IAEA Marine Environment Laboratories

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C. IMAP MONITORING GUIDELINES FOR CI18

C-1. Monitoring Guidelines/Protocols for sampling and determination of biomarkers in marine molluscs (such as *Mytilus* sp.) and fish (such as *Mullus barbatus*)

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1 Guidelines for sampling and sample preservation of marine molluscs and fish

1.1 Introduction

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).

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.

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; d) Stress on stress (SoS), a not mandatory biomarker suitable to reveal the reduced capacity of the organisms to survive to the action of further environmental stressors.

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].It should be noted that LMS in *Mullus barbatus* may be considered for inclusion as a mandatory parameter only if the capacities will be strengthened enough though the Inter-laboratory comparison to guarantee the correct use of this biomarker.

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.

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 determination of the different biomarkers as provided in UNEP/MED WG.509/28 and UNEP/MED WG.509/29.

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

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. 509/23). Whenever possible the biomarkers and chemical analysis should be done on the same samples. This will allow better integration of biological effects and contaminants monitoring. 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/MAP, 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

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[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.

[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/MAP. 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.

fraction (Goldberg et al., 1978[8]; Bayne, 2009[9]; Viarengo et al., 2000[10]).

In biomonitoring programmes, wild native mussels sampling can be organized; 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, when wild mussels are used, it is recommended to evaluate the stage of gonadal development in the sampled molluscs, a parameter that can greatly change the physiological status of the organism.

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; however, this does not eliminate sampling of wild native mussels. A longer time of exposure (2 months or longer) may be applied; however, in this case it is necessary to evaluate the stage of gonad development to reduce the effects of the confounding factors. 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 parameter to readily identify extremely polluted areas, where the high concentration of toxic chemicals may cause lethal pathological alterations in the animals.[12]

The mussels have to be caged in containment structures (e.g. polyethylene bags, or better, non-plastic bags, mounted on PVC tubing) for a period of at least of 30 days [13] (Sforzini et al., 2018[14]). It is important that the mussels used for caging experiments are collected from a clean site, and that before to start the experiment.

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.).

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

a. Mussel collection

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.

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[15]; Moore et al., 2004). The selected frequency for the biomonitoring activities is also in line with the practice exercised during MEDPOL IV Biomonitoring.

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[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.

[12] It should be noted that a stress due to the caging bags was not found during realization of MEDPOL IV biomonitoring activities.

[13] A period of 30 days is best for collecting data related to the analysis of biomarkers only; however, if samples are also taken for chemical analysis a period of at least 60 days should be ensured, along with providing information on gonad development.

[14] 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.

[15] 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

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[16]). Therefore, the sampling period should avoid periods when the ambient seawater temperature is above 24 °C.

Divers must collect the live mussels (wild or caged) manually at 5-7 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.

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

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}); when possible, the data of the Chla concentration in the water may be also evaluated. If necessary, tidal values (m) should be also reported. All the information should be related to the sampling day.

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.

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 (UNEP/MAP, 2019). 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 determination of heavy and trace elements and organic contaminants[17]. In this regard it should be noted that additional chemical analysis is not necessary, but the integrative approach uses the data obtained during the chemical monitoring activities.

b. Mussel transport

After collection, the animals can be used for sample preparation directly in the field; therefore, on board clean space/laboratory facilities are necessary; 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. The transport should be undertaken within a period of 8 hrs; however, it should be noted that in some cases transportation cannot be completed within this period of time. In this case the laboratory should collect some preliminary data showing that no changes occur in control animals maintained for the selected period of time in the transport conditions. A specific common testing of the period of transportation should be exercised between the Parties.

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.

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 determination of biomarkers in these organisms will also permit the measurement of the

[16] 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.

[17] It should be noted that in some cases the logistic problems and physiological impacts on animals may be caused by maintaining mussels for 24 h in clean, aerated seawater to eliminate gut contents

harmful biological effects of the complex contaminant mixtures present in the marine coastal environment.

The integrated chemical-biological assessments of the effects of the contaminants present in the marine environment supports provision of data needed for GES assessment. As for the chemical monitoring, sample collection for biomarkers should be focused on selected locations such as hotspots and control or reference sites.

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

a. Materials

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.

b. Equipment

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

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[18].

The chemicals and solution[19] 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.

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

d. Tissue dissection

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

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. When possible, this rapid dissection should be done as soon as possible shortly after molluscs sampling (this should be done on board in clean condition).

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[20]; 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[21]; Bolognesi and Fenech, 2012[22]).

Haemolymph cells for the evaluation of LMS (NRRT assay -Lowe et al., 1995[23]; 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 (Figure 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.

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

[18] In line with present experience of Spain, alternatively use of filtered sea water, collected at the clean/reference sites in national coastal waters, at the salinity found at the sampling site, can be considered

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

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

[21] 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

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

[23] 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.

Digestive glands for the evaluation of LMS (cytochemical assay on cryostat sections -Moore, 1976[24], 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)

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

- Mussels biometrics: length (to 0.1 cm), weight (to 0.1 g), soft tissue weight (to 0.1 g); dry soft tissue mass (to 0.1 g), dry shell mass (to 0.1g);
- Condition Index (CInd): this parameter should be evaluated in a simple way as: CInd = 100 x Dry soft tissue weight (to 0.01 g) / Whole animal dry weight (to 0.1 g); alternatively, dry soft tissues mass / dry shell mass. More accurate (and complex) approaches are available such as: CInd = 100 x Dry weight (to 0.1 g) / Internal shell volume (to 0.1 cm^3) (ICES, 2011; Lutz, 1980[25]; Aldrich and Crowley; 1986[26]; Davenport and Chen, 1987[27]; Hansson et al., 2017).

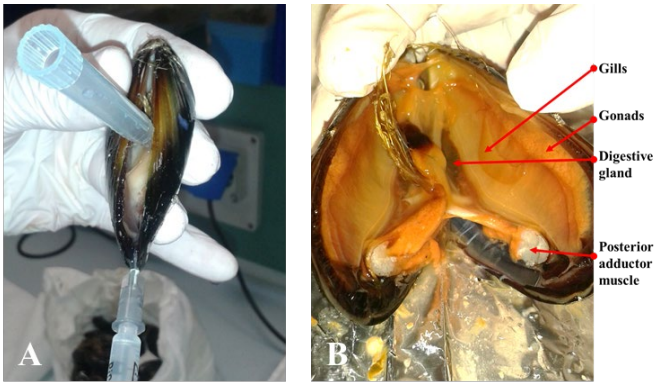

Figure 1. A) Haemolymph extraction from mussel posterior adductor muscle; B) mussel tissue identification.

The presence of parasites in the soft tissues should be also reported (Francisco, C. J et al. 2010[28], Robledo, J. A. F et al. 1994[29], Figueras, A. J et al. 1991[30]).

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.

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 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.

1.3 Technical note for the sampling and sample preservation of marine fish (*Mullus barbatus*) for biomarkers determination

The aim of the MED POL Biomonitoring Programme is to provide a clear picture of the quality of the marine

[24] 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.

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

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

[27] 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.

[28] Francisco, C. J., Hermida, M. A., & Santos, M. J. (2010). Parasites and symbionts from Mytilus galloprovincialis (Lamark, 1819) (Bivalves: Mytilidae) of the Aveiro estuary Portugal. Journal of Parasitology, 96(1), 200-205

[29] Robledo, J. A. F., Santarém, M. M., & Figueras, A. (1994). Parasite loads of rafted blue mussels (Mytilus galloprovincialis) in Spain with special reference to the copepod, Mytilicola intestinalis. Aquaculture, 127(4), 287-302

[30] Figueras, A. J., Jardon, C. F., & Caldas, J. R. (1991). Diseases and parasites of rafted mussels (Mytilus galloprovincialis Lmk): preliminary results. Aquaculture, 99(1-2), 17-33

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.

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).

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.509/23). The sampled fish have to be alive and in good conditions. Whenever possible, it is recommendable that once fish is on board, to keep it alive in aerated tanks using clean sea water before tissue dissection processing takes place.

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[31]; Porte et al., 2002[32]; Regoli et al., 2002[33]; Viarengo et al., 2007a[34]; Martínez-Gómez et al., 2012[35], 2017[36]) In this regard, it should be considered that the sex difference is essential for contaminants determination, therefore analyses, when possible, should be done on same sex pooled samples. 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[37]). 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.

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*).

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

a. Selection of the sampling areas and sampling frequency

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[38]; Follesa and Carbonara, 2019[39]).

Although the sex difference can influence various physiological parameters, the biomarkers selected for

[31] 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.

[32] 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.

[33] 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.

[34] Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E., Koehler, A., 2007a. 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.

[35] 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.

[36] Martínez-Gómez, C., Fernández, B., Robinson, C. D., Campillo, J. A., León, V. M., Benedicto, J., & Vethaak, A. D. (2017). Assessing environmental quality status by integrating chemical and biological effect data: The Cartagena coastal zone 26as a case. Marine environmental research, 124, 106-117.

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

[38] 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

[39] 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.

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[40]; Ferrer-Maza et al., 2015[41]).

b. Fish collection

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[42]; Sieli et al., 2011[43]). 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.

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

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 (UNEP/MAP, 2019). 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 sampling and sample preparation of marine biota for the determination of CI17: heavy and trace elements and organic contaminants.

The integrated chemical-biological assessments of the effects of the contaminants present in the marine environment supports provision of data for GES assessment. As for the chemical monitoring, sample collection should be focused on selected locations such as hotspots and reference stations. Whenever possible, same specimen should be used for biomarker and chemical analysis. However, it must also be considered that the research team responsible for chemical analysis sampling is usually different from the one responsible for biomarker analysis sampling.

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}). It should be noted that the environmental data (water temperature, salinity and dissolved oxygen should be recorded at the same depth that fish have been collected. Whenever possible, the use of a CTD device is highly recommended.

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.

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

a. Materials

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

The following equipment is needed: Liquid nitrogen storage container (Dewar); Freezers -80°C; Ruler;

[40] 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.

[41] 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.

[42] 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

[43] 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.

Weight scale (readability 0.1 g - 0.01 g); Video camera / Smartphone video camera.

c. Chemicals and solutions

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

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 determination. To successfully obtain blood cells from the caudal vein it is recommendable to extract them immediately after collection, once the fish are on board and before any other tissue sampling has been conducted and before blood starts clotting. Fish are opened by robust scissors and the tissues are removed by using dissecting fine scissors and dissecting forceps.

Liver samples for the evaluation of Lysosomal membrane stability (cytochemical assay of LMS on cryostat sections -Köhler, 1991[44]; Köhler and Pluta, 1995[45]; UNEP/RAMOGE, 1999; 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.)).

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.

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). The brain tissue of *Mullus barbatus* can be considered as an additional tissue for evaluation of AChE activity, but not as an alternative to evaluation of AChE activity in muscle. However, it should be noted that the preliminary studies and available scientific literature confirm a high activity in brain tissue of *Mullus barbatus*.

Immediately after fish sampling, 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[46]). 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)

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

- Fish biometrics: total length (to 0.1cm), total weight (to 0.1 g), eviscerated weight (to 0,1 g);
- Fulton's condition factor, K (Bagenal and Tesch, 1978[47]). K =100 x body eviscerated weight (to 0.1 g) / total length^3 (to 0.1 cm) (see also Martinez-Gomez et al 2012). 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) /eviscerated weight (to 0.1 g), where eviscerated weight corresponds to the total weight without all internal organs (stomach, liver, gonad, intestine). 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) / eviscerated weight (to 0.1 g)). As known, liver plays a central role in fish metabolism and numerous studies have highlighted that toxic chemical may affect liver size and its functions. It has been also demonstrated in numerous field studies that fish

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

[45] 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.

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

[47] 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.

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[48]). To establish M. barbatus age in a more precise manner it is necessary to evaluate the otoliths as described by ICES (2017[49]) and Carbonara et al. (2018).

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

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 determination, 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.

2 Guidelines for the determination of lysosomal membrane stability (LMS) in marine molluscs and fish

2.1 Introduction

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 where a cause and effect relationship has been established (CI18).

There are different approaches for the study of the biological effects of the contaminants usually categorized on the basis of the level of biological organisation. For IMAP Common Indicator 18 the optimal approach is based on a use of biomarkers on selected organisms typical of the marine coastal waters. Biomarkers are biological parameters which changes may identify a pollutant-induced stress syndrome. The advantage of the use of biomarkers is that these sublethal parameters are early-warning indicators of the effects of the chemical contamination; therefore, it is possible to highlight the initial noxious effects of contaminants on organisms, before any effects at the population/community level are evident. The use of biomarkers allows to provide valuable information to decision makers to promptly implement the necessary measures to reduce damage at the ecosystem level.

The Monitoring Guidelines/ Protocols related to the biomarker determination in marine molluscs (such as *Mytilus* sp.) and fish (such as *Mullus barbatus*) provide a step-by-step guidance on the methodologies for the evaluation of the selected biomarkers, as well as for the interpretation of the results related to sample preparation and analysis of biomarkers. They are aimed at supporting comparable quality assurance of the data, as well as comparability between sampling areas in different national monitoring programmes.

The Monitoring Guidelines/ Protocols related to the biomarker determination in marine molluscs (such as *Mytilus* sp.) and fish (such as *Mullus barbatus*) follow on UNEP/MAP Manual for biomarker determination (UNEP/RAMOGE, 1999). They are also aligned with the Guidelines for biomarker determination, which were developed by other Regional Organisations, such as OSPAR (2013[50]) and ICES (Davies & Vethaak, 2012[51]).

2.2 Technical note for the determination of Lysosomal membrane stability (LMS) a) on cryostat sections in mussel digestive gland and fish liver and b) *in vivo* evaluation in mollusc haemocytes

Lysosomes are cytoplasmic vesicles; these single membrane organelles are characterized by their content of more than 50 types of acid hydrolases that are able to catabolise almost all of the different cellular components. The acidic pH of the lysosomal matrix is maintained by the activity of a proton pump present in the lysosomal membrane, and by the presence of an internal component of acidic proteins (Alberts et al., 2002[52]). The lysosomal vacuolar system comprises: newly formed (from the Golgi apparatus) Primary lysosomes (matrix pH ~ 6) of about 0.5 µm dimension within which the hydrolytic enzymes are not active; Secondary, active, lysosomes (matrix pH 4-5) that may

[48] 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.

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

[50] OSPAR Commission, 2013. Background document and technical annexes for biological effects monitoring, Update 2013. 239 pp.

[51] Davies, I.M.; Vethaak, D. (Ed.) (2012). Integrated marine environmental monitoring of chemicals and their effects. ICES Cooperative Research Report, 315. ICES: Copenhagen. ISBN 978-87-7482-120-5. 277 pp. Part of: ICES Cooperative Research Report. ICES: Copenhagen. ISSN 1017-6195.

[52] Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., 2002. Molecular Biology of the Cell, 4th edition. New York: Garland Science.

reach dimensions of several µm; and Tertiary lysosomes with reduced size and hydrolytic activity, containing non-degradable residues (often reported as Ceroid-Lipofuscin or Lipofuscin) that can be eliminated by exocytosis from cells that have this capacity.

The lysosomes have various functions in different cell types, and in different organisms; but they are always involved in the digestion of the nutritional components ingested into the cells by endocytosis orphagocytosis, and in autophagic activity (self-digestion) in relation to protein turnover and the degradation of damaged cellular components (Klionsky and Emr, 2000[53]; Cuervo, 2004[54]; Moore, 2008[55]; Moore et al., 2015[56]). The lysosomal vascular system can accumulate both organic lipophilic xenobiotics, and inorganic hydrophilic chemicals (Viarengo, 1989[57]; Moore et al., 2007[58]; Sforzini et al., 2018a[59]). It should be noted that the toxic chemicals that penetrate into the cells may damage membranes, organelles, soluble proteins etc. As mentioned above, lysosomes are normally involved in the removal and degradation of damaged cellular components; and, therefore, for this reason, toxic chemicals will contribute to increasing the autophagic activity. This pathophysiological reaction represents a fairly standard aspect of the toxic effects of the contaminants at cellular level; and may be highlighted by various parameters, such as changes in the number of lysosomes and their enzyme content, changes in fusion events and consequent increase of the lysosomal volume, as well as changes in the matrix pH and membrane permeability. This latter effect, if severe, may lead to the release of acidic hydrolases into the cytosol, an event that could, as an extreme consequence, provoke cell death.

Among the numerous biomarkers developed to study the effects of the toxic chemicals on the lysosomal vascular system, the evaluation of the lysosomal membrane stability (LMS) was found to represent the best choice (Viarengo et al., 2007a; Moore et al., 2008[60]). This biomarker is considered to have an excellent dose-response relationship, a high sensitivity, minimal chemical specificity (most toxic chemicals affect LMS) and there are no methodological concerns for both the methods proposed (Neutral Red Retention Time, NRRT, as well as cytochemical analysis of frozen cryostat sections) that are very simple and robust (Moore et al., 2008). Confounding factors usually do not represent a serious problem (Svendsen et al., 2004[61]); however, taking into account that the lysosomal vascular system is responsive to the variations of environmental parameters (such as sudden temperature and salinity changes, food availability and hypoxia/anoxia – Moore et al., 2008) and to the physiological changes related to gonad maturation in the spawning period, awareness and adequate precautions need to be considered in the realization of a biomonitoring programme (see Confounding factors).

Finally, it is important to highlight that LMS is not only an internationally recognised biomarker of stress, diagnostic of pathophysiological alterations at the cellular tissue level, but is also the only cellular biomarker found to be prognostic for possible effects at the population level (Moore et al., 2012[62]). In fact, Allen and Moore (2004[63]) have clearly shown the existence of a direct relationship between LMS and the Scope for Growth (SFG) of mussels. SFG is a parameter that evaluates the capability of the animals to adequately utilize the energy from food for growth and reproduction; therefore, as demonstrated by Widdows et al. (1981[64]), a decrease of this parameter reflects possible changes at the population level. The data reported in Figure 2 clearly show that the decrease of LMS is associated to a decrease in the SFG of the organisms, a precursor of possible effects at population level.

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[53] Klionsky, D.J., Emr, S.D., 2000. Autophagy as a regulated pathway of cellular degradation. Science 290, 1717-1721

[54] Cuervo, A.M., 2004. Autophagy: in sickness and in health. Trends Cell Biol. 14, 70-77.

[55] Moore, M.N., 2008. Autophagy as a second level protective process in conferring resistance to environmentally induced oxidative stress. Autophagy 4, 254-256

[56] Moore, M.N., Shaw, J.P., Ferrar Adams, D.R., Viarengo, A., 2015. Anti-oxidative cellular protection effect of fasting-induced autophagy as a mechanism for hormesis. Mar. Environ. Res. 107, 35-44.

[57] Viarengo, A., 1989. Heavy metals in marine invertebrates: mechanisms of regulation and toxicity at the cellular level. Aquat. Sci. Review 1, 295-317.

[58] Moore, M.N., Viarengo, A., Donkin, P., Hawkins, A.J., 2007. Autophagic and lysosomal reactions to stress in the hepatopancreas of blue mussels. Aquat. Toxicol. 84, 80-91.

[59] Sforzini, S., Moore, M.N., Oliveri, C., Volta, A., Jha, A., Banni, M., Viarengo, A., 2018a. Role of mTOR in autophagic and lysosomal reactions to environmental stressors in molluscs. Aquat. Toxicol. 195, 114-128.

[60] Moore, M.N., Koehler, A., Lowe, D. & Viarengo, A., 2008. Lysosomes and autophagy in aquatic animals. In: Methods in Enzymology (D. Klionsky, Ed), 451, 582-620. Academic Press/Elsevier, Burlington.

[61] Svendsen, C., Spurgeon, D.J., Hankard, P.K., Weeks, J.M., 2004. A review of lysosomal membrane stability measured by neutral red retention: is it a workable earthworm biomarker? Ecotoxicol Environ Saf. 57, 20-29.

[62] Moore, M.N., Viarengo, A., Somerfield, P.J., Sforzini, S., 2012. Linking lysosomal biomarkers and ecotoxicological effects at higher biological levels. In Ecological Biomarkers: Indicators of Ecotoxicological Effects (Editors: C. Amiard-Triquet, J.C. Amiard, P.S. Rainbow). Pp. 107-130.

[63] Allen, J.I., Moore, M.N., 2004. Environmental prognostics: is the current use of biomarkers appropriate for environmental risk evaluation. Mar. Environ. Res. 58, 227-232.

[64] Widdows, J., Bayne, B., Donkin, P., Livingstone, D., Lowe, D., Moore, M., & Salkeld, P., 1981. Measurement of the responses of mussels to environmental stress and pollution in Sullom Voe: A base-line study. Proc. R. Soc. Edin. Section B. Biological Sciences 80, 323-338.

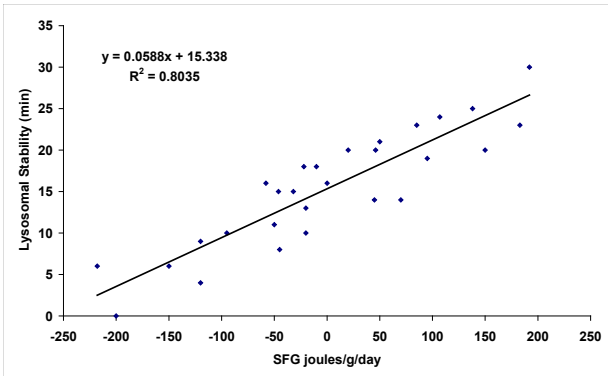

Figure 2. Lysosomal Stability (LMS) as an indicator of whole organism health and possible effects at population level. LMS shows a significant linear relationship with Scope of Grows in the marine mussel *Mytilus edulis*. Data is a compositive of several field and laboratory experiments (Allen & Moore, 2004)

Recently, it has been demonstrated in lab and field studies that toxic chemicals that affect LMS also inhibit mTOR (mechanistic Target of Rapamycin) activity (Sforzini et al., 2018a, b). mTOR is an evolutionarily conserved serine/threonine protein kinase, plying a key role in the growth and reproduction of the organisms by regulating important cellular processes such as RNA and protein synthesis, energy metabolism, cytoskeleton organization, lysosomal membrane permeability, endocytosis and autophagy (Soulard et al., 2009[65]; Laplante and Sabatini, 2012[66]; Sforzini et al., 2018a). For these reasons, the dephosphorylation of mTOR (complex 1: mTORC1, and complex 2: mTORC2) renders the cells catabolic, thus reducing the SFG of the animals. As reported in Sforzini et al. (2018b[67]), the polycyclic aromatic hydrocarbons (PAHs) accumulated in the digestive gland of mussels caged for 28 days in the highly contaminated Porto Torres harbour (Sardinia, Italy) induce a dephosphorylation of mTORC1 associated with a decrease of LMS and an increase of the lysosomal/cytoplasmic (L/C) volume ratio.

The lysosomal changes observed in field and lab experiments clearly indicate that increased autophagic activity is not compensated by the protein synthesis and that the mussel digestive gland cells become catabolic (Sforzini et al., 2018a, b). In these animals, the enhancement of the lysosomal content of neutral lipid seems to indicate that the mitochondrial energy production by fatty acid oxidation is reduced (Sforzini et al., 2018a). These findings confirm and clarify why a decrease of LMS is indicative and prognostic for a larger set of phenomena related to mTOR inhibition that may lead to a reduction of the SFG of the animals.

For these reasons, LMS, a simple and robust biomarker, was adopted as a mandatory test in the MED POL Biomonitoring programme.

Under this Technical Note, the Monitoring Guidelines for Biomarker Determination of Marine Molluscs (such as Mytilus sp.) and Fish (Mullus barbatus) for IMAP Common Indicator 18 provides the following two Protocols: i) Protocol for tissue section preparation, enzymatic determination of lysosomal membrane stability (LMS) on cryostat sections in mussel digestive gland and fish liver and for the evaluation and interpretation of the results; and ii) Protocol for *in vivo* determination of lysosomal membrane stability (LMS) in mussel haemocytes, and for the evaluation and interpretation of the results.

2.2.1 Protocol for tissue section preparation, enzymatic determination of lysosomal membrane stability (LMS) on cryostat sections in mussel digestive gland and fish liver and evaluation and interpretation of the results

a. Principle

The cytochemical procedure for the evaluation of the LMS is based on the determination of the activity of the lysosomal enzyme N-acetyl-β-hexosaminidase. Lysosomal destabilisation is measured as an increase of the membrane permeability to the enzyme substrate (naphthol AS-BI N-acetyl-β-glucosaminide) visualized by the reaction with the enzyme in presence of diazonium salt. The changes of the stability of the lysosomal membranes are determined by exposure of the cryostat sections to an acidic solution: with this treatment, lysosomes from healthy animals remain not permeable to the substrate for longer periods (more than 20 min and up to 40 min), but the membrane of the lysosomes in the cells of stressed organisms result labilised in a shorter time, depending on the severity of the pollutant-induced stress syndrome.

b. Materials

The following materials are needed to support optimal application of the Protocol: Glass beakers; Glass graduated cylinders; Hellendahl staining jars; 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; Coverslips, no.1 (0.13 - 0.16 mm), 60 x 24 mm, Menzel-Gläser.

c. Equipment

[65] Soulard, A., Cohen, A., Hall, M.N., 2009. TOR signaling in invertebrates. Curr. Opin. Cell Biol. 21, 825-836.

[66] Laplante, M., Sabatini, D.M., 2012. mTOR signaling in growth control and disease. Cell 149, 274-293.

[67] Sforzini, S., Oliveri, C., Orrù, A., Chessa, G., Jha, A., Viarengo, A., Banni, M., 2018b. 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.

The following chemicals and solutions are needed for optimal application of this protocol: High quality Cryostat; Shaking water thermostatic bath (up to 40 °C); Good quality bright-field microscope (10×, 20×, 40× objectives) equipped with a linear colour video camera; pH meter; Magnetic stirrer.

d. Chemicals and solutions

The following chemicals and solutions are needed for optimal application of this protocols: Lysosomal membrane labilising buffer (Solution A): 0.1 M Na-citrate Buffer, 2.5% NaCl w:v, pH 4.5.

Substrate incubation medium (Solution B) needs to be prepared just 5 minutes before use by applying the following procedure: 20 mg of naphthol AS-BI N-acetyl-β-D-glucosaminide ($C_{26}H_{27}BrN_2O_8$) are dissolved in 2.5 ml of 2-methoxyethanol and made up to 50 ml with solution A, containing also 3.5 g Polypep ($C_{14}H_{11}Cl_2N_3O \cdot ½ZnCl_2$; low viscosity polypeptide to act as a section stabiliser; Polypep is not easy to dissolve; therefore, it needs to dissolve Polypep in the solution A time before the addition of the substrate)[68].

Diazonium dye (Solution C) is prepared by applying the following procedure: 0.1 M Na-phosphate buffer, pH 7.4, containing 1 mg/ml of diazonium dye Fast Violet B salt (Sigma Aldrich, F1631) (or Fast Red Violet LB Salt -Sigma Aldrich, F3381; or Fast Blue RR -Sigma Aldrich, 201545) (Note: saturated solution, to be stored in dark).

Aqueous Mounting Medium (e.g. glycerol gelatine) to mount the sections[69].

e. Tissue section preparation

Using a high-quality motorized cryostat (cabinet temperature below -28 °C), 10 µm thick sections are cut using a 15° knife angle. The sections are transferred to "warm" slides (at room temperature of about 20 °C) to flash-dry them. The slides can be stored in the cryostat for at least 4 hours before use. Before the analysis, the tissue sections are gradually acclimated to room temperature (at least 30 min at 4 °C and 30 min at room temperature).

f. Enzymatic determination of LMS

The application of the following procedure is essential according to Moore (1976), UNEP/RAMOGE (1999), Moore et al. (2004), Martínez-Gómez et al. (2015), Köhler, et al. (2002)[70] and Broeg, K et al. (2002)[71]. The slides containing the sections are placed in a Hellendahl jar containing solution A for different times (3, 5, 10,15, 20, 30, 40 minutes) at 37 °C in shaking water-bath (60 rpm) in order to find out the range of pre-treatment time needed to completely labilise the lysosomal membrane i.e. labilisation period (LP). The set of slides are transferred into the solution B and incubated for 20 minutes at 37 °C in a Hellendahl jar in a shaking water-bath. The slides are then washed in Hellendahl jar filled with filtered sea water at room temperature or with a saline solution (3% NaCl) at room temperature for 2 to 3 minutes. Subsequently the slides are transferred into the solution C containing the diazonium coupler for 10 min at room temperature, and then rinsed in a Hellendahl jar filled with running tap water for 5 minutes. Finally, the sections are mounted in aqueous mounting medium.

g. Result evaluation

The slides are viewed under a microscope and divide the analysis of each section in four areas (quarters) for statistical interpretation. Lysosomes will stain reddish-purple due to the reactivity of the substrate with N-acetyl-*β*-hexosaminidase (Fig. 3a and b).

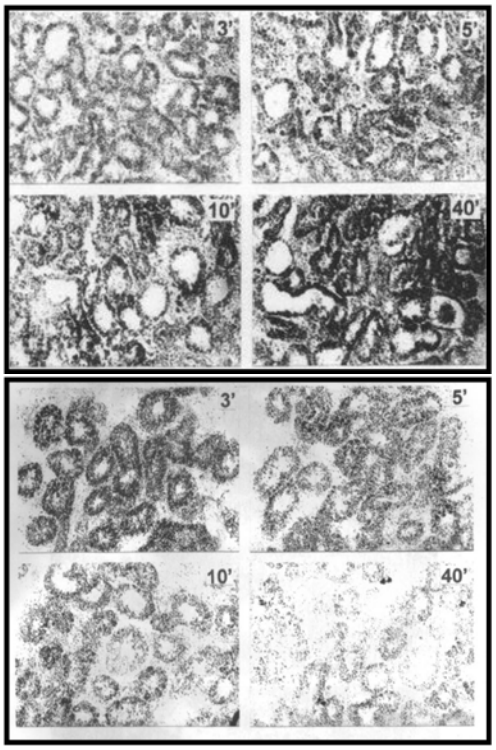

Figure 3a. LMS cryostat sections of *Mytilus* sp digestive gland, left panel: mussels sampled in an unpolluted site, right panel: mussels sampled in a polluted site.

[68] Use of same solutions across region is recommended, as feasible, in order to reduce quantitative differences in the results respectively to increase reproducibility of the results of analytical determination of biomarkers.

[69] For a few chemicals there is the indication of the supplier and is highly recommended to use exactly those in order to obtain comparable results. If not specified, the reagents must be of analytical grade.

[70] Köhler, A., Wahl, E., & Söffker, K. (2002). Functional and morphological changes of lysosomes as prognostic biomarkers of toxic liver injury in a marine flatfish (Platichthys flesus (L.)). Environmental Toxicology and Chemistry: An International Journal, 21(11), 2434-2444

[71] Broeg, K., Köhler, A., & Westernhagen, H. V. (2002). Disorder and recovery of environmental health monitored by means of lysosomal stability in liver of European flounder (Platichthys flesus L.). Marine environmental research, 54(3-5), 569-573.

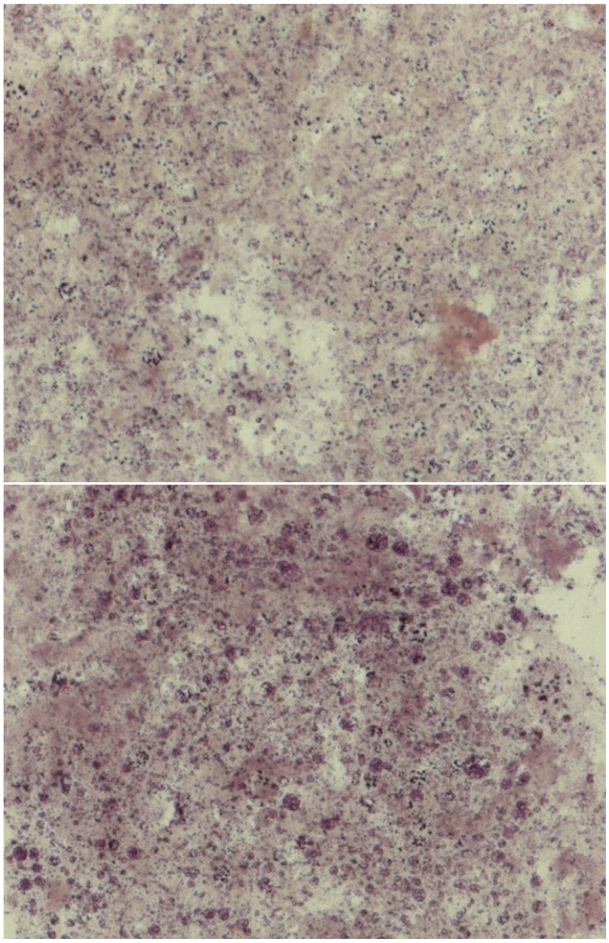

Figure 3b. LMS cryostat sections of fish (flounder) liver; animals sampled in a polluted site; left Panel: Lysosomal stain at 2 min, right Panel : Lysosomal stain at 6 min. LMS in healthy fish sampled in a pristine marina area is of about 35 min.

Evaluation of LP is done as shown in Figure 4. The staining intensity can be assessed visually by microscopic examination; it is also possible to collect microscopic images by a video camera and analyse them using an image analyser. Three min are used as the minimal pre-treatment time since the sections without pre-treatment may provide sometimes stronger staining.

Finally, LP from test samples is compared with those obtained from mussels sampled in the reference area and the gradient of cytotoxicity is determined. Reduction in the LP along the expected pollution gradient would indicate cellular stress due to pollution. Any decrease in staining intensity in successive sections following that with maximal staining may be due to loss of enzyme by diffusion from fully labilised lysosomes. If there are two peaks of staining intensity, then consider only the first staining peak as the LP; this fact may be due to the different properties of the lysosomes present in the cells.

For mussel digestive gland and fish liver, timing intervals of 3, 5, 10, 15, 20, 30 and 40 minutes are normally utilised (Moore, 1976, Köhler, 1991; Köhler and Pluta, 1995). The data can then be statistically analysed using the non-parametric Mann-Whitney U-test (Speigel, 1961[72]) and compared with reference data.

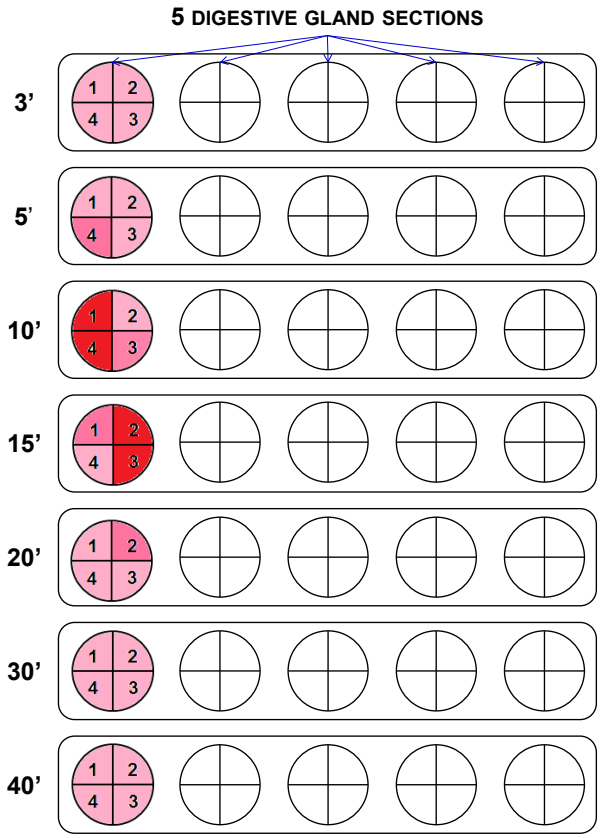

Figure 4. Evaluation of LP: for each section one quarter is analysed and the incubation time in the acid buffer is determined which produces the maximal staining reactivity. The analysis is repeated for the remaining three quarters and the data averaged. This value represents the LP of the first digestive gland section. The LPs for the other animals (in this case n = 5) are similarly obtained. Example: Maximum Staining Intensity (red): Quarter 1 = 10 min; Quarter 2= 15 min; Quarter 3 = 15 min; Quarter 4 = 10 min; LP value for specimen 1 = mean of 4 quarter = 12.5 min

It is important to note that, using cryostat tissue sections not pre-incubated in the acidic solution it is possible, by image analysis, to obtain the data concerning the ratio between the lysosomes and cytoplasm volumes. This parameter could be associated with that of LMS to evaluate if the organisms are "catabolic": that is when the increase of the autophagic process in the cells is no longer compensated by an adequate level of protein synthesis (Sforzini et al., 2018a, b).

At the end of the analysis, the results of the LMS evaluation must be listed in an additional page of the Biomarker Analysis Register (indicating also the data of the analysis and the name of the researchers involved). If the slide were analysed using a video camera, the exact location of the file of the biomarker analysis must be indicated in the Biomarker Analysis Register together

[72] Speigel, M.R., 1961. Statistics. Schaum's Outline Series. Mc Graw-Hill Book Company, 359 p.

with the information concerning the -80°C freezer in which the chucks are stored after the analysis.

h. Interpretation of the results

The analysis of the literature data confirms that the Background Assessment Levels (BAC) and Environmental Assessment Criteria (EAC) for LMS in mussels are essentially those proposed in Decision IG.23/6 on 2017 Mediterranean Quality Status Report (UNEP/MAP 2017[73]) and report of Davies and Vethaak, 2012.

i) LMS evaluated by the histochemical method: BAC = 20 min, EAC = 10 min.

ii) LMS values higher than 20 min should be consider typical of mussels in healthy conditions.

iii) LMS values from 20 min to 10 min identify animals showing a stress condition and mussels characterized by LMS values lower than 10 min should be considered pathologically stressed.

iv) For LMS in fish liver (*M. barbatus*), there are not as yet sufficient data to adequately quantify the BAC and EAC values: in this case, the values of LMS obtained in fish from the monitored sampling sites should be always compared with those obtained in fish living in relatively pristine control areas.

i. Confounding factors

In mussels LMS may be affected by extreme values of the environmental parameters: for this reason the animals should not be sampled in winter (low temperature and food deprivation), in summer periods when the seawater temperature is too high (the T at the sampling site should be always recorded) and the animals should be always sampled at about 4 m deep to avoid to collect animals that suffer long hypoxic periods -Moore et al.[74], 1980, 2007; ICES, 2011; OSPAR Commission, 2013).

In addition, it is important to know that those low salinities may affect the biomarker response, a fact that may become relevant in the biomonitoring programmes using caged mussels in areas such as estuaries. Moreover, mussels have different physiological conditions during the different seasons. For this reason, the animals should be sampled always outside the spawning period: in fact, during these periods, the animals are often in a poor condition with reduced LMS values. However, spawning in fish (*Mullus barbatus*) has only minimal effects on lysosomal activity and does not mask the effects that toxic chemicals may have on LMS (Köhler, 1991).

j. Reporting data

As provided in IMAP Guidance Fact Sheet for CI 18, the unit agreed for Lysosomal Membrane Stability (LMS) in bivalve molluscs such as mussel or fish (*M. barbatus*) is PT minutes (Cryostat section enzymatic method).

2.2.2 Protocol for *in vivo* determination of lysosomal membrane stability (LMS) in mussel haemocytes and evaluation and interpretation of the results

Neutral red (NR) is an eurhodin dye that is able to freely permeate the cell membrane in its lipophilic form. Within cells the compound is trapped by protonisation in its hydrophilic form in the lysosomes and accumulated in these organelles, where it can be visualised by a bright-field microscopy as red colour or by a fluorescence microscopy using a Rhodamine emission filter. The amount of Neutral Red trapped in the lysosomes depends on the pH of the organelles, in part due to the efficiency of their membrane associated proton pump (Seglen, 1983[75]). The neutral red retention time (NRRT) assay reflects the efflux of the dye from the lysosomes into the cytosol following damage to the membrane and/or the impairment of the H+ ion pump (Lowe et al., 1992). These impairments of the lysosomal membrane will result in a reduction of the dye retention in the organelles. Studies indicate that, similarly to the cytochemical method described above, the NRRT assay is sensitive to the main classes of chemical pollutants (Lowe, 1988; Moore et al., 2008).

The following protocol has been specifically adapted to be used on mussels, but it can be used on the cells of other molluscs.

a. Materials

The following materials are needed to support optimal application of the Protocol: Volume adjustable pipette, 20-200 µl and 200-1000 µl; Pipette tips, 20-200 µl and 200-1000 µl; Microcentrifuge tubes, snap cap, 2.0 ml; 2 L glass beaker; Microscope slides, 76x26 mm, 1 mm thick, pre cleaned/ready to use, Menzel-Gläser, Superfrost; Coverslips, no.1 (0.13 - 0.16 mm), 60 x 24 mm, Menzel-Gläser.

b. Equipment

The following equipment is needed: Good quality bright-field microscope (possibly an inverted microscope) with 10×, 20× and 40× objectives with a linear colour video camera; Humidity chambers (NR is a photosensitive dye, therefore the humidity chambers should be covered with an aluminium foil to prevent the light entry); Aquarium air pump and bubbler; pH meter; Magnetic stirrer.

c. Chemicals and solutions

[73] UNEP/MAP (2017) Mediterranean Quality Status Report Decision IG.23/6 2017

[74] Moore, M.N., Koehn, R.K., Bayne, B.L., 1980. Leucine aminopeptidase (aminopeptidase-1), N-acetyl-β-hexosaminidase and lysosomes in the mussel Mytilus edulis L., in response to salinity changes. J. Exp. Zool. 214, 239-249.

[75] Seglen. P.o. 1983. Inhibitors of Lysosomal functions. Meth. Emzymol. 96, 737-765.

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 (g/Kg), however, in the Mediterranean Sea the salinity can reach 44 PSU (g/Kg).

Physiological saline solution should be prepared as follows: 20 mM (4.77 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 are dissolved in 1 litre of deionised water. The solution is air bubbled for 10 minutes and then adjusted to pH 7.9 (or to the sea water pH) with 1M NaOH. The solution is stored in a refrigerator, but used at room temperature.

Neutral Red (NR) dye should be prepared as follows: the stock solution is prepared by dissolving 20 mg of NR powder (Sigma Aldrich, N4638) in 1 ml di dimethyl sulfoxide (DMSO). 5 µl of stock solution are transferred into 995 µl of physiological saline (working solution). NR stock solution is kept in the dark and in fridge (0-4 °C) when not utilized; the stock solution can be used for one month. The working solution must be prepared freshly before the analysis. N.B. For few chemicals there is the indication of the supplier and is highly recommended to use exactly those in order to obtain comparable results. If not specified, the reagents must be of analytical grade.

d. Practical evaluation

The Neutral Red (NR) method is recommended according to Lowe et al. (1995). The method for mussel haemolymph collection is reported in the Protocol for the dissection and storage of tissue samples from marine molluscs (such as *Mytilus* sp.) of technical note for the collection, transport and sampling of marine molluscs (such as *Mytilus* sp.) for biomarker analysis.

The NR methods requires applying the following procedure: 2 µL of Poly-L-lysine solution (0.1 % (w/v) in H_2O) (Sigma Aldrich, P8920) are put on a microscope slide and spread out with a coverslip. Leave to dry in a humidity chamber. 40 µL of haemolymph-saline mixture is dispensed on the slide, in the same position where the poly-l-lysine was added and incubated in a humidity chamber for 30 minutes to allow the cells to attach. Carefully, the excess solution is drained from the slide by placing the slide on its side and letting the liquid run off. 40 µL of the neutral red working solution is added and the slide is left in a humidity chamber for 15 min (maintained 15-16 °C during the analysis). A coverslip is applied and the preparation is inspected under a microscope. This first inspection corresponds to time 0' in Table 1.

e. Result evaluation

To evaluate results there is a need to visually look at the slides every 15 minutes for the first hour and then every 30 minutes for the next two hours thereafter (NR is a photosensitive dye, therefore, the light exposure time during the sample analysis should be as short as possible) (UNEP/RAMOGE, 1999; Moore et al., 2004). See Figure 5.

The time at which in 50% of the cells lysosomes release neutral red is then determined. Derive a mean value for each specimen and then a global mean for all specimens pertaining to the same pool. Samples from monitored field sites are compared with those taken from reference field sites and the gradient of cytotoxicity is determined. An increase in leaching rates will indicate cellular stress due to pollution.

Table 1: Example for result evaluation, with "+" more than 50% of the cells retain neutral red in the lysosomes; and "-" less than 50% of the cells retain neutral red in the lysosomes.

Samples	0	15	30	60	90	120	150	180	NRRT
Control	+	+	+	+	+	+	+	+	180
Treatment	+	+	±	-	-	-	-	-	30

It is also possible to collect digital images of the haemocytes (objectives 20× or 40×): this will allow to evaluate the NRRT at a later stage, a fact that may be important when there is the need to analyse numerous samples. This approach also allows to evaluate the reduction of NR accumulated in lysosomes. In addition, the cells' images and the collected data could be sent to an external lab (the Reference Centre) to check the quality of the results.

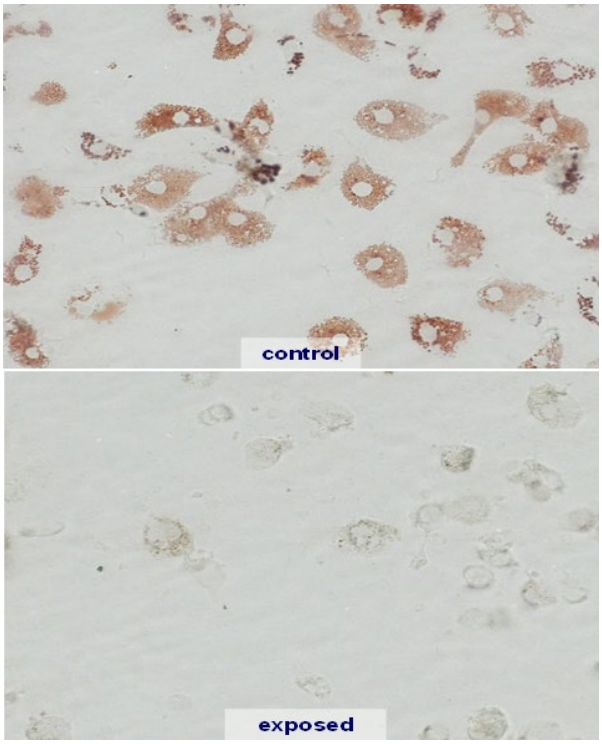

Figure 5: Images of neutral red retention time (NRRT) assay to show lysosomal membrane stability of mussel haemocytes. More detailed information and images about NRRT in mussel haemocytes can be found in Martínez-Gómez et al., 2015.

At the end of the analysis the results of the LMS evaluation must be listed in an additional page of the Biomarker Analysis Register (indicating also the data of the analysis and the name of the researchers involved). If the slide were analysed using a video camera, the exact location of the file of the biomarker analysis must be indicated in the Biomarker Analysis Register.

Recently, Martínez-Gómez et al. (2015) suggested that lysosomal size alterations should be associated with the NRRT to calculate the Percentage of LMS. This index takes into account lysosomal changes such as enlargement but no leakage, leakage and enlargement but colourless lysosomes and rounded up fragmenting cells (Martínez-Gómez et al., 2015). Although we have as yet limited data from field biomonitoring studies, the image analysis of the NRRT samples will allow the collection of microscopy images that could be used in the future for this improvement of this analysis. On the other hand, the % LMS, as indicated in ICES TIMES Num 56 (Martínez-Gómez et al., 2015) is highly recommended and it should be considered for future assessing LMS by NRRT assay. Consequently, (Field % LMS) Units (%) Tissue (haemocytes) was included as an optional field in the proposed Reporting Formats for CI18 (UNEP/MED WG.509/33).

The data can then be statistically analysed using the non-parametric Mann-Whitney *U*-test (Speigel, 1961) and compared with reference data.

f. Interpretation of the results

The analysis of the literature data confirms that the Background Assessment Levels (BAC) and Environmental Assessment Criteria (EAC) for LMS in mussels are essentially those proposed previously (Davies and Vethaak, 2012), and included into Decision IG23/6 on the 2017 Mediterranean Quality Status Report (2017 MED QSR), respectively LMS evaluated by the in vivo NRRT method: BAC = 120 min, EAC = 50 min.

g. Confounding factors

In mussels LMS may be affected by extreme values of the environmental parameters. For this reason, the animals should not be sampled in winter (low temperature and food deprivation) and in summer periods when the seawater temperature is too high; the T at the sampling site should always be recorded. The animals should always be sampled at about 4 m deep to avoid collecting animals that suffer long hypoxic periods (Moore et al., 1980, 2007; ICES, 2011; OSPAR Commission, 2013).

In addition, it is important to know that those low salinities may affect the biomarker response. This is a fact that may become relevant in the biomonitoring programmes using caged mussels in areas such as estuaries. Moreover, mussels have different physiological conditions during the different seasons. For this reason, the animals should be sampled always outside the spawning period: in fact, during these periods, the animals are often in a poor condition with reduced LMS values.

h. Reporting data

The unit for the agreed toxicological test NRRT assay under IMAP CI18 for bivalve molluscs such as mussel is “minute”.

3 Guidelines for determination of micronuclei (MNi) frequency, Acetylcholinesterase (AChE) activity and Stress on Stress (SoS) in marine molluscs and fish

3.1 Introduction

This working document is the continuation of Monitoring Guideline/Protocols for Biomarker Analysis of Marine Molluscs (such as Mytilus sp.) and Fish (Mullus barbatus) for IMAP Common Indicator 18 provided in UNEP/MED WG. 492/4. It details protocols for the following biomarkers: i) acetylcholinesterase (AChE) activity; ii) micronuclei (MNi) frequency; and iii) stress on stress (SoS).

3.2 Technical note for the determination of micronuclei (MNi) frequency in fish (*Mullus barbatus*) blood cells and in mussel (*Mytilus* sp.) gill cells and haemocytes

Micronuclei are small DNA-containing bodies that can be present near the cell nucleus during interphase resulting from both chromosome breakage and spindle dysfunction. The micronucleus (MN) test is suitable for the evaluation of the genotoxic activity of xenobiotic agents and of complex environmental mixtures in the laboratory, as well as in field studies (Al-Sabti and Metcalfe, 1995[76]; Hayashi et al., 1998[77]; Bolognesi and Fenech, 2012).

The types of genotoxic damage that could contribute to micronuclei production include:

a) unrepaired DNA strand-breaks induced by environmental and endogenous genotoxic agents which may result in acentric chromosome fragments.
b) products from interactions with kinetochore proteins, centromeres and spindle apparatus that could lead to unequal chromosome distribution or whole chromosome loss at anaphase.

Studies indicate that the relative occurrence of micronuclei provides an indication of accumulated

[76] Al-Sabti, K., Metcalfe, C.D., 1995. Fish micronuclei for assessing genotoxicity in water. Mutat. Res. 343, 121-135.

[77] Hayashi, M., Ueda, T., Uyeno, K. et al., 1998. Development of genotoxicity assay systems that use aquatic organisms. Mutat. Res. 399, 125-133.

genetic damage throughout the life span of the cells, and even during short phases of contamination. These considerations suggested the suitability of this test to monitor the extent of genotoxic damage in marine organisms in a time-integrated manner. It has been demonstrated that fish respond to toxic agents in a similar way to higher vertebrates and can be used as bioindicator to monitor the genotoxic effects of substances that are also potentially hazardous to humans (Al-Sabti and Metcalfe, 1995; Barsiene et al., 2004[78]; Bolognesi and Hayashi, 2011; Bolognesi and Cirillo, 2014[79]). The MN test, due to its potential for application to any proliferating cell population regardless of the karyotype, has been successfully established in many fish species that are often characterized by a low amount of DNA per cell, large numbers of small chromosomes and low mitotic activity.

Different fish cell types have been considered for the MN analysis: gill, fin, kidney and hepatic cells and peripheral erythrocytes. However, the complexity of the protocol for the isolation of cells from gill, fin, kidney and liver involve the killing of the animals and thus limits their application for environmental monitoring. Nucleated erythrocytes are the most commonly used cells in the fish MN test. The erythrocyte MN test was validated in a number of studies in laboratory and in the field.

A dose-response increase in MN frequency has been observed after exposure to ionizing radiations and to a large number of genotoxic pollutants such as aflatoxins, polycyclic aromatic hydrocarbons, chlorinated hydrocarbons, heavy metals and pesticides. The use of DNA-reacting fluorescent dyes is particularly useful to detect small MN. Different kinds of nuclear alterations (NAs) are also observed in fish erythrocytes such as buds, broken eggs, lobed, notched, vacuolated and karyolitic nuclei. The mechanisms responsible for NAs are not yet fully understood. A number of them, such as buds, are considered to be indicators of genotoxic damage and, therefore, they may complement the scoring of MN in routine genotoxicity surveys. Other NAs, such as lobed and notched nuclei, are mainly associated with cytotoxicity and need to be recorded separately (Bolognesi and Hayashi, 2011).

In molluscs, the MN assay has been applied in various species of bivalves, under both field and laboratory conditions: haemocytes and gill cells are the targets most frequently considered. The validation process for the MN assay in the genus *Mytilus* started in 1987 (Majone et al., 1987[80]). Dose related induction of micronuclei (MNi) by different pollutants has been reported in mussels exposed under laboratory conditions and in field studies (Barsiene et al., 2006, Bolognesi and Hayashi, 2011; Bolognesi and Fenech, 2012).

The large majority of studies evaluated only the MN frequency. More recently, the results on the frequency of other parameters included in the "cytome" approach, such as nuclear abnormalities or different types of cells, have been reported showing associations with pollutant levels (Bolognesi and Fenech, 2012). Further investigations and data collection are needed using standardized experimental protocols and scoring criteria for identifying the different types of cell and nuclear anomalies, in order to define the role of these biomarkers in environmental biomonitoring.

In line with above elaborated under this Technical Note, the Monitoring Guidelines for Biomarker Determination of Marine Molluscs (such as *Mytilus sp.)* and Fish (such as *Mullus barbatus*) for IMAP Common Indicator 18 provides the following two Protocols: i) Protocol for the determination of micronuclei (MNi) frequency in fish (*Mullus barbatus*) blood cells and evaluation and interpretation of the results; and ii) Protocol for the determination of micronuclei (MNi) frequency in mussel gill cells and haemocytes and evaluation and interpretation of the results.

3.2.1 Protocol for the determination of micronuclei (MNi) frequency in fish blood cells and evaluation and interpretation of the results

a. Materials

Application of this protocol requires availability of the following material: Pasteur pipette rubber bulbs; Petri dishes; Volume adjustable pipette, 20-200 µl and 200-1000 µl; Pipette tips, 20-200 µl and 200-1000 µl; Single-use syringe, Luer Lock,10-20 ml; Single-use syringe, 5 ml; Corning stripettes, disposable serological plastic pipette, 5 ml; Glass Pasteur pipettes, 150 mm; Microscope slides, 76x26 mm, 1 mm thick, pre cleaned/ready to use, Menzel-Gläser, Superfrost, wiped with ethanol and allowed to dry before use; Coverslips, no.1 (0.13 - 0.16 mm), 60 x 24 mm, Menzel-Gläser; Coplin jar, glass, for 10 slides; Microscope slide staining container, glass; slide staining rack; Storage microscope slides boxes.

b. Equipment

The equipment needed for the determination includes: Microscope with good quality optics for bright-field examination of stained slides at 1000 × magnification; Fluorescence microscope ocular (10×) and objective (100×) final magnification of 1000 ×.

c. Chemicals and Solutions

[78] Barsiene, J., Lazutka., Syvokiene, J., Dedonyte, V., Rybakovas, A., Bagdonas, E., Biornstad, A., Andersen, O.K., 2004. Analysis of micronuclei in blue mussels and fish from Baltic and North Seas. Environ. Toxicol. 19, 365-371.

[79] Bolognesi, C., Cirillo, S., 2014. Genotoxicity biomarkers in aquatic bioindicators. Zoology 60, 273-284.

[80] Majone, F., Brunetti, R., Gola, I., Levis, A.G., 1987. Persistence of micronuclei in the marine mussel, *Mytilus galloprovincialis*, after treatment with mitomycin C. Mutat. Res. 191, 157-161.

For the determination of micronuclei (MNi) frequency in fish (*Mullus barbatus*) blood cells and for the evaluation and interpretation of the results, the following chemical and solutions are used: Sodium heparin; Methanol, Methyl alcohol, absolute, Assay: 99,8%; Giemsa's azur-eosin-methylene blue solution (Sigma Aldrich, 1.09204); Eukitt, quick-hardening mounting medium for microscopy, or DPX Mountant for histology; DAPI (4',6-Diamidino-2-Phenylindole, Dilactate); Mowiol 4-88, glycerol, Tris.

GIEMSA staining procedure follows the below described procedure (Bolognesi and Fenech, 2012):

- Sorensen buffer, pH 6.8: Prepare two solutions (sol. A and sol. B);
- Sol. A: 9.073 g/L of potassium dihydrogen phosphate dehydrate (KH_2PO_4) [CAS No: 7778-770];
- Sol. B: 11.87 g/L of di-Sodium hydrogen phosphate dehydrate ($Na_2HPO_4 . 2H_2O$) [CAS No: 100028-24-7];
- To obtain 100 ml of Sorensen buffer solution, pH 6.8, 53.4 ml of solution A are mixed with 46.6 ml of solution B. The final solution (A+B) is utilised to prepare the GIEMSA staining solution and rinsing solution.
- 50 ml of Giemsa's, azur-eosine-methylene blue solution is filtered with filter paper. Protect from light;
- 200 ml of Giemsa's staining solution (3% vol/vol) are prepared by adding 6 ml of filtered Giemsa and 6 ml of Sorensen buffer to 188 ml of distilled water and put it into a slide staining container.

Mowiol mounting medium preparation follows the below described procedure:

- 6 g glycerol and 2.4 g Mowiol 4-88 are added to a 50 ml tube;
- 6 ml distilled water are added, mixed and left for 2 h RT;
- 12 ml 0.2 M Tris buffer solution (pH 8.5) are added;
- The tube is incubated in hot water (50-55 °C) for 10 minutes and stirred occasionally to allow Mowiol to dissolve (this can be repeated over several hours, if necessary);
- The solution is centrifuged at 5000 x *g* for 15 minutes to remove any undissolved solids;
- 1-2 ml aliquots of the Mowiol mounting medium are stored in microcentrifuge tubes at -20 °C;
- At 4 °C, the solution is stable for 1 month;
- Coverslipped slides are left in the dark overnight to harden before the analysis. This solution normally hardens overnight after slide preparation and does not require the coverslips to be sealed with nail polish[81].

d. Practical evaluation

The method for blood cell collection and slide preparation is provided in the Protocol for the dissection and storage of tissue samples from marine fish (*Mullus barbatus*) of the Technical Note for the collection, sampling and sample preservation of marine fish (*Mullus barbatus*) for biomarker analysis (C-1, Chapter 1.3.)

Slide staining procedure includes the following steps:

- Fixed slides are stained with 3% Giemsa solution for 10 min;
- The slides are rinsed 2 times in washing solution (Sorensen buffer 1.5%);
- The slides are air dried at room temperature;
- The slides are placed on tissue paper to be coverslipped;
- Two large drops of Eukitt or DPX (use a plastic dropper) are put on coverslips;
- The slides are inverted and placed on the coverslips. Allow the mounting medium to spread; The slides are turned so that the coverslips are on top and press the coverslips gently to expel any excess medium and air bubbles.

Alternatively, the slides can be stained with DAPI (300 nM in PBS) for 2-3 min and then mounted with Mowiol mounting medium. Put two large drops of Mowiol mounting medium on the slide and place on the coverslip; press the coverslip gently to expel any excess mounting medium and air bubbles.

The slides must be stored in a container at room temperature (Giemsa stained slides) or in a 0-4 °C fridge (DAPI stained slides). Every slide must be identify indicating the data of preparation and a code allowing to know the biometric characteristics of the mussels analysed and the name of the researcher who performed the analysis. All the information should be added in the Biomarker Analysis Register in which the position of the container in the lab should also clearly reported.

e. Result evaluation

Slide scoring is based on the following procedure:

- Coded and randomized slides are scored blind by a single observer;
- About 5000 erythrocytes per animal are analysed in slides stained with DAPI by a fluorescence microscope under 1000× magnification;
- About 5000 erythrocytes per animal are analysed in slides stained with Giemsa by a light microscope under 1000× magnification.

Criteria for micronuclei scoring are as follows:

- Diameter of micronucleus of 1/3-1/30 of the diameter of the main nucleus;
- Micronuclei are on the same optical plane as the main nucleus;
- Micronuclei are round or oval;
- Micronuclei are not linked or connected to the main nucleus;

[81] For a few chemicals there is the indication of the supplier and is highly recommended to use exactly those in order to obtain comparable results. If not specified, the reagents must be of analytical grade.

- Micronuclei may touch but not overlap the main nucleus and the micronuclear boundary should be distinguishable from the nuclear boundary;
- Chromatin structure is similar to that of the main nucleus.

There is a need to consider below listed nuclear abnormalities that could be also enumerated as a complement to the evaluation of the genotoxic effects of environmental chemicals:

- Bud: small nuclear bodies connected with the main nucleus or as small protrusion of the nuclei; Buds usually have 1/3-1/16 diameter of the main nucleus;
- Broken eggs: nuclear bodies connected with the main nucleus or as small protrusion of the nuclei with a diameter more than ½ the main nucleus;
- Blebbed Nuclei: small evaginations of the nuclear membrane;
- Lobed Nuclei: large evaginations of the nuclear membrane;
- Binucleated cells.

At the end of the analysis the results of the MNi evaluation must be listed in an additional page of the Biomarker Analysis Register, indicating also the data of the analysis and the name of the researchers involved. If the slides were analysed using a video camera, the exact location of the file of the biomarker analysis must be indicated in the Biomarker Analysis Register together with the information concerning the fridge in which the slides are stored after the analysis. Moreover, the following information should be included on the score sheet for the micronucleus assay in fish cells:

- Name of the person scoring the slides;
- Code number of each slide;
- Total number of total cells scored;
- Number of cells scored (fish: > 5000)/slide;
- Number of micronuclei (MNi) and micronucleated cells (MNcells) per 1000.

The data can then be statistically analysed using the non-parametric Mann-Whitney *U*-test (Speigel, 1961) and compared with reference data.

f. Interpretation of the results

The analysis of the bibliographical data indicates that the baseline values of MNi frequency in Mullus barbatus blood cells may vary from 0.1 MN/1000 cells (Martinez Gomez et al., 2010) to 0.7 MN/1000 cells (Bolognesi et al., 2006; Viarengo et al., 2007). Davies and Vethaak (2012) suggested a background response of < 0.32 MN/1000 cells and an elevated response > 0.32 MN/1000 cells. In the Decision IG.23/6 of the Mediterranean Quality Status Report (UNEP/MAP, 2017) no values for BAC in M. barbatus is reported. This is related to the fact that there are a few data concerning the MNi frequency values in M. barbatus sampled in the different Mediterranean areas. The value of MNi frequency in the blood cells of M. barbatus from unpolluted areas (controls) is one of the main requisites for the assay's application in environmental biomonitoring. However, as mentioned below (Confounding factors), due to the differences in the sea water temperature in the different Mediterranean areas, the amount of MNi in the cells of control animals may vary greatly. Therefore, in absence of BAC values, the MNi frequencies obtained in the biomonitoring studies need to be compared with the MNi values obtained by the same lab in the controls.

Future biomonitoring programme to be established for IMAP CI 18 should provide MNi intercalibrated data from animals sampled in the different Mediterranean regions in order to use these data for evaluation of correct BAC values.

g. Confounding factors

As reported for LMS, the animals show different physiological conditions in the different seasons (UNEP/RAMOGE, 1999; ICES, 2001; Moore et al., 2004; Viarengo et al., 2007; OSPAR Commission, 2013). For this reason, the animals should not be sampled in the summer and in the winter and always out from the spawning period: in fact, in these periods fish are often in a poor condition showing reduced detoxification of pollutants and DNA repair capacity. It is important to highlight that water temperature was shown to have a direct effect on the mitotic rate and on the level of DNA damage and consequently on the extent of MN expression with different baseline MN values for different water temperatures (Barsiene et al., 2004). Therefore, exogenous factors other than genotoxic pollutants, such as climatic variations modulating the induction of genotoxic damage, have to be considered in the data analysis; therefore, as mentioned above, it is always necessary to compare the MN data obtained to those of the reference animals sampled under similar environmental conditions.

h. Reporting data

The unit for the agreed toxicological test MNi frequency under IMAP CI18 for fish (*Mullus barbatus*) is: MNi/1000 cells.

3.2.2 Protocol for the determination of micronuclei (MNi) frequency in mussel gill cells and haemocytes and evaluation and interpretation of the results

a. Materials

Application of this protocol requires availability of the following material: 15 ml centrifuge tubes, polypropylene, sterile, conical bottom; Microcentrifuge tubes, snap cap, 2.0 ml; Corning stripettes, disposable serological plastic pipette, 10 ml; Corning stripettes, disposable serological plastic pipette, 5 ml; Glass Pasteur pipettes, 150 mm; Pasteur pipette rubber bulbs; Petri dishes; Volume adjustable pipette, 20-200 µl and 200-1000 µl; Pipette tips, 20-200 µl and 200-1000 µl; Single-use syringe, Luer Lock,10-20 ml; Single-use syringe, 5 ml; Dissecting forceps, fine and medium; Dissecting scissors; Fine scissors 14 cm length; Scalpel blades and handles; Ice and ice bucket; Swinnex filter holders 25 mm; Nylon Net filters, type NY8H, 180 µm pore size; Nylon Net filters, type NY80, 80 µm pore size; Counting chambers (e.g. Thoma or Burcker);

Microscope slides, 76x26 mm, 1 mm thick, pre cleaned/ready to use, Menzel-Gläser, Superfrost, wiped with ethanol and allowed to dry before use; Coverslips, no.1 (0.13 - 0.16 mm), 60 x 24 mm, Menzel-Gläser; Coplin jar, glass, for 10 slides; Microscope slide staining container, glass; slide staining rack; Storage microscope slides boxes.

b. Equipment

The equipment needed for the determination includes: Bench top centrifuge, capable of spinning at 1000 × g; Rotary mixer for tubes; Chemical safety cabinet; Magnetic stirrer; Vortex; Vacuum pump; pH meter; Freezer -20 °C; Microscope with good quality optics for bright-field examination of stained slides at 1000× magnification; Fluorescence microscope ocular (10×) and objective (100×) final magnification of 1000 ×.

c. Chemicals and Solutions

For the determination of micronuclei (MNi) frequency in mussels (*Mytilus* sp.) gill cells and haemocytes and for the evaluation and interpretation of the results, the following chemical and solutions are used: PBS, P3813-10 Pak, SIGMA-Aldrich; HANKS' Balanced Salts (HBSS), without sodium bicarbonate and phenol red, H1387, SIGMA-Aldrich; Dispase I (neutral protease, grade I), 04942086001, 10 x 2 mg (Roche); Methanol, Methyl alcohol, absolute, Assay: 99,8%; Glacial Acetic Acid, puriss., Assay: 99.8-100.5%; Giemsa's azure eosin methylene blue solution (Sigma Aldrich, 1.09204); Eukitt, quick-hardening mounting medium for microscopy, or DPX Mountant for histology; DAPI (4',6-Diamidino-2-Phenylindole, Dilactate). To prepare Mowiol mounting medium: Mowiol 4-88, glycerol, Tris.

Preparation of HANKS' balanced salts solution (HBSS) 2X, pH 7.4 is based on the following procedure (Bolognesi and Fenech, 2012):

- 1 litre of solution 2X is prepared by adding the content of two packages of HANKS' balanced salts (HBSS), (SIGMA-Aldrich cat No. H-1387) to 800 ml of distilled water, gently stirring until dissolved. Do not heat. The original package is rinsed with a small amount of water to remove all traces of powder;
- 0.7 g sodium bicarbonate is added to the solution;
- Stir until dissolved. While stirring, the pH of the solution is adjusted if necessary (pH 7.4);
- Additional water is added to bring the solution to the final volume.

A Dispase solution (grade I, > 6 U/mg, Roche) 0.1 mg/ml in HANKS' 2X is prepared by dissolving 5 mg of the lyophilized enzyme in 50 ml of HANKS' 2X solution at room temperature. Use fresh solution for each experiment.

100 ml of fixative are prepared by mixing methanol with glacial acetic acid in the ratio of 3:1. The fixative should be freshly prepared each time and used at 4 °C. This procedure should be undertaken in a well-ventilated fume hood. GIEMSA staining solution and Mowiol mounting medium are prepared as described for the MNi analysis in fish cells[82].

d. Practical evaluation

The method for mussel haemolymph collection is provided in Protocol for the dissection and storage of tissue samples from marine molluscs (such as *Mytilus* sp.) of the Technical note for the collection, transport and sampling of marine molluscs (such as *Mytilus* sp.) for biomarker analysis (C-1, Chapter 1.2.). After obtaining the haemolymph sample, the needle is discharged and the content is expelled in a centrifuge tube. The obtained cell suspensions are centrifuged at 1000 rpm (220 ×g) for 5 min.

The method for mussel gill collection is provided in the Protocol for the dissection and storage of tissue samples from marine molluscs (such as *Mytilus* sp.) of the Technical note for the collection, transport and sampling of marine molluscs (such as *Mytilus* sp.) for biomarker analysis.

e. Gill cell preparation

Plastic Millipore filter holders (Swinnex) or stainless-steel filter holder are used: nylon filters (180 nm and 80 nm) were assembled in two different filter holders to be used in sequence:

- Step 1: Mussels (6-10 animals/experimental group) are dissected, gills are removed and placed in a coded test tube/animal;
- Step 2: Gills are minced. 2 ml of dispase enzyme (0.1 mg/ml) in Hank's 2X are added. The enzymatic incubation is 10 min at room temperature in a rotating stirrer;
- Step 3: 7 ml of Hank's solution 2X are added to each test tube. The obtained cell suspension is filtered using a syringe connected with the filter apparatus. The filtered cell suspensions are collected in centrifuge test tubes. The quality of the cell suspension is checked using an inverted microscope. The cell suspensions are centrifuged at 1000 rpm (228×g) 5min at room temperature.

The procedure for slide preparation requires the following steps:

- After removing the supernatant, the pellet is suspended in fixative solution (methanol: acetic acid = 3:1) in a volume of 1-2 ml based on the number of cells;
- After at least 20 min the cellular suspensions are dropped on frozen slides (-20 °C). The slides are air dried at room temperature.

Slide staining procedure is as follows:

- The microscope slides with the fixed cells are immersed for 5 min at room temperature in Coplin jars or staining dishes containing 3% Giemsa solution;

[82] For a few chemicals there is the indication of the supplier and is highly recommended to use exactly those in order to obtain comparable results. If not specified, the reagents must be of analytical grade.

- The slides 2 times are rinsed in the washing solution (Sorensen buffer 1.5%);
- The slides are air dried at room temperature;
- The slides to be coverslipped are placed on tissue paper;
- Two large drops of Eukitt or DPX (use a plastic dropper) are put on coverslips;
- The slide is inverted and the coverslip is place on. Allow the mounting medium to spread. The slide is turned so that the coverslip is on top, and the coverslip is pressed gently to expel any excess mounting and air bubbles.

Alternatively, the slides can be stained with DAPI (300 nM in PBS) for 2-3 min and then mounted with Mowiol mounting medium. Two large drops of Mowiol mounting medium are put on the slide and the coverslip is placed on; the coverslip is pressed gently to expel any excess of mounting medium and air bubbles. The slides must be stored in a container at room temperature (Giemsa stained slides) or in a 0-4 °C fridge (DAPI stained slides). Every slide must be identify indicating the data of preparation and a code allowing to know the biometric characteristics of the mussels analysed and the name of the researcher who performed the analysis. All the information should be added in the Biomarker Analysis Register in which the position of the container in the lab should also clearly reported.

f. Result evaluation

Slide scoring requires use of the following equipment: Optical microscope ocular (10×) and objective (100×) final magnification of 1000×. Coded and randomized slides were scored blind by a single observer. At least 2000 cells have to be scored for micronuclei evaluation in mussel haemocytes; only agranular haemocytes should be selected for MNi evaluation. Criteria for cell scoring are as follows:

- Haemocytes: haemocytes with well spread nuclear chromatin. The cytoplasmic boundary or membrane of the cell should be intact and clearly distinguishable from the cytoplasmic boundary of adjacent cells;
- Gill cells: agranular epithelial-like cells with well spread nuclear chromatin. The cytoplasmic boundary or membrane of the cell should be intact and clearly distinguishable from the cytoplasmic boundary of adjacent cells.

Criteria for micronuclei scoring are as follows:

- Diameter of micronucleus is smaller than 1/3 of the diameter of the main nucleus;
- Micronuclei are on the same optical plane as the main nucleus;
- Micronuclei are round or oval;
- Micronuclei are not linked or connected to the main nucleus;
- Micronuclei may touch but not overlap the main nucleus and the micronuclear boundary should be distinguishable from the nuclear boundary;
- Chromatin structure is similar to that of the main nucleus.

At the end of the analysis the results of the MNi evaluation must be listed in an additional page of the Biomarker Analysis Register (indicating also the data of the analysis and the name of the researchers involved). If the slide were analysed using a video camera, the exact location of the file of the biomarker analysis must be indicated in the Biomarker Analysis Register together with the information concerning the fridge in which the slides are stored after the analysis.

Moreover, the following information should be included on the score sheet for the micronucleus assay in mussel cells:

- Name of the person scoring the slides;
- Code number of each slide;
- Total number of total cells scored;
- Number of cells scored (mussels: > 2000);
- Number of micronuclei (MNi) and micronucleated cells (MNcells) per 1000.

The data can then be statistically analysed using the non-parametric Mann-Whitney U-test (Speigel, 1961) and compared with reference data.

g. Interpretation of the results

Although Decision IG23/6 on the 2017 Mediterranean Quality Status Report (2017 MED QSR) and Davies and Vethaak (2012) define BAC value of 1 MN/1000 cells in mussels (Mytilus galloprovincialis), it is worth mentioning that a wider analysis of the bibliographical data clearly indicates that the baseline values of MN frequency in haemocytes and gill cells of mussels varies with the water temperature ranging from 0.37 MN/1000 cells at water temperature of 5 °C (Barsiene et al., 2004, 2006) to 6 MN/1000 cells at temperature 20 °C (Bolognesi and Fenech, 2012). The availability of BAC values in mussels from unpolluted areas (controls) is one of the main requisites for the assay's application in environmental biomonitoring. In absence of generally accepted BAC values, the MN frequencies obtained in the biomonitoring studies needs to be compared with the range of MN values obtained by the same lab in the controls.

Future biomonitoring programme to be established for IMAP CI 18 should provide MNi intercalibrated data from animals sampled in the different Mediterranean regions in order to use these data for evaluation of correct BAC values.

h. Confounding factors

It is well documented the animals show different physiological conditions in the different seasons (UNEP/RAMOGE, 1999; ICES, 2001; Moore et al., 2004; Viarengo et al., 2007a; OSPAR Commission, 2013). For this reason, the animals should be not sampled in the summer and in the winter and always out from the spawning period: in fact, in these periods mussels and fish are often in a poor condition showing reduced detoxification of pollutants and DNA repair capacity. Water temperature was shown to have a direct effect on the cell mitotic rate and consequently on the extent of MN expression with different baseline MN values for different water temperatures (Barsiene et al., 2004; 2006). Exogenous factors other than genotoxic

pollutants, such as climatic variations modulating the induction of genotoxic damage, have to be considered in the data analysis (ICES, 2011); therefore, as mentioned above, it is always necessary to compare the MN data obtained to those of the reference animals sampled under similar environmental conditions.

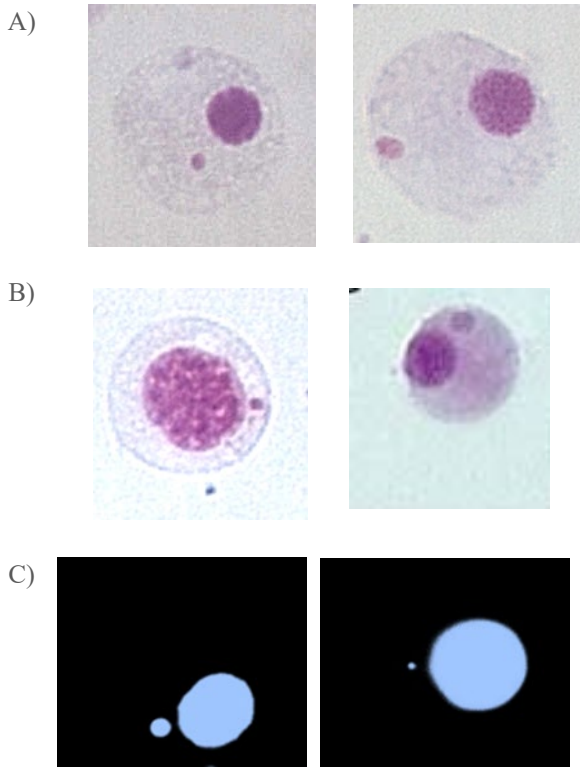

Figure 6: Images of different cell types from mussels (*Mytilus galloprovincialis*) stained using 3% Giemsa. A) Agranular gill aciliated cells with MNi, B) Agranular Hemocytes with MNi, C) Agranular gill cells with MNi, stained using DAPI.

In the papers reported in the References, a photo gallery of the various cell types, MN and nuclear anomalies is present (Barsiene et al., 2006; Bolognesi and Fenech, 2012; Davies and Vethaak, 2012).

i. Reporting data

As provided in IMAP Guidance Fact Sheet for CI 18, the unit agreed for Micronucleus assay toxicological test under IMAP CI18 is number of cases, ‰ in haemocytes i.e. MNi/1000 cells in bivalve molluscs such as mussel.

3.3 Technical note for the determination of Acetylcholinesterase (AChE) activity in mussel gills and fish muscle

Acetylcholinesterase (AChE) is the enzyme present in the plasma membrane of numerous cell types of most animals, which catalyses the reaction:

Acetylcholine → choline + acetic acid.

AChE activity was proposed as a biomarker of exposure to anticholinergic compounds such as Carbamates and Organophosphorus Pesticides (OP) (Bocquené et al., 1993[83]; Escartín and Porte, 1997[84]; Boucquené and Galgani, 1998; Burgeot et al., 2001[85]; Galloway et al., 2002[86]). In vertebrate tissues, this enzyme activity was found to be extremely sensitive to these two classes of pesticides and, consequently, these chemicals are able to affect numerous physiological functions of the animals, such as respiration, feeding, swimming, etc. For these reasons, this biomarker could also be considered a biomarker of stress; in fact, the inhibition of this enzyme activity could alter the capacity of the animals to adapt to their environment. It also has been demonstrated that numerous environmental contaminants such as PAHs, PCBs, metals, etc. may affect AChE activity (Bocquené et al., 1993; Escartín and Porte, 1997; Solé et al., 2010[87]).

However, it should be noted that the sensitivity of AChE activity to Carbamates and OP may vary greatly in different organisms. In particular, in marine mussels, the sensitivity of AChE activity to pesticides is similar to that of biomarkers such as lysosomal membrane stability (LMS), a well-known biomarker of stress (Rickwood and Galloway, 2004[88]). In the bivalve molluscs, a decrease of the AChE activity can only give an indication of possible environmental contamination by pesticides and so, should be considered as a general stress biomarker.

In line with above elaborated, under the Technical Note, the Monitoring Guidelines for Biomarker Analysis of Marine Molluscs (such as *Mytilus sp.*) and Fish (such as *Mullus barbatus*) for IMAP Common Indicator 18

[83] Bocquené, G. Galgani, F., Burgeot, T., Le Dean, L., Truquet, P., 1993. Acetylcholinesterase levels in marine organisms along French coasts. Mar. Poll. Bull. 26, 101-106.

[84] Escartín, E., Porte, C., 1997. The use of cholinesterase and carboxylesterase activities from *Mytilus galloprovincialis* in pollution monitoring. Environ. Toxicol. Chem. 16, 2090-2095.

[85] Burgeot, T., Bocquené, G., His, E., Vincent, F., Geffard, O., Beira, R., et al., 2001. Monitoring of biological effects of pollutants: field application. In: Garrigues Ph., Barth, H., Walker, C.H., Narbonne, J.F., editors. Biomarkers in marine organisms: a practical approach. Amsterdam: Elsevier, pp. 179-213.

[86] Galloway, T.S., Millward, N., Browne, M.A., Depledge, M.H., 2002. Rapid assessment of organophosphorous/carbamate exposure in the bivalve mollusc *Mytilus edulis* using combined esterase activities as biomarkers. Aquat Toxicol. 61, 169-180.

[87] Solé, M., Baena, M., Arnau, S., Carrasson, M., Maynou, F., Cartes, J.E., 2010. Muscular cholinesterase activities and lipid peroxidation levels as biomarkers in several Mediterranean marine fish species and their relationship with ecological variables. Environ. Int. 36, 202-211.

[88] Rickwood, C.J., Galloway, T.S., 2004. Acetylcholinesterase inhibition as a biomarker of adverse effect. A study of *Mytilus edulis* exposed to the priority pollutant chlorfenvinphos. Aquat Toxicol. 67, 45-56.

provides the Protocol for tissue homogenate preparation and for enzymatic determination of AChE activity, as well as evaluation and interpretation of the results.

3.3.1 Protocol for tissue homogenate preparation and for enzymatic determination of AChE activity, as well as evaluation and interpretation of the results

a. Principle

As indicated in the IMAP Guidance Factsheets (UNEP/MED WG.467/5, 2019), the method for the biochemical evaluation of the AChE activity is based on the capacity of the enzyme to use as specific substrate Acetylthiocholine (ACTC):

Acetylthiocholine ⟶ Thiocholine + Acetic acid

The thiocholine released by the AChE activity is detected by the reaction with 5,5'-dithio-bis-[2-nitrobenzoic acid] (DTNB), a reagent specific for thiol detection, leading to the formation of 5- mercapto-2-nitrobenzoate that has a yellow colour and a maximum of absorbance at 412 nm. This method for the evaluation of AChE activity was initially described by Ellman et al. (1961[89]). The method here reported, based on Ellman et al. (1961), was adapted to obtain the best analytical conditions as reported by Bocquené and Galgani (1998) and Galloway et al. (2002).

b. Materials

The following materials are needed to ensure optimal implementation of this Protocol: Volume adjustable pipette, 20-200 µl and 200-1000 µl; Pipette tips, 20-200 µl and 200-1000 µl; 15 ml centrifuge tubes, polypropylene, sterile, conical bottom; Microcentrifuge tubes, snap cap, 2.0 ml; 100 mL, 200 mL glass beaker; Glass graduated cylinders; 1-3 mL Spectrophotometer Cuvettes (10 mm light path).

a. Equipment

The following equipment is needed: Homogenization apparatus (a Potter apparatus for soft tissue such as gills and an Ultra Turrax apparatus for muscle homogenization); Refrigerated centrifuge (20 000 x g); Spectrophotometer UV-Visible; Thermostatic ice container; Weight scale (0.01 g).

b. Chemicals and Solutions

The chemicals and solution needed for application of this protocol are as follows: 0.02 M sodium phosphate buffer pH 7 (added with 0.1 % Triton X-100 before use) (the phosphate buffer can be stored at 0-4 °C); 10 mM 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) (Sigma Aldrich, D8130) in Tris 0.1 M pH 8 (this solution can be stored at 0-4 °C for one week); 0.1 M Acetylthiocholine (ACTC) iodide (Sigma Aldrich, A5751) (ACTC substrate can be stored at -20 °C, the ACTC solution should be prepared freshly before the use); Bradford Reagent (Sigma Aldrich, B6916); BSA - Albumin, bovin serum, fraction V, fatty acid free (Sigma Aldrich, 126575 : for a few chemicals there is the indication of the supplier and is highly recommended to use exactly those in order to obtain comparable results. If not specified, the reagents must be of analytical grade).

c. Mussel gills homogenate preparation

Extraction is performed on fresh or frozen tissue (1:4 W:V) using 0.02 M phosphate buffer pH 7.0 (+ 0.1% Triton X-100). The tissue (from 0.1 to1 g) is homogenized for one min using a Potter homogenizer. Extracts are then centrifuged at 10000 × g for 30 minutes at 4 °C and an aliquot of the supernatant is used in the assay. The supernatant can be stored at -20 °C or below (for 12 months) without significant loss of activity.

d. Muscle fish homogenate preparation

The procedure is the same as described for mussel gills. Only the initial step of the homogenate preparation is different because it needs to use an Ultra-Turrax apparatus to prepare the muscle homogenate. If a blender is not available, the tissue can be treated with liquid nitrogen in a porcelain mortar to reduce it in a powder with a pestle. An aliquot of the muscle powder preparation is then homogenized as described above and properly diluted (1/5-1/10) before using it in the analysis.

e. Determination of AChE activity

The procedure for determination of AChE activity can be summarized as follows:

- 60 µl of 10 mM DTNB (0.5 mM final concentration) and 100 µl of supernatant (about 200-500 µg proteins) are added in a total volume of 1200 µl 0.02 M phosphate buffer (pH 7); all the reagents must be brought to 20 C° before the start of the analysis; also, the temperature of incubation and reaction must be kept at room or controlled temperature, such as +20°C.
- After 5 min incubation to allow the DTNB to react with the sulfhydryl groups of the amino acids in the sample, 31.2 µl of 0.1 M ACTC (2.6 mM final concentration) are added to start the enzymatic reaction;
- The enzymatic reaction rate was quantified using a spectrophotometer (412 nm) against a blank without ACTC substrate. In order to subtract the spontaneous hydrolysis of substrate, a second blank is performed without sample in the reaction mixture;
- The reaction for the analysis of the homogenate is usually run for 1-5 min; the time may vary in relation to the enzymatic activity of the sample

[89]Ellman, G.L., Courtney, K.D., Andres, V. Jr, Feather-Stone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 7, 88-95.

that can change in different animals as well in animals in different physiological states.

f. Protein determination

It is suggested to evaluate the protein content using the method of Bradford (1976[90]). This procedure for protein determination can be summarized as follows:

- Protein concentration is evaluated in accordance with Bradford (1976). The method consists of mixing 1 part of the protein sample with 30 parts of the Bradford Reagent. The sample may be a blank, a protein standard, or an unknown sample. The blank consists of the homogenisation buffer with no protein. The protein standard consists of a known concentration of the bovine serum albumin - BSA (Albumin, bovin serum, fraction V, fatty acid free)- protein solubilised in the homogenisation buffer; the concentrations used for the preparation of BSA standards range from 0.025 to 0.8 mg/ml (stock solution 1 mg/ml);
- The assay is performed directly in a cuvette by adding 0.05 ml of sample, standard and blank to 1.5 ml of Bradford Reagent (acclimatisation at 20 C°) in the dark. The S10 sample is diluted (1:5 - 1:10) with the homogenization buffer before performing the assay;
- The absorbance values recorded for the samples are interpolated to the standard values to obtain the mg of protein contained in 1 mL of sample and multiplied by the dilution factor (in this case 5 - 10) to finally obtain the mg / mL of proteins.

g. Result evaluation

The results of evaluation need to be derived on the following equation for Calculation of AChE activity:

AChE activity (nmol min $^{-1}$ * mg protein^{-1}) = (ΔOD_{412} min^{-1} sample – ΔOD_{412} blank) * V_{tot} /(0.0136*L*Vs *tot prot. conc.) = ($\Delta OD_{412\ min-1}$ sample – $\Delta OD_{412\ min-1}$ blank) * Vtot /(0.0136*L*mg protein) .[91]

where: $\Delta OD_{412\ min-1}$ sample – $\Delta OD_{412\ min-1}$ blank = change in optical density (absorbance) per minute of sample at 412 nm, corrected for spontaneous hydrolysis (blank);
Vtot = total assay volume (ml);
0.0136 = µmolar extinction coefficient of TNB ($\mu M^{-1}cm^{-1}$);
L = light path (which is 1 cm for multi well and cuvette);
Vs = sample volume (ml);
tot prot. conc. = total protein concentration in the enzymatic extract (mg ml^{-1});
mg protein = Vs*tot. prot. conc.

A few examples for results evaluation are listed here-below:

- 100 µl of a mussel gill extract give a rough activity of 0.200 OD min^{-1};
- The protein concentration of the extract is 5 mg/ml (5 µg/µl);
- 100 µl of mussel gill extract = 100 × 5 = 500 µg = 0.5 mg;
- AChE activity, expressed in U min^{-1} mg protein^{-1}, is: 0.200 OD min–1 * Vtot 1.2 ml / 0.5 mg protein = 0.48 U min^{-1} mg protein^{-1};
- U min^{-1} mg protein^{-1} / 0.0136 (molar extinction coefficient) = nmol of substrate hydrolysed min^{-1} mg protein^{-1};
- 0.48 U min^{-1} mg protein^{-1} / 0.0136 = 35.3 nmol ACTC hydrolysed min^{-1} mg protein^{-1};
- i.e.: 1 ΔOD_{412} min^{-1} mg protein^{-1} corresponds to the hydrolysis of 73.53 nmol of ACTC;
- Expressed in nmol of substrate hydrolysed, the specific activity is: 0.48 U min–1 mg protein–1 × 73.53 nmol = 35.3 nmol ACTC hydrolysed min^{-1} mg protein^{-1}.

At the end of the determination, the results of the evaluation of the AChE activity must be listed in an additional page of the Biomarker Analysis Register, indicating also the data of the analysis and the name of the researchers involved. In the Biomarker Analysis Register, the information concerning the – 80 °C freezer in which the homogenates of the different samples are stored after the analysis should be also recorded.

h. Interpretation of the results

Background Assessment Levels (BAC) and Environmental Assessment Criteria (EAC) for AChE activity are previously proposed in Decision IG.23/6 on 2017 Mediterranean Quality Status Report:

- AChE activity (nmol/min/mg protein) in mussel gills in French Mediterranean waters: BAC = 29, EAC = 20;
- AChE activity (nmol/min/mg protein) in mussel gills in Spanish Mediterranean waters: BAC = 15, EAC = 10.

So far, data of AChE activities in *M. galloprovincialis* and *M. barbatus* from reference areas from the Mediterranean Sea are very limited. It has been proposed that the baseline level should be defined on a regional basis, using available long-term data (which is not yet widely available). BACs in mussel gills from Spanish Mediterranean waters were calculated using values obtained from at least two reference areas (on the basis of chemical analysis in mussel tissues) and from at least three years sampling (data submitted to MED POL database), along with records of salinity and temperature of the ambient water at the sampling time. EAC are usually derived from toxicological data and, in this case,

[90] Bradford, M M, 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal. Biochem. 72, 248-254.

[91] Note: OD_{412} = Optical Density = Absorbance at 412 nm wavelength.

they were calculated by subtracting 30% from BAC values (Davies and Vethaak, 2012).

In the past, the labs have not used common protocols for mussels collection and transport, tissue sampling and storage; moreover, the methodologies used for the analysis of the AChE activity were not intercalibrated between those lab providing data and the methods used to calculate the protein content (i.e. Bradford versus Lowry) could be playing also a role in the final value of AChE activities. The BAC and EAC values in the gills of mussels sampled in coastal areas showing relatively similar climatic characteristics should be more similar, although South-East-South of the Spanish Mediterranean Coast is a different marine region than the French Mediterranean coast. A well-organised Q.A. programme based on the intercalibration activity, the use of the same analytical protocol (here reported) and the same reagents is required to clarify the differences in the experimental results obtained in the organisms sampled in different Mediterranean areas. Moreover, it should be noted that it may be difficult to find unpolluted sites along coasts in areas characterized by extensive agricultural activities, a fact that may be important in the estimation of the BAC values.

The data reported clearly demonstrate that for AChE activity, the results should be interpreted on the basis of the enzymatic activity values found in the reference mussels sampled in a well-established relatively uncontaminated coastal area. The reported data indicate that a reduction of 30% of the value obtained in the control animals may represent a correct EAC value. It is important to emphasize that the use of caged mussels, obtained from a production farm, usually minimises this problem and facilitates the interpretation of the results.

Although present assessment criteria do not provide values of AChE activity in fish, it is worth mentioning the values of AChE activity in *M. barbatus* muscle as proposed by Davies and Vethaak (2012) based on the analytical data of Burgeot et al. (1996[92]): BAC = 155 nmol/min/mg protein and EAC = 109 nmol/min/mg protein. However, Solé et al. (2010) reported an AChE value of 53.3 nmol/min/mg protein for the unpolluted site of Besòs (Spain). Given no BAC or EAC values for *Mullus barbatus* have been proposedin Decision 22/7 on IMAP and 23/6 Decision on 2017 Mediterranean Quality Status Report., UNEP/MAP will consider these values in the scope of further work that will be undertaken to upgrade the assessment criteria.

As mentioned above, also in the case of *M. barbatus*, the problem of the differences in the AChE analytical results could be clarified in the framework of the Q.A. activities (Viarengo et al., 2000). Moreover, the results of AChE activity should be interpreted on the basis of the values obtained in the reference fish sampled in a well-established relatively uncontaminated area. A reduction of 30% of the value obtained in the control animals may represent a correct EAC value.

i. Confounding factors

The animals show different physiological status during the different seasons (UNEP/RAMOGE, 1999; ICES, 2001; Moore et al., 2004; Viarengo et al., 2007a; OSPAR Commission, 2013). For this reason, the animals should be not sampled in the summer and in the winter and always outside the spawning period. As for other enzymatic activities, climatic changes, and in particular the values of water temperature, can affect the level of the AChE activity (Hogan, 1970[93]).

The AChE activity level may vary between juveniles and adult fish; therefore, a particular attention must be given to using animals with similar biometrics characteristics, indicating fish of similar age (Galgani et al., 1992[94]). In *Mytilus edulis* from the Baltic Sea, the AChE values vary twofold depending on the sampling season, in relation to the temperature changes (Leiniö and Lehtonen, 2005[95]).

The AChE activity was found to be affected by algal toxin (Dailianis et al., 2003[96]; Kankaanpää et al., 2007[97]). It is therefore suggested to report in the Biomarker Analysis Register the information about the presence of an algal bloom when the animals are collected.

j. Reporting data

As provided in IMAP Guidance Fact Sheet for CI 18, the unit for Acetylcholinesterase (AChE) activity assay in bivalve molluscs (such as *Mytilus* sp.) or fish (*M. barbatus*) is: nmol/min/mg protein.

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[92] Burgeot, T., Bocquené, G., Porte, C., Pfhol Leszkowicz, A., Santella, R.M., Raoux, C., Dimeet, J., et al. 1996. Bioindicators of pollutant exposure in the northwestern part of the Mediterranean Sea. Mar. Ecol. Prog. Ser. 131, 125-141.

[93]Hogan, J.W., 1970. Water temperature as a source of variation in specific activity of brain acetylcholinesterase of bluegills. Bull. Environ. Contam. Toxicol. 5, 347-353.

[94] Galgani F., Bocquené G., Cadiou, Y., 1992. Evidence of variation in cholinesterase activity in fish along a pollution gradient in the North Sea. Mar. ecol. Prog. Ser. 91, 1–3).

[95] Leiniö, S. and Lehtonen, K. K. 2005. Seasonal variability in biomarkers in the bivalves *Mytilus edulis* and *Macoma balthica* from the northern Baltic Sea. Comp. Biochem. Physiol. C 140, 408–421.

[96]Dailianis, S., Domouhtsidou, G.P., Raftopoulou, E., Kaloyianni, M., Dimitriadis, V.K., 2003. Evaluation of neutral red retention assay, micronucleus test, acetylcholinesterase activity and a signal molecule (cAMP) in tissues of *Mytilus galloprovincialis* (L.), in pollution monitoring. Marine Environmental Research 56, 443–470.

[97]Kankaanpää, H., Leiniö, S., Olin, M., Sjövall, O., Meriluoto, J., Lehtonen, K. K., 2007. Accumulation and depuration of cyanobacterial toxin nodularin and biomarker responses in the mussel *Mytilus edulis*. Chemosphere 68, 1210–1217.

3.4 Technical note for the determination of Stress on Stress (SoS) in mussels

This biomarker is based on the definition of "stress": stress is a measurable alteration of the organism's physiology induced by an environmental change that results in a reduced capacity of the individual to adapt to further environmental variations (Bayne, 1986[98]). This concept was practically applied to mussels superimposing exposure to air, a natural stressor, over the harmful effects of chemicals contamination in their environment. Marine mussels are often naturally exposed to air (Bayne, 2009) for short periods of time (hours) but they can also survive for days out of water. This ability to sustain prolonged emersion periods is due to their capacity to reduce the water loss by valve (shell) closure; and the muscle contraction required for this is supported by a shift of the energy metabolism from aerobic to anaerobic, typical of these organisms (De Zwaan and Zandee, 1972[99]; Bayne, 2009). The toxic chemicals, by altering the cellular functions and increasing energy requirement for the detoxification mechanisms, or directly affecting the energy metabolism, can reduce the ATP availability for basic physiological functions, and in particular for muscular contraction, thus leading to animal death in a short time.

Numerous experimental studies have confirmed that this biomarker at the whole animal level is suitable for identifying the effects of low concentrations of contaminants in the water. In particular, it was demonstrated that inorganic contaminants such as heavy metals (Cu and Cd) or organic aromatic compounds such as 9,10-dimethyl 1,2-benzo anthracene (DMBA) and PCBs (Aroclor 1254) and organochemicals at submicromolar concentrations affect the SoS response in mussels in a dose dependent manner; and that the toxic effect is significantly increased in the molluscs exposed to chemical mixtures (Eertman et al., 1993[100]; Viarengo et al., 1995[101]; Marcheselli et al., 2011[102]).

It is important to point out that PAHs, one of the more ubiquitous groups of environmental contaminants, may affect SoS in mussels (*Mytilus trossulus*) sampled from field contaminated areas (Thomas et al., 1999[103]). These findings confirm the general applicability of this stress biomarker as being sensitive to the various classes of pollutants in the laboratory, as well under field conditions. Although it was demonstrated that other biomarkers such as LMS or Scope for Growth are more sensitive, it should be noted that the methodology for SoS evaluation is very simple, low cost and does not need expensive equipment. Moreover, this biomarker has a clear dose-response relationship and shows a typical decreasing trend that lends itself to easy toxicological interpretation; although some hormetic effects at minimal toxicant concentrations were reported by Eertman et al. (1995[104]).

In line with above elaborated, under the Technical Note, the Monitoring Guidelines for Biomarker Analysis of Marine Molluscs (such as *Mytilus sp*.) and Fish (such as *Mullus barbatus*) for IMAP Common Indicator 18 provides the Protocol for the evaluation of SoS and for the interpretation of the results.

3.4.1 Protocol for the evaluation of SoS and interpretation of the results

a. Equipment

For optimal application of this Protocol the following equipment is needed: Thermostatic bag; Aquarium or laboratory or incubator chamber at controlled temperature.

b. Field sampling

The mussels may be caged for 30 days in the different field sites or collected from wild populations; in both cases, the sampled animals should be submerged and with a shell size of about 4-5 cm. It is important to stress that, in the case of wild animals sampled from different areas, the size of the molluscs has to be similar. It is necessary to take into account that younger animals (smaller mussels) have a longer survival time in air. Moreover, during the sampling procedure, water temperature, salinity and dissolved oxygen at the sampling site should be recorded. During mussel collection, byssal threads need to be cut with scissors in order to reduce the injury to the animals.

Some additional information can be useful when wild mussels are being used, such as the evaluation of the Condition Index (Crosby and Gale, 1990[105]; Mann,

[98] Bayne, B.L., 1986. In: The Role of the Oceans as a Waste Disposal Option, ed. G. Kullemberg. Riedel, NY, pp. 617-634.

[99] De Zwaan, A., Zandee D.I., 1972. The utilization of glycogen and accumulation of some intermediates during anaerobiosis in Mytilus edulis L. Comp. Biochem. Physiol. Part B 43, 47-54.

[100] Eertman, R.H.M., Wagenvoort, A.J., Hummel, H., Smaal, A. C., 1993. "Survival in air" of the blue mussel *Mytilus edulis* L. as a sensitive response to pollution-induced environmental stress. J. Exp. Mar. Biol. Ecol. 170, 179-195.

[101] Viarengo, A., Canesi, L., Pertica, M., Mancinelli, G., Accomando, R., Smaal, A.C., Orunesu, M., 1995. Stress on Stress Response: A Simple Monitoring Tool in the Assessment of a General Stress Syndrome in Mussels. Mar Environ Res 39, 245-248.

[102] Marcheselli, M., Azzoni, P., Mauri, M., 2011. Novel antifouling agent-zinc pyrithione: Stress induction and genotoxicity to the marine mussel *Mytilus galloprovincialis*. Aquat. Toxicol. 102, 39-47.

[103]Thomas, R.E., Harris, P.M., Rice, S.D., 1999. Survival in air of *Mytilus trossulus* following long-term exposure to spilled Exxon Valdez crude oil in Prince William sound. Comp. Biochem. Physiol. Part C 122, 147-152.

[104] Eertman, R.H.M., Groenink, C.L.F.M.G., Sandee, B., Hummel, H., 1995. Response of the blue mussel *Mytilus edulis* L. following exposure to PAHs or contaminated sediments. Mar. Environ. Res. 39, 169-173.

[105] Crosby, M.P., Gale, L.D., 1990. A Review and Evaluation of Bivalve Condition Index Methodologies with a Suggested Standard Method. J. Shellfish Res. 9, 233-237.

1992[106]), and the degree of gonadal maturation (Bayne, 2009). In general mussels should be sampled out of the spawning period: indeed, just after the spawning, the animals are stressed and show a lower time of survival in air.

The sampled mussels should be rapidly transferred in a thermal insulated container with cotton towelling soaked with marine water to maintain an adequate humidity level. A temperature of about 4 °C should be maintained by using ice packs in the container.

c. Determination of SoS

Air exposure experiment: In the laboratory at least 10 x 4 animals from each site are subjected to anoxia by air exposure at 18 °C in humidified chambers. Mussels are placed over a moistened filter paper to guarantee the correct humidity level (the additional stressor should be the air exposure and not the water loss of the animals). Survival is assessed daily. Death symptoms are considered to be open valves and absence of muscular activity (open valve squeezing does not restore valves closure). Dead animals are recorded until 100% mortality is reached.

d. Result evaluation

The table 2 reported below shows a typical mortality recording sheet for every 10 animals exposed to air for the LT_{50} evaluation (LT = lethal time; LT_{50} = number of days required to observe 50% mortality).

SoS: date of experiment (xx/yy/zz), Site …., Site code …., Sampling date ….

Environmental data: Water salinity (‰), Temperature (°C), pH, O_2 (mg/L).

Date	Day	Dead	Alive	% Alive
xx/yy/zz	0	0	10	100
xx/yy/zz	1	0	10	100
xx/yy/zz	2	1	9	90
xx/yy/zz	3	0	9	90
xx/yy/zz	etc.	etc.	etc.	etc.
xx/yy/zz	16	10	0	0

Data analysis

LT_{50} values are used to evaluate the statistical differences between controls and animals from sites at different pollution levels. Survival curves and LT_{50} values can be estimated using the Kaplan-Meier method (Kaplan and Meier, 1958[107]) and the Spearman-Karber test (Hamilton et al., 1977[108]). At the end of the analysis, the results of the evaluation of the SoS must be listed in an additional page of the Biomarker Analysis Register (indicating also the data of the analysis and the name of the researchers involved).

e. Interpretation of the results

The indications for data interpretation related to SoS, considering related BAC and EAC values as established in Decision IG. 23/6 on 2017 Mediterranean Quality Status Report in line with the ICES Cooperative Research Report No. 315.277pp are as follows: LT50 (days) – BAC 10 and EAC 5. SoS values higher than 10 days indicate healthy molluscs; values between 5 and 10 days indicate stressed animals; values lower than 5 days highlight mussels highly stressed in a pathological situation.

The analysis of the data reported by different authors such as Smaal et al. 1991[109] and Viarengo et al. (1995) indicate LT50 of 7.5 - 8 days. Recent data published in the literature on SoS found values of SoS ranging between 6 and 8 days in resident mussels (17ºC ambient water) with concentrations of CBs, p-p-DDEs, Chrysene, Hg and Pb above BACs in their tissues (Martínez-Gómez et al., 2017). The discrepancies noticed in the literature data emphasize the importance of an intercalibration activity in the framework of the QA programme in order to establish correct BAC and EAC values for SoS in the Mediterranean Sea. The data that will be collected through implementation of IMAP CI 18 will support establishing the correct SoS BAC and EAC for the Mediterranean area. Presently, the correct interpretation of data related to SoS should be based on comparing the field data with the results of SoS obtained in mussels sampled in a reference coastal area.

f. Confounding factors

The animals show different physiological status in the different seasons (UNEP/RAMOGE, 1999; ICES, 2001; Moore et al., 2004; Viarengo et al., 2007a; OSPAR Commission, 2013). For this reason, the animals should be not sampled in the summer or in the winter, and always outside the spawning period: in fact, in these periods the mussels are often in a poor condition and show a reduced survival time in air. This fact clearly indicates that, when possible, the use of caged mussels represents the best solution to obtain standardized, more comparable and reproducible data.

It should be noted that younger animals (smaller size) have longer times of survival in air (Thomas et al., 1999). Finally, the temperature value of the chamber for the SoS experiments should be routinely checked: lower

[106]Mann, R., 1992. A comparison of methods for calculating condition index in eastern oysters *Crassostrea virginica* (Gmelin, 1791). VIMS Articles. 720.

[107] Kaplan, E. L., Meier, P., 1958. Nonparametric estimation from incomplete observations. J. Am. Stat. Ass. 53, 457-481.

[108] Hamilton, M.A., Russo, R.C., Thuston, R.V., 1977. Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. Environ. Sci. Technol. 11, 714-719.

[109] Smaal A.C., Wagenvoort, A., Hemelraad, J., Akkerman, I., 1991. Response to stress of mussels (Mytilus edulis) exposed in Dutch tidal waters. Comp. Biochem. Physiol. C. 100, 197-200.

temperatures in the chamber (together with the temperature of the sea water at the sampling site, as mentioned above) allow the molluscs to survive in air for longer periods of time (Thomas et al., 1999).

Reporting data

The unit for the agreed toxicological test SoS under IMAP CI18 in bivalve molluscs such as mussel is: LT_{50} (days).

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