UNITED NATIONS

EP

UNEP/MED WG.492/3

UNEP

UNITED NATIONS
ENVIRONMENT PROGRAMME
MEDITERRANEAN ACTION PLAN

26 March 2021
Original: English

Meeting of the Ecosystem Approach Correspondence Group on Pollution Monitoring

Videoconference, 26-28 April 2021

Agenda item 3: Monitoring Guidelines/Protocols for IMAP Common Indicator 18

Monitoring Guideline/Protocols for Sampling and Sample Preservation of Marine Molluscs (such as *Mytilus* sp.) and Fish (such as *Mullus barbatus*) for IMAP Common Indicator 18

Note by the Secretariat

In line with the Programme of Work 2020-2021 adopted by COP21, UNEP/MAP-MED POL Programme has prepared the Monitoring Guidelines related IMAP Common Indicator 18, along with the Monitoring Guidelines related to data quality assurance and reporting.

With the set of Monitoring Guidelines related to IMAP Common Indicators 13, 14, 17 and 20 reviewed by the Integrated Meetings of the Ecosystem Approach Correspondence Groups on Monitoring (1-3 December 2020), these new monitoring guidelines form a coherent manual to guide technical personnel of IMAP competent laboratories of the Contracting Parties for the implementation of standardized and harmonized monitoring practices related to a specific IMAP Common Indicator (i.e. sampling methods, sample preservation and transportation, sample preparation and analysis, along with quality assurance and reporting of monitoring data). These guidelines present a summary of the best available known practices employed in marine monitoring bringing together integrated comprehensive analytical practices 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 relevant experiences.

This Monitoring Guideline for Sampling and Sample Preservation of Marine Molluscs (such as *Mytilus* sp.) and Fish (such as *Mullus barbatus*) for IMAP Common Indicator 18 provides the following two Technical Notes:

1) Technical Note for the sampling and sample preservation of marine molluscs (such as *Mytilus* sp.) for biomarker analysis, which includes the following two Protocols:
   i) Protocol for the collection and transport of marine molluscs (such as *Mytilus* sp.)
   ii) Protocol for the dissection and storage of tissue samples from marine molluscs (such as *Mytilus* sp.);
2) Technical Note for the sampling and sample preservation of marine fish (such as *Mullus barbatus*) for biomarker analysis, which includes the following two Protocols:
   i) Protocol for the collection of marine fish *(*such as *Mullus barbatus*)
   ii) Protocol for the dissection and storage of tissue samples from marine fish (such as *Mullus barbatus*).

These Monitoring Guidelines/Protocols, including this one for sampling and sample preservation of Marine Molluscs (such as *Mytilus* sp.) and Fish (such as *Mullus barbatus*) establish a sound ground for further regular update of monitoring practice for the purpose of successful IMAP implementation.

The Meeting of CorMon on Pollution Monitoring (26-28 April 2021) is expected to discuss this document and endorse its submission for consideration of the Meeting of MEDPOL Focal Points that will be held in May 2021.

Table of Contents

List of Abbreviations / Acronyms

AChE	Acetylcholinesterase
CI	Common Indicator
CI	Condition Index
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
GES	Good Environmental Status
HELCOM	Baltic Marine Environment Protection Commission - Helsinki Commission
IAEA	International Atomic Energy Agency
IMAP	Integrated Monitoring and Assessment Programme of the Mediterranean Sea and Coast and Related Assessment Criteria
LMS	Lysosomal membrane stability
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
MNi	Micronuclei
OSPAR	Convention for the Protection of the Marine Environment for the North-East Atlantic
QA/QC	Quality Assurance/Quality Control
SoS	Stress on stress

1 Introduction

1. A fundamental aspect related to IMAP Ecological Objective 9 concerns the monitoring of the concentrations of different classes of harmful chemicals evaluated in relevant matrices i.e. sediment, sea water, and biota (CI17). These data need to be associated to the results concerning the level of the biological effects of the toxic contaminants that may be present in the marine environment where a cause and effect relationship has been established (CI18).

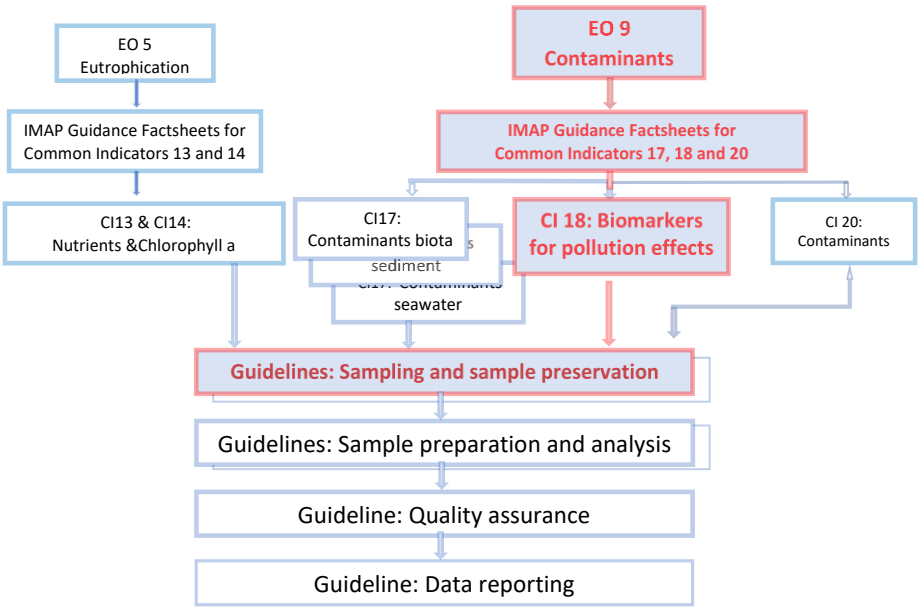
2. From the initial phase of the UNEP/MAP-MED POL Monitoring Programme, it was decided to attempt to highlight the early effects of the toxic contaminants on the marine life using biomarkers i.e. biological parameters which variations may highlight a pollutant-induced stress syndrome in the studied organisms.

3. At the present stage of IMAP implementation the following biomarkers are selected for regular monitoring[1]: a) lysosomal membrane stability (LMS), a biomarker able to highlight an increased autophagy, diagnostic of the effects of toxic chemicals and prognostic of possible effects at population level; b) acetylcholinesterase (AChE) activity, a biomarker diagnostic of possible neurotoxic effects; c) micronuclei (MNi) frequency, a biomarker able to highlight the genotoxic effects of the contaminants; and d) stress on stress (SoS), a biomarker suitable to reveal the reduced capacity of the organisms to survive to the action of further environmental stressors.

4. These biomarkers can be used in many different organisms. However, to ensure a comparability of the obtained results, the Molluscs (such as *Mytilus* sp.) and the fish (such as *Mullus barbatus)* were therefore selected for the biomarkers analysis[2].

5. An important aspect for the collection of the animals is that both molluscs and fish must be living organisms, unstressed by the collection procedure and the handling/transport, before being dissected to obtain the tissues used for the biological analysis.

6. This Monitoring Guideline/ Protocols provides appropriate methodologies for sampling and transport of Mytilus sp. and Mullus barbatus, as well as for their tissue preparation under controlled conditions to ensure the representativeness and the integrity of the biological samples used for the analysis of the different biomarkers as provided in UNEP/MED WG.492/4 and UNEP/MED WG.492/5.



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

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

2 Technical note for the sampling and sample preservation of marine molluscs (such as *Mytilus* sp.) for biomarker analysis

7. The marine molluscs used to perform the biomarker toxicological evaluations should be also used for the chemical analysis as described in the Monitoring Guidelines/Protocols for Sampling and Sample Preservation of Marine Biota for IMAP Common Indicator 17: Heavy and Trace Elements and Organic Contaminants (UNEP/MAP WG. 482/13). The molluscs must be alive and maintained in good conditions. Molluscs (*Mytilus* sp.) are internationally recognized for decades of research and biomonitoring as ideal organisms for monitoring the marine coastal environment (OSPAR 1997[2]; UNEP, 1997[3]; UNEP/RAMOGE, 1999[4]; Moore et al., 2004[5]; Martínez-Gómez et al., 2015[6]; Hansson et al., 2017[7]; etc.). Mussels are sessile, filter-feeding intertidal molluscs able to continuously sample the water column; and to accumulate in their tissues the chemicals present in the dissolved and the particulate fraction (Goldberg et al., 1978[8]; Bayne, 2009[9]; Viarengo et al., 2000[10]).

8. In biomonitoring programmes, it is possible to use wild mussels; however, in this case, it is important to know that the chemicals accumulated in the tissues may reflect pollution events happened months or years prior to the sampling. Moreover, in the case of a large monitoring programme it is also important to take into account the fact that mussels from different populations may have different growth rates and gonad maturation stages due to the specific environmental conditions of the sampling areas (i.e. food availability, sea water temperature, salinity, etc.). Consequently, for these reasons, it is important to evaluate the stage of gonadal development in the sampled molluscs, a parameter that can greatly change the physiological status of the organisms.

9. In order to reduce the sampling problems that can occur from the use of wild organisms, it is possible to use caged farmed mussels instead (Viarengo et al., 2007[11]). The animals will be genetically homogeneous (being collected in the same farm) and at a similar stage of gonad development as they come from the same population; and the same size will correspond to the same age of the animals. Moreover, the contaminant background will be minimal and similar in all the animals. After a month of caging at the sampling site (i.e. a period of time that guarantees a quite similar stage of gonadal development in the mussels caged in the various sites along the coast), the toxic effects observed in the mussels will be directly related to the amount of harmful chemicals accumulated in the mussel tissues. For these reasons, the use of caged organisms, when possible, is highly recommended. In this regard, it is important to highlight that the use of caged mussels also allows the evaluation of their survival rate after one month of exposure in the polluted areas: the incidence of mussel death is a very important

[2] OSPAR, 1997. JAMP Guidelines for General Biological Effects Monitoring (OSPAR Agreement 1997-7). OSPAR Commission, Monitoring guidelines. Ref. No: 1997-7. 20 pp.
[3] UNEP, 1997. The MED POL Biomonitoring Programme Concerning the Effects of Pollutants on Marine Organisms Along the Mediterranean Coasts. UNEP(OCA)/MED WG.132/3, Athens.
[4] UNEP/RAMOGE: Manual on the Biomarkers Recommended for the MED POL Biomonitoring Programme. UNEP, Athens, 1999
[5] Moore, M.N., Lowe, D. and Köhler, A. 2004. Biological effects of contaminants: Measurement of lysosomal membrane stability. ICES Techniques in Marine Environmental Sciences. No. 36. 39 pp.
[6] Martínez-Gómez, C., Bignell, J. and Lowe, D., 2015. Lysosomal membrane stability in mussels. ICES Techniques in Marine Environmental Sciences No. 56. 41
[7] Hansson, T., Thain, J., Martínez-Gómez, C., Hylland, K., Gubbins, M., Balk L., 2017. Supporting variables for biological effects measurements in fish and blue mussel. ICES Techniques in Marine Environmental Sciences. No. 60. 22 pp. http://doi.org/10.17895/ices.pub.2903.
[8] Goldberg, E.D., Bowen, V.T., Farrington, J.W., Harvey, G., Martin, J.H., Parker P.L., Risebrough, R.W., Robertson, W., Schneider, E., Gamble, E., 1978. The Mussel Watch. Environmental Conservation 5, 101-125.
[9] Bayne, B.L., 2009. Marine Mussels: Their Ecology and Physiology. Cambridge University Press 528 p.
[10] Viarengo, A.; Lafaurie, M.; Gabrielides, G.P.; Fabbri, R.; Marro, A., Roméo, M., 2000. Critical evaluation of an intercalibration exercise undertaken in the framework of the MED POL biomonitoring program. Mar. Environ. Res. 49, 1-18.
[11] Viarengo, A., Dondero, F., Pampanin, D.M., Fabbri, R., Poggi, E., Malizia, M., Bolognesi, C., Perrone, E., Gollo, E., Cossa, G.P., 2007. A biomonitoring study assessing the residual biological effects of pollution caused by the HAVEN wreck on marine organisms in the Ligurian Sea (Italy). Arch Environ Contam Toxicol. 53, 607-616.

parameter to readily identify extremely polluted areas, where the high concentration of toxic chemicals may cause lethal pathological alterations in the animals.

10. The mussels have to be caged in containment structures (e.g. polyethylene bags mounted on PVC tubing) for a period of 30 days (Sforzini et al., 2018[12]). It is important that the mussels used for caging experiments are collected from a clean site, and that before to start the experiment.

11. Under this Technical Note, the Monitoring Guidelines for Sampling and Sample Preservation of Marine Molluscs (such as Mytilus sp.) and Fish (Mullus barbatus) for IMAP Common Indicator 18 provides the following two Protocols: i) Protocol for the collection and transport of marine molluscs (such as Mytilus sp.) and ii) Protocol for the dissection and storage of tissue samples from marine molluscs (such as Mytilus sp.).

2.1 Protocol for the collection and transport of marine molluscs (such as *Mytilus* sp.)

a. Mussel collection

12. Mussels are intertidal organisms and, therefore, the sampling area may cover the entire length of the coastline if caged mussels are used. In the case of the sampling of mussels from wild populations, only the rocky zones will be adequate for the settlement of these bivalve molluscs.

13. The mussel sampling frequency suggested is once a year; the most adequate sampling periods are during the post winter months, but before or after the spawning period. Usually, in most Mediterranean coastal areas, the two periods are April-June and September-November; but the sampling periods may vary depending on the climatic characteristics of the various Mediterranean regions (ICES, 2011[13]; Moore et al., 2004).

14. *M. galloprovincialis* is a eurythermal species displaying a tolerance to a wide range of temperatures (from near-freezing to ~ 31 °C). Physiological studies of *M. galloprovincialis* indicate its acute upper thermal tolerance (e.g., as indicated by cardiac failure) can range from 26 °C to 31 °C, depending on the acclimation temperature and salinity (Braby and Somero, 2006[14]). Therefore, the sampling period should avoid periods when the ambient seawater temperature is above 24 °C.

15. Divers must collect the live mussels (wild or caged) manually at 4-5 m water depth. Mussel byssus threads should be cut from the substrate, since pulling the animals from the rocks (threading) can result in damage to internal tissues and induce an additional stress response in mussels. In case mussels living at the water/air interface are used, the contamination by lipophilic contaminants present in the water surface may alter the evaluation of the chemicals contents in the mussels soft tissues; moreover, the higher variability of this environment may influence the physiological status of the molluscs.

16. Mussel batches (both wild or caged animals) must consist of a standardized shell size usually 4-5 cm. A sufficient number of mussels is required to allow for biomarker analysis; the collection of 80-100 animals is suggested for the analysis of Lysosomal membrane stability (LMS), Micronuclei (MNi) frequency, Acetylcholinesterase (AChE) activity and Stress on Stress (SoS).

17. During the mussel collection a report should be prepared containing all sampling information data related to a) the sampling data as day, month and year, b) the number of molluscs sampled, c) the depth of collection (m), d) the georeferencing as Lat.-Long. (decimal degrees), e) location on the shoreline and the type of coast, f) type of site as reference or pollution gradient as distance from a polluted site (km), g) environmental data such as water temperature (C°), salinity (dimensionless) and dissolved oxygen (μmol L^{-1}). If necessary, tidal values (m) should be also reported.

[12] Sforzini S, Oliveri C, Orrù A, Chessa G, Jha A, Viarengo A, Banni M., 2018. Application of a new targeted low density microarray and conventional biomarkers to evaluate the health status of marine mussels: A field study in Sardinian coast, Italy. Sci. Total Environ. 628-629, 319-328.

[13] ICES, 2011. Report of the Study Group on Integrated Monitoring of Contaminants and Biological Effects (SGIMC), 14–18 March 2011, Copenhagen, Denmark. ICES CM 2011/ACOM:30. 265 pp

[14] Braby, C.E., Somero, G.N., 2006. Following the heart: temperature and salinity effects on heart rate in native and invasive species of blue mussels (genus *Mytilus*). J. Exp. Biol. 209, 2554-2566.

18. For caged mussels it is necessary to include information on depth of deployment (m), time of immersion (days), water column depth (m) and source of mussels.

19. For the interpretation of the biological effects of the chemical contaminants, it is important not only to evaluate their concentrations in the environmental matrices but also to estimate the amount of priority contaminants accumulated in the mollusc tissues. In that respect it is recommended to monitor same priority contaminates for CI 18 as they have been agreed for monitoring of CI 17 in biota matrix respectively Cd, HgT, Pb, PAHs, PCBs, Hexachlorobenzene, Lindane and ΣDDTs[15]. In this last case, 50 additional mussels should be collected for the chemical analysis, taken to the laboratory, and maintained at the field T, in clean, aerated seawater (at least 1 L/animal) for 24 h to eliminate gut contents. Then the soft tissues should be processed as described in the protocols related to the different chemical analysis reported in the Guidelines for sample preparation and analysis of marine biota for the analysis of CI17: heavy and trace elements and organic contaminants.

b. Mussel transport

20. After collection, the animals can be used for sample preparation directly in the field; however, the most usual procedure is to transport them to the laboratory. In this case, the animals are transported in a thermal insulated bag containing some ice cubes, the molluscs themselves being enveloped in a cotton tissue soaked with sea water; this ensures that the temperature in the container remains around 0-4 °C with a high humidity level. However, the transport period should be no longer than 8 hrs.

21. In the laboratory, the Collection Report must be placed in the Biomarker Analysis Register; the animals must be immediately sampled by the researcher(s) in charge of the biomonitoring programme and the samples adequately coded. In the Register the names of the researchers involved must be reported together with all the information concerning the location of the fridge in which the samples are stored.

22. Finally, it is also important to take into account that in the south-east of the Mediterranean basin there are coastal areas where *Mytilus* sp. are not present. In these areas, the use of the clams *Paratapes textilis* or *Pinctada radiata* is recommended. These bivalve molluscs are benthic organisms that live in sand and, therefore, will give broadly similar information, as would be obtained from mussels, about the effects of the contaminants present in the suspended organic material (the most important component of the diet of these filter-feeding molluscs) and those released from the sediments into the interstitial water. Although not exactly the same as the information obtained with mussels (i.e., intertidal organisms exposed to the contaminants present in the water column), the analysis of the biomarkers in these organisms will also permit the measurement of the harmful biological effects of the complex contaminant mixtures present in the marine coastal environment.

23. In this way, the integrated chemical-biological assessments of the effects of the contaminants present in the marine environment might better support the achievement of GES. As for chemical monitoring, sample collection should focus on selected locations such as hotspots and control or reference sites.

2.2 Protocol for the dissection and storage of tissue samples from marine molluscs (such as *Mytilus* sp.)

a. Materials

24. Application of this protocol requires availability of the following materials: Scalpel blades and handles; Dissecting forceps, fine and medium; Dissecting fine scissors; 1 mL syringes; 20 mL syringes with 21G (40 mm) needle; Syringe filters 0.45 μm; 15 mL centrifuge tubes, polypropylene, sterile, conical bottom; Microcentrifuge tubes, snap cap, 2.0 mL; Volume adjustable pipette, 20-200 μL and 200-1000 μL; Pipette tips, 20-200 μL and 200-1000 μL; 2 L glass beaker; Ice and ice bucket; Thermos ice packs; Cryostat chucks; Aluminum foil / Parafilm; Plastic container (200-400 mL); Thermostatic plastic container (3-4 L); Labeling tape; Permanent marker; Paper sheets and pen.

[15] UNEP/MAP (2019). UNEP/MED WG.467/5. IMAP Guidance Fact Sheets: Update for Common Indicators 13, 14,18, 18, 20 and 21: Nes proposal for Candidate Indicators 26 and 27

b. Equipment

25. The following equipment is needed: pH meter; Magnetic stirrer; Aquarium air pump and bubbler; Liquid nitrogen storage container (Dewar); Freezers -80°C; Ruler; Weight scale (readability 0.1 g - 0.01 g).

c. Solutions and chemicals

26. The use of filtered sea water (0.45 μm) collected at the animals' sampling sites is recommended; alternatively, it is possible to use a physiological saline where the salinity and pH is the same as the conditions at the sampling sites. The salinity of the solution described below is about 30.5 PSU, however, in the Mediterranean Sea the salinity can reach up to 44 PSU.

27. The chemicals and solution[16] needed for application of this protocol are as follows: Physiological saline: 20 mM (4.7 g) HEPES; 436 mM (25.48 g) NaCl; 53 mM (13.06 g) $MgSO_4$; 10 mM (0.75 g) KCl; 10 mM (1.47 g) $CaCl_2$. The NaCl concentration should be adjusted to take into account the sea water salinity at the sampling site. These components need to be dissolved in 1 litre of deionised water (using 2 L glass beaker and a magnetic stirrer). Then air bubble of the solution for 10 minutes is needed, and then adjustment to pH 7.9 (or to the sampling site's sea water pH) with 1M NaOH. There is a need to store the solution in a refrigerator, but to use it at room temperature.

28. Additionally, liquid nitrogen and n-Hexane are needed as reagents.

d. Tissue dissection

29. As mentioned above, preservation, storage and transportation to the laboratory from remote locations are key factors to undertake toxicological measurements in living organisms.

30. Molluscs (where possible *Mytilus* sp.) are opened by insert a scalpel halfway along their ventral surface; tissues are removed by using dissecting fine scissors and dissecting forceps and the tissues utilised for biomarker analysis.

31. Gills for the evaluation of AChE activity may be used as fresh tissue or rapidly frozen in liquid nitrogen and stored at -80 °C until the time of the analysis (Bocquené and Galgani, F. 1998[17]; UNEP/RAMOGE, 1999); gills for the evaluation of MNi frequency are removed, places in 15 mL centrifuge tubes and immediately processed (UNEP/RAMOGE, 1999; Barsiene et al., 2006[18]; Bolognesi and Fenech, 2012[19]).

32. Haemolymph cells for the evaluation of LMS (NRRT assay -Lowe et al., 1995[20]; UNEP/RAMOGE, 1999; Moore et al., 2004; Martínez-Gómez et al., 2015) are prepared in the following analytical procedure: a scalpel halfway is inserted along the ventral surface of the mussel and the valves are partially opened; a pipette tip (1000 μL) is inserted to allow the inset of the insulin syringe in the posterior adductor muscle of the mussel (Fig. 1A). N.B. it needs to remove the needle from the 1 mL syringe: the syringe needs to be fitted with a 21 G (0.5 mm inner diameter), 40 mm needle (a needle from a 20 mL syringe). The water is drained from the shells. The syringe is filled with 0.5 mL of physiological saline and then 0.5 mL of haemolymph are aspirated from the posterior adductor muscle of the mussel. After obtaining the haemolymph sample, the needle is discharged, and the contents is expelled into 2 mL microcentrifuge tube.

33. Haemolymph cells for the evaluation of MNi frequency are obtained as described above for LMS (UNEP/RAMOGE, 1999; Bolognesi and Fenech, 2012).

[16] If not specified, the reagents must be of analytical grade

[17] Bocquené, G., Galgani, F. 1998. Biological effects of contaminants: Cholinesterase inhibition by organophosphate and carbamate compounds. ICES Techniques in Marine Environmental Sciences, No. 22

[18] Barsiene, J., Schiedek, D., Rybakovas, A., Syvokiene, J., Kopecka, J., Forlin, L., 2006. Cytogenetic and cytotoxic effects in gill cells of the blue mussel Mytilus spp. from different zones of the Baltic Sea. Mar. Pollut. Bull. 53, 469-478

[19] Bolognesi, C., Fenech, M., 2012. Mussel micronucleus cytome assay, Nat. Protoc. 17, 1125-1137.

[20] Lowe, D.M., Soverchia C., Moore M.N., 1995. Lysosomal membrane responses in the blood and digestive cells of mussels experimentally exposed to fluoranthene. Aquatic Toxicol. 33, 105-112.

34. Digestive glands for the evaluation of LMS (cytochemical assay on cryostat sections -Moore, 1976[21], UNEP/RAMOGE, 1999; Moore et al., 2004; Martínez-Gómez et al., 2015) are obtained following this procedure: 5 small pieces of digestive gland (4-5 mm^3) are rapidly excised from the mid part of the organ obtained from five different animals and placed on an aluminium cryostat chuck (aligned in a straight row across the center). The chuck should be pre-labelled and pre-cooled in ice. 10 animals will be analysed by preparing 2 chucks for the same field sample. While dissecting the tissue, the chuck must be leaved on ice. Then the chuck is placed for 40 seconds in a small plastic box (200-400 mL) containing pre-cooled n-hexane (hexane super-cooling prevents the formation of ice in the tissues and, hence, it reduces structural damage to the subcellular components) at -70 °C using liquid nitrogen filled in a thermostatic plastic container (3-4 L) (the temperature of about -70 °C is visualized by the solidification of the n-hexane, a certain amount of liquid n-hexane in the presence of a solid component will ensure the correct temperature for the sample treatment). The chuck is sealed with 2-3 pieces of Parafilm/aluminium foils and immediately stored at -70 °C (at this temperature the tissue preparations maintain their integrity for months).

e. Additional parameters to be recorded in this step (in the field or at the laboratory)

35. The following additional parameters need to be recorded in this step both in the field or at the laboratory:

- Mussels biometrics: length (to 0.1 cm), weight (to 0.1 g), soft tissue weight (to 0.1 g);
- Condition Index (CI): this parameter should be evaluated in a simple way as: CI = 100 x Dry weight (to 0.01 g) / Whole animal weight (to 0.1 g); although more accurate (and complex) approaches are available such as: CI = 100 x Dry weight / Internal shell volume (ICES, 2011; Lutz, 1980[22]; Aldrich and Crowley; 1986[23]; Davenport and Chen, 1987[24]; Hansson et al., 2017).

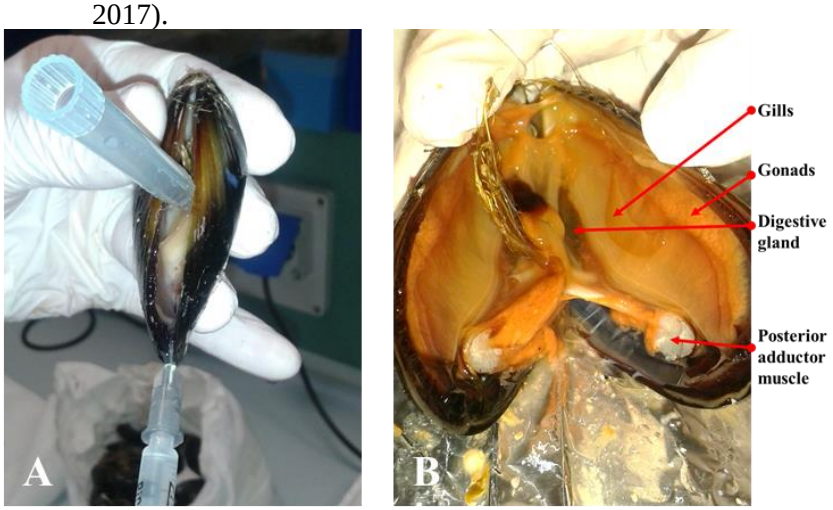


Fig. 1. A) Haemolymph extraction from mussel posterior adductor muscle; B) mussel tissue identification.

36. The presence of parasites in the soft tissues should be also reported.

[21] Moore, M.N., 1976. Cytochemical demonstration of latency of lysosomal hydrolases in digestive gland cells of the common mussel *Mytilus edulis*, and changes induced by thermal stress. Cell Tissue Res. 175, 279-287.
[22] Lutz, R.A. 1980. Mussel Culture and Harvest: A North American Perspective, Elsevier Science Publishers, B.V. Amsterdam, 305pp.
[23] Aldrich, J.C., Crowly, M. 1986. Conditions and variability in *Mytilus edulis* L. from different habitats in Ireland. Aquaculture 52: 273–286.
[24] Davenport, J., Chen, X. 1987. A comparison of methods for the assessment of condition in the mussel (*Mytilus edulis* L.). J. Molluscan Stud., 53: 293–297.

37. Sampled molluscs and their gonads should be recorded by a high definition video camera (or smartphone video camera) to document the reproductive status of the animals. Samples of gonads should be frozen in liquid nitrogen and stored at -70 °C to be available, if necessary, for examination.

38. At the end of the procedure related to sample preparation and storage, a report must be prepared indicating the list and the code of the samples for the different biomarker analysis, the -80 °C fridge used for the storage of the samples and the location in the fridge of the different samples, as well as the list reporting the data related to all the additional parameters evaluated. The report must to be added to the Register for the Biomarker Analysis. In the Report, it needs also to indicate the data of the samples` preparation and storage and the name of the researchers involved in the work.

3 Technical note for the sampling and sample preservation of marine fish (*Mullus barbatus*) for biomarker analysis

39. The aim of the MED POL Biomonitoring Programme is to provide a clear picture of the quality of the marine coastal environment in the Mediterranean area. An important aspect for achieving this target is the selection of the sentinel organisms to be used for the evaluation of the toxic effects of the marine contaminants.

40. The use of the same organisms throughout the different Mediterranean areas ensures more comparable ecotoxicological results. *Mullus barbatus* was selected as sentinel organisms on the basis of the results of numerous studies and of the previous activities in the framework of the MED POL biomonitoring programmes (UNEP/RAMOGE, 1999).

41. The marine fish *M. barbatus* sampled to perform the biomarker toxicological evaluations should be also used for the chemical analysis as described in the Monitoring Guidelines/Protocols for Sampling and Sample Preservation of Marine Biota for IMAP Common Indicator 17: Heavy and Trace Elements and Organic Contaminants for CI17 (UNEP/MAP WG. 482/13). The sampled fish have to be alive and in good conditions.

42. The red mullet, *Mullus barbatus* (L.), is a fish that is widely distributed along all Mediterranean coast (www.fao.org/fishery/species/3208/en) and it was used in past years as a sentinel organism to evaluate the accumulation of toxic chemicals, as well as to study the harmful biological effects of environmental pollutants (Mathieu et al., 1991[25]; Porte et al., 2002[26]; Regoli et al., 2002[27]; Viarengo et al., 2007[28]; Martínez-Gómez et al., 2012[29], 2017). Its lifestyle (i.e. non-migratory animals, relatively localised in the coastal areas) and its feeding habits (e.g. their diet consists mainly of small benthic organisms such as crustaceans, molluscs and worms - www.fao.org/fishery/species/3208/en) render this fish as a suitable sentinel organism. M. barbatus is a batch spawner; the existence of a seasonal, depth-related movement in this species has been well described (Machias and Labropoulou, 2002[30]). Their toxic chemical intake reflects well the pollution level of the sediment from the inner and medium continental shelves and of the overlaying water column.

[25] Mathieu, A., Lemaire, P., Carriere, S., Drai, P., Giudicelli, J., Lafaurie, M., 1991. Seasonal and sex-linked variations in hepatic and extrahepatic biotransformation activities in striped mullet (Mullus barbatus). Ecotoxicol. Environ. Saf. 22, 45-57.
[26] Porte, C., Escartín, E., García de la Parra, L.M., Biosca, X., Albaigés, J., 2002. Assessment of coastal pollution by combined determination of chemical and biochemical markers in *Mullus barbatus*. Mar. Ecol. Prog. Ser. 235, 205-216.
[27] Regoli, F., Pellegrini, D., Winston, G.W., Gorbi, S., Giuliani, S., Virno-Lamberti, C., Bompadre, S., 2002. Application of biomarkers for assessing the biological impact of dredged materials in the Mediterranean: the relationship between antioxidant responses and susceptibility to oxidative stress in the red mullet (*Mullus barbatus*). Mar. Pollut. Bull. 44, 912-922.
[28] Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E., Koehler, A., 2007. The use of biomarkers in biomonitoring: a 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. Comp. Biochem. Physiol. C 146, 281-300.
[29] Martínez-Gómez, C., Fernández, B., Benedicto, J., Valdés, J., Campillo, J. A., León, V. M., Vethaak, A. D., 2012. Health status of red mullets from polluted areas of the Spanish Mediterranean coast, with special reference to Portmán (SE Spain). Mar. Environ. Res. 77, 50-59.
[30] Machias, A., Labropoulou, M., 2002. Intra-specific variation in resource use by red mullet, *Mullus barbatus*. Estuar. Coast. Shelf Sci. 55, 565-578.

43. Under this Technical Note, the Monitoring Guidelines for Sampling and Sample Preservation of Marine Molluscs (such as Mytilus sp.) and Fish (Mullus barbatus) for IMAP Common Indicator 18 provides the following two Protocols: i) Protocol for the collection of marine fish (*Mullus barbatus*) and ii) Protocol for the dissection and storage of tissue samples from marine fish (*Mullus barbatus*).

3.1 Protocol for the collection of marine fish (*Mullus barbatus*)

a. Selection of the sampling areas and sampling frequency

44. *M. barbatus* is a benthic species that inhabits the sandy and muddy bottoms of the Mediterranean continental shelf (www.fao.org/fishery/species/3208/en). The mature organisms are usually distributed in the first 3-5 km from the coast at depths ranging from a few meters to 500 meters (Carlucci et al., 2009[31]; Follesa and Carbonara, 2019[32]).

45. Although the sex difference can influence various physiological parameters, the biomarkers selected for environmental assessment may be evaluated using both male and female fish, as long as the specimens used are sampled according to a standardised sampling protocol in order to minimise confounding factors. However, the animals should always be sampled outside the reproductive periods (i.e. September-October or March-April –see the Guidelines for biomarker analysis CI18) (Carbonara et al., 2015[33]; Ferrer-Maza et al., 2015[34]).

b. Fish collection

46. *M. barbatus* are collected by gill net fishing or trawling using a square-meshed net of 40 mm or, if justified, by a diamond meshed net of 50 mm as required by the EU legislation (EC 1967/2006[35]; Sieli et al., 2011[36]). The gill net fishing time should be no longer of 30 min; and the trawling time no longer of 15 minutes using a speed ≤ 3 knots in order to minimise possible alterations of the physiological status of living fish. Fish having a length of 12-16 cm should be selected for the biomarker analysis.

47. Fish are killed on board and the tissues for the biomarker analysis are sampled as described in the Protocol for the dissection and storage of tissue samples from marine fish (*Mullus barbatus*).

48. For the interpretation of the biological effects of the chemical contaminants, it is important not only to evaluate their concentrations in the environmental matrices but also to estimate the amount of prioritized contaminants accumulated in fish tissues or whole body. In that respect it is recommended to monitor same priority contaminates for CI 18 as they have been agreed for monitoring of CI 17 in biota matrix respectively Cd, HgT, Pb, PAHs, PCBs, Hexachlorobenzene, Lindane and ΣDDTs[37]. In this last case, fish should be collected, taken to the laboratory and processed as described in the protocols related to the different chemical analysis for CI 17 as reported in the Guidelines for sample

[31] Carlucci, R., Lembo, G., Maiorano, P., Capezzuto, F., Marano, C.A., Sion, L., Spedicato, M.T., Ungaro, N., Tursi, A., Gianfranco, D., 2009. Nursery areas of red mullet (*Mullus barbatus*), hake (*Merluccius merluccius*) and deep-water rose shrimp (*Parapenaeus longirostris*) in the Eastern-Central Mediterranean Sea. Estuar. Coast. Shelf Sci. 83, 529-538

[32] Follesa, M.C., Carbonara, P., eds. 2019. Atlas of the maturity stages of Mediterranean fishery resources. Studies and Reviews n. 99. Rome, FAO. 268 pp.

[33] Carbonara, P., Intini, S., Modugno, E., Maradonna, F., Spedicato, M. T., Lembo, G., Zupa, W., Carnevali, O., 2015. Reproductive biology characteristics of red mullet (*Mullus barbatus* L., 1758) in Southern Adriatic Sea and management implications. Aquat. Living Resour. 28, 21-31.

[34] Ferrer-Maza, D., Muñoz, M., Lloret, J., Faliex, E., Vila, S., Sasal, P., 2015. Health and reproduction of red mullet, *Mullus barbatus*, in the western Mediterranean Sea. Hydrobiologia 753, 189-204.

[35] EC COUNCIL REGULATION No 1967/2006 of 21 December 2006 concerning management measures for the sustainable exploitation of fishery resources in the Mediterranean Sea, amending Regulation (EEC) No 2847/93 and repealing Regulation (EC) No 1626/94

[36] Sieli, G., Badalucco, C., Di Stefano, G., Rizzo, P., D'Anna, G., Fiorentino, F. 2011. Biology of red mullet, *Mullus barbatus* (L. 1758), in the Gulf of Castellammare (NW Sicily, Mediterranean Sea) subject to a trawling ban. J Appl Ichthyol. 27:1218-1225.

[37] UNEP/MAP (2019). UNEP/MED WG.467/5. IMAP Guidance Factsheets: Update for Common Indicators 13, 14, 17, 18, 20 and 21: New proposal for Candidate indicators 26 and 27.

preparation and analysis of marine biota for the analysis of CI17: heavy and trace elements and organic contaminants.

49. In this way, the integrated chemical-biological assessments of the effects of the contaminants present in the marine environment might better support the achievement of GES (Good Environmental Status). Sample collection should focus on selected locations such as hotspots and reference stations.

50. During the fish collection a report (Collection Report) should be prepared containing sampling information data related to a) the sampling data as day, month and year, b) the number of fish sampled, c) the depth of collection (m), d) the georeferencing as Lat.-Long (decimal degrees), e) type of bottom, f) type of site as reference or pollution gradient as distance from a polluted site (km), g) environmental data such as water temperature(C°), salinity (dimensionless) and dissolved oxygen (μmol L^{-1}).

51. In the lab, the Collection Report must be left in the Biomarker Analysis Register; the Report should also contain the names of the researchers involved in fish collection.

3.2 Protocol for the dissection and storage of tissue samples from marine fish (*Mullus barbatus*)

a. Materials

52. Application of this protocol requires availability of the following materials: Dissecting forceps, fine and medium; Dissecting robust and fine scissors; Single-use syringe, 5 ml; Volume adjustable pipette, 20-200 μl and 200-1000 μl; Pipette tips, 20-200 μl and 200-1000 μl; Microscope slides, 76x26 mm, 1 mm thick, pre cleaned/ready to use, Menzel-Gläser, Superfrost, wiped with ethanol and allowed to dry before use; Ice and ice bucket; Thermos ice packs; Cryostat chucks (anodized-aluminium support to cut cryostat sections of the biological samples); Aluminium foil / Parafilm; Thermostatic plastic container (200-400 ml); Labelling tape; Permanent marker; Paper sheets and pen.

b. Equipment

53. The following equipment is needed: Liquid nitrogen storage container (Dewar); Freezers -80°C; Ruler; Weight scale (readability 0.1 g - 0.01 g); Video camera / Smartphone video camera.

c. Chemicals and solutions

54. The chemicals and solution needed for application of this protocol are as follows: Sodium heparin; Methanol, Methyl alcohol, absolute, Assay: 99,8%; Ethanol; Liquid nitrogen.

N.B. If not specified, the reagents must be of analytical grade.

d. Tissue dissection

55. Immediately after collection, living fish (*Mullus barbatus*) are killed on board by severing the spinal cord and rapidly dissected to obtain the tissues for the selected biomarker analysis. Fish are opened by robust scissors and the tissues are removed by using dissecting fine scissors and dissecting forceps.

56. Liver samples for the evaluation of Lysosomal membrane stability (cytochemical assay of LMS on cryostat sections -Köhler, 1991[38]; Köhler and Pluta, 1995[39]; UNEP/RAMOGE, 1999;

[38] Köhler, A., 1991. Lysosomal perturbations in fish liver as indicators for toxic effects of environmental pollution. Comp. Biochem. Physiol. 100C, 123-127.
[39] Köhler, A. Pluta, H.J., 1995. Lysosomal injury and MFO activity in the liver of flounder (*Platichthys flesus* L.) in relation to histopathology of hepatic degeneration and carcinogenesis. Mar. Environ. Res. 39, 255-260.

Martínez-Gómez et al., 2015) are processed essentially as described for mussel digestive glands (Protocol for the dissection and storage of tissue samples from marine molluscs (such as *Mytilus* sp.)).

57. The only difference is that chucks are frozen directly in liquid nitrogen for 40 s. Rapidly excise 5 small pieces (4-5 mm^3) from the mid part of the organ obtained from five different animals and place them on an aluminium cryostat chuck (aligned in a straight row across the centre). The chuck should be pre-labelled and pre-cooled in ices. 10 animals will be analysed by preparing 2 chucks for the same field sample. While dissecting the tissue, leave the chuck on ice. Then place it for 40 s in a small thermostatic plastic box containing liquid nitrogen. Seal the chuck with 2-3 pieces of Parafilm/aluminium foil and immediately store at -70 °C (at this temperature the tissue preparations maintain their integrity for months). LMS is a very sensitive parameter: a special attention should be given to use fish undergoing a minimal stress during fishing.

58. Muscle for the evaluation of AChE activity may be used as fresh tissue or rapidly frozen in liquid nitrogen and stored at -70 °C before the analysis (at this temperature the tissue preparations maintain their integrity for months).

59. Blood cells for the evaluation of Micronuclei frequency are collected from the caudal vein of intact fish using a syringe containing sodium heparin (1000 units/mL), mixed and immediately smeared on clean glass slides (Bolognesi and Hayashi, 2011[40]). The slides are dried overnight and subsequently fixed with methanol for at least 20 min.

e. Additional parameters to be recorded in this step (in the field or at the laboratory)

60. The following additional parameters need to be recorded in this step both in the field or at the laboratory:

- Fish biometrics: total length (to 0.1cm), weight (to 0.1 g);
- Fulton's condition factor, K (Bagenal and Tesch, 1978[41]). K = weight (to 0.1 g) / total length (to 0.1 cm)3. The condition factor reflects the nutritional state or "well-being" of an individual fish and is sometimes interpreted as an index of growth rate (Bagenal and Tesch, 1978; ICES, 2011);
- Measurement of GSI: GSI = (gonad weight (to 0.01 g) x 100) /(total body weight (to 0.1 g) - stomach content - gonad weight (to 0.01 g)). The gonad size is an important indicator of the reproductive status and GSI allows to evaluate when fish, in relation to their size (or age), are sexually immature or adult, or if the animals show retarded gonad development as compared to normal sexual development (Hansson et al., 2017; ICES, 2011);
- Liver Somatic Index (LSI or HSI). LSI = (liver weight (to 0.1g) x 100) / (total body weight (to 0.1 g) - stomach content - liver weight (to 0.1 g)). As known, liver plays a central role in fish metabolism and numerous studies have highlighted that toxic chemicals may affect liver size and its functions. It has been also demonstrated in numerous field studies that fish accumulation of contaminants may affect the LSI value (Hansson et al., 2017; ICES, 2011).
- Age: 12-16 cm length is a dimension typical of 1-2 years old fish (Carbonara et al. 2018[42]). To establish M. barbatus age in a more precise manner it is necessary to evaluate the otoliths as described by ICES (2017[43]) and Carbonara et al. (2018).

61. Sampled fish and their gonads should be recorded by a high definition video camera (or smartphone video camera) to document the reproductive status of the animals. Samples of gonads

[40] Bolognesi, C., Hayashi, M., 2011. Micronucleus assay in aquatic animals. Mutagenesis 26, 205-213.
[41] Bagenal, T.B. and Tesch, F.W. 1978. Age and Growth. Pages 101-136, in T.B. Bagenal, edit. Methods for assessment of fish production in freshwaters, 3rd edition. Blackwell Scientific Publications, Oxford, England.
[42] Carbonara P., Intini S., Kolitari J., et al. 2018. A holistic approach to the age validation of *Mullus barbatus* L., 1758 in the Southern Adriatic Sea (Central Mediterranean). Sci. Rep. 8: 13219.
[43] ICES, 2017. Workshop on Ageing Validation methodology of *Mullus* species (WKVALMU), 15-19 May 2017, Conversano, Italy. ICES CM 2017/ SSGIEOM:31. 74 pp.

should be frozen in liquid nitrogen and stored at -70 °C to be available, when necessary, for examination.

62. At the end of the procedure related to samples preparation and storage, a report must be prepared indicating the list and the code of the samples for the different biomarker analysis, the -80 °C freezer used for the storage of the samples including the exact location in the freezer, as well as the list reporting the auxiliary data related to all the additional parameters evaluated. The report must be added to the Register for the Biomarker Analysis. The Report must also include the data of the sample preparation and storage and the name of the researchers involved in the work.

Annex I
References

References

Aldrich, J.C., Crowly, M. 1986. Conditions and variability in *Mytilus edulis* L. from different habitats in Ireland. Aquaculture 52: 273–286.

Bagenal, T.B. and Tesch, F.W. 1978. Age and Growth. Pages 101-136, in T.B. Bagenal, edit. Methods for assessment of fish production in freshwaters, 3rd edition. Blackwell Scientific Publications, Oxford, England.

Barsiene, J., Schiedek, D., Rybakovas, A., Syvokiene, J., Kopecka, J., Forlin, L., 2006. Cytogenetic and cytotoxic effects in gill cells of the blue mussel *Mytilus* spp. from different zones of the Baltic Sea. Mar. Pollut. Bull. 53, 469-478.

Bayne, B.L., 2009. Marine Mussels: Their Ecology and Physiology. Cambridge University Press 528 p.

Bocquené, G., Galgani, F. 1998. Biological effects of contaminants: Cholinesterase inhibition by organophosphate and carbamate compounds. ICES Techniques in Marine Environmental Sciences, No. 22.

Bolognesi, C., Fenech, M., 2012. Mussel micronucleus cytome assay, Nat. Protoc. 17, 1125-1137.

Bolognesi, C., Hayashi, M., 2011. Micronucleus assay in aquatic animals. Mutagenesis 26, 205-213.

Braby, C.E., Somero, G.N., 2006. Following the heart: temperature and salinity effects on heart rate in native and invasive species of blue mussels (genus *Mytilus*). J. Exp. Biol. 209, 2554-2566.

Carbonara P., Intini S., Kolitari J., et al. 2018. A holistic approach to the age validation of *Mullus barbatus* L., 1758 in the Southern Adriatic Sea (Central Mediterranean). Sci. Rep. 8: 13219.

Carbonara, P., Intini, S., Modugno, E., Maradonna, F., Spedicato, M. T., Lembo, G., Zupa, W., Carnevali, O., 2015. Reproductive biology characteristics of red mullet (*Mullus barbatus* L., 1758) in Southern Adriatic Sea and management implications. Aquat. Living Resour. 28, 21-31.

Carlucci, R., Lembo, G., Maiorano, P., Capezzuto, F., Marano, C.A., Sion, L., Spedicato, M.T., Ungaro, N., Tursi, A., Gianfranco, D., 2009. Nursery areas of red mullet (*Mullus barbatus*), hake (*Merluccius merluccius*) and deep-water rose shrimp (*Parapenaeus longirostris*) in the Eastern-Central Mediterranean Sea. Estuar. Coast. Shelf Sci. 83, 529-538.

Davenport, J., Chen, X. 1987. A comparison of methods for the assessment of condition in the mussel (*Mytilus edulis* L.). J. Molluscan Stud., 53: 293–297.

EC COUNCIL REGULATION No 1967/2006 of 21 December 2006 concerning management measures for the sustainable exploitation of fishery resources in the Mediterranean Sea, amending Regulation (EEC) No 2847/93 and repealing Regulation (EC) No 1626/94.

Ferrer-Maza, D., Muñoz, M., Lloret, J., Faliex, E., Vila, S., Sasal, P., 2015. Health and reproduction of red mullet, *Mullus barbatus*, in the western Mediterranean Sea. Hydrobiologia 753, 189-204.

Follesa, M.C., Carbonara, P., eds. 2019. Atlas of the maturity stages of Mediterranean fishery resources. Studies and Reviews n. 99. Rome, FAO. 268 pp.

Goldberg, E.D., Bowen, V.T., Farrington, J.W., Harvey, G., Martin, J.H., Parker P.L., Risebrough, R.W., Robertson, W., Schneider, E., Gamble, E., 1978. The Mussel Watch. Environmental Conservation 5, 101-125.

Hansson, T., Thain, J., Martínez-Gómez, C., Hylland, K., Gubbins, M., Balk L., 2017. Supporting variables for biological effects measurements in fish and blue mussel. ICES Techniques in Marine Environmental Sciences. No. 60. 22 pp. http://doi.org/10.17895/ices.pub.2903.

ICES, 2011. Report of the Study Group on Integrated Monitoring of Contaminants and Biological Effects (SGIMC), 14–18 March 2011, Copenhagen, Denmark. ICES CM 2011/ACOM:30. 265 pp.

ICES, 2017. Workshop on Ageing Validation methodology of *Mullus* species (WKVALMU), 15-19 May 2017, Conversano, Italy. ICES CM 2017/ SSGIEOM:31. 74 pp.

Köhler, A., 1991. Lysosomal perturbations in fish liver as indicators for toxic effects of environmental pollution. Comp. Biochem. Physiol. 100C, 123-127.

Köhler, A. Pluta, H.J., 1995. Lysosomal injury and MFO activity in the liver of flounder (*Platichthys flesus* L.) in relation to histopathology of hepatic degeneration and carcinogenesis. Mar. Environ. Res. 39, 255-260.

Lowe, D.M., Soverchia C., Moore M.N., 1995. Lysosomal membrane responses in the blood and digestive cells of mussels experimentally exposed to fluoranthene. Aquatic Toxicol. 33, 105-112.

Lutz, R.A. 1980. Mussel Culture and Harvest: A North American Perspective, Elsevier Science Publishers, B.V. Amsterdam, 305pp.

Machias, A., Labropoulou, M., 2002. Intra-specific variation in resource use by red mullet, *Mullus barbatus*. Estuar. Coast. Shelf Sci. 55, 565-578.

Martínez-Gómez, C., Bignell, J. and Lowe, D., 2015. Lysosomal membrane stability in mussels. ICES Techniques in Marine Environmental Sciences No. 56. 41 pp.

Martínez-Gómez, C., Fernández, B., Benedicto, J., Valdés, J., Campillo, J. A., León, V. M., Vethaak, A. D., 2012. Health status of red mullets from polluted areas of the Spanish Mediterranean coast, with special reference to Portmán (SE Spain). Mar. Environ. Res. 77, 50-59.

Mathieu, A., Lemaire, P., Carriere, S., Drai, P., Giudicelli, J., Lafaurie, M., 1991. Seasonal and sex-linked variations in hepatic and extrahepatic biotransformation activities in striped mullet (*Mullus barbatus*). Ecotoxicol. Environ. Saf. 22, 45-57.

Moore, M.N., 1976. Cytochemical demonstration of latency of lysosomal hydrolases in digestive gland cells of the common mussel *Mytilus edulis*, and changes induced by thermal stress. Cell Tissue Res. 175, 279-287.

Moore, M.N., Lowe, D. and Köhler, A. 2004. Biological effects of contaminants: Measurement of lysosomal membrane stability. ICES Techniques in Marine Environmental Sciences. No. 36. 39 pp.

OSPAR, 1997. JAMP Guidelines for General Biological Effects Monitoring (OSPAR Agreement 1997-7). OSPAR Commission, Monitoring guidelines. Ref. No: 1997-7. 20 pp.

Porte, C., Escartín, E., García de la Parra, L.M., Biosca, X., Albaigés, J., 2002. Assessment of coastal pollution by combined determination of chemical and biochemical markers in *Mullus barbatus*. Mar. Ecol. Prog. Ser. 235, 205-216.

Regoli, F., Pellegrini, D., Winston, G.W., Gorbi, S., Giuliani, S., Virno-Lamberti, C., Bompadre, S., 2002. Application of biomarkers for assessing the biological impact of dredged materials in the Mediterranean: the relationship between antioxidant responses and susceptibility to oxidative stress in the red mullet (*Mullus barbatus*). Mar. Pollut. Bull. 44, 912-922.

Sforzini S, Oliveri C, Orrù A, Chessa G, Jha A, Viarengo A, Banni M., 2018. Application of a new targeted low density microarray and conventional biomarkers to evaluate the health status of marine mussels: A field study in Sardinian coast, Italy. Sci. Total Environ. 628-629, 319-328.

Sieli, G., Badalucco, C., Di Stefano, G., Rizzo, P., D'Anna, G., Fiorentino, F. 2011. Biology of red mullet, *Mullus barbatus* (L. 1758), in the Gulf of Castellammare (NW Sicily, Mediterranean Sea) subject to a trawling ban. J Appl Ichthyol. 27:1218-1225.

UNEP, 1997. The MED POL Biomonitoring Programme Concerning the Effects of Pollutants on Marine Organisms Along the Mediterranean Coasts. UNEP(OCA)/MED WG.132/3, Athens.

UNEP/RAMOGE: Manual on the Biomarkers Recommended for the MED POL Biomonitoring Programme. UNEP, Athens, 1999.

UNEP/MAP (2019) UNEP/MED WG.467/5. IMAP Guidance Factsheets: Update for Common Indicators 13,14, 17, 18, 20 and 21: New proposal for candidate indicators 26 and 27.

Viarengo, A., Dondero, F., Pampanin, D.M., Fabbri, R., Poggi, E., Malizia, M., Bolognesi, C., Perrone, E., Gollo, E., Cossa, G.P., 2007. A biomonitoring study assessing the residual biological effects of pollution caused by the HAVEN wreck on marine organisms in the Ligurian Sea (Italy). Arch Environ Contam Toxicol. 53, 607-616.

Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E., Koehler, A., 2007. The use of biomarkers in biomonitoring: a 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. Comp. Biochem. Physiol. C 146, 281-300.

Viarengo, A.; Lafaurie, M.; Gabrielides, G.P.; Fabbri, R.; Marro, A., Roméo, M., 2000. Critical evaluation of an intercalibration exercise undertaken in the framework of the MED POL biomonitoring program. Mar. Environ. Res. 49, 1-18.