. When collecting mussels by ship, a commercial mussel dredge can be used. When collecting mussels by hand, personnel should wear gloves. Clean containers consisting of material suitable for the subsequent

analyses should be used for transportation. Details of the requirements for recording the relevant sampling parameters and information on sampling methods are given in Technical Annexes 1 and 2.

5.3 Seabird eggs

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

6. Storage and pre-treatment

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

6.1 Fish

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

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

6.2 Shellfish

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

6.3 Seabird eggs

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

7. Analytical procedures

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

7.1 Organic contaminants

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

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

7.2 Metals

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

8. Quality assurance

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

51. To minimise the risk of contamination or the loss of determinands during sampling, storage, pre-treatment or analysis (and so to avoid the generation of false data) QA measures should be applied to the sample from first contact to final measurement and data reporting. All detailed QA data should

be recorded in accordance with the QA procedures laid down in the relevant documents. Laboratories should work according to EN 17025.

9. Data reporting

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

10. References

EC, 2000. Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy. Official Journal of the European Communities L 327/1-72.

<http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2000:327:0001:0072:EN:PDF>

EC, 2008. Directive 2008/105/EC of the European Parliament and of the Council of 16 December 2008 on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Parliament and of the Council. Official Journal of the European Union L 348/84-97

EC, 2008. Directive 2008/56/EC of the European Parliament and of the Council of 17 June 2008 establishing a framework for community action in the field of marine environmental policy (Marine Strategy Framework Directive). Official Journal of the European Union L164/19-40.

<http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:164:0019:0040:EN:PDF>

EC, 2010. Report on the recommendations for the Good Environmental Status of European Seas regarding contaminants and pollution effects under the Marine Strategy Framework Directive. MSFD GES Task Group 8

Gilbert, R.O., 1987. Statistical Methods for Environmental Pollution Monitoring. Van Nostrand Reinhold, New York.

ICES: Data submenu under: ICES: <http://www.ices.dk/indexfla.asp>

Nicholson, M.D., Fryer, R.J. and Larsen, J.R., 1997. A Robust Method for Analysing Contaminant Trend Monitoring Data. ICES Techniques in Marine Environmental Sciences, No. 20. 12 pp.

OSPAR, 2010: Joint Assessment and Monitoring Programme 2010 – 2014. OSPAR Agreement 2010-4

OSPAR, 2009: Background Document on CEMP Assessment Criteria for the QSR 2010. Publication No. 461978-1-907390-08-1

OSPAR, 2008: CEMP Assessment Manual: Co-ordinated Environmental Monitoring Programme Assessment Manual for contaminants in sediment and biota. Publication No. 377978-1-906840-18-1

Tema Nord, 1995. Manual for Nordic Environmental Specimen Banking.

Annex V:

References

Annex V : References

1. EC (2010). Guidance Document No: 25 Guidance on chemical monitoring of sediment and biota under the Water Framework Directive
2. EC (2014). Guidance Document No: 32 Guidance on biota monitoring under the Water Framework Directive
3. EC Council Regulation No 1967/2006 concerning management measures for the sustainable exploitation of fisheries resources in the Mediterranean Sea
4. EC Directive 2008/105/EC (2008) on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Parliament and of the Council
5. Galgani, F., Chiffolleau, 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>.
6. 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>.
7. 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
8. ICES/OSPAR (2018). CEMP Guidelines for Monitoring Contaminants in Biota. Technical Annexes: 1) organic contaminants; 2) metals; 3) parent and alkylated PAHs; 8) chlorobiphenyls
9. UNEP/MAP (2019a). 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
10. UNEP (2019b). UNEP/MED WG.463/6. Monitoring Protocols for IMAP Common Indicators related to pollution
11. UNEP (2019c) UNEP/MED WG.467/8. Data Standards and Data Dictionaries for Common Indicators related to pollution and marine litter.
12. US EPA (2000). Guidance for Assessing Chemical Contaminant Data for Use in Fish Advisories Volume 1 Fish Sampling and Analysis. Third Edition.