UNITED NATIONS

EP

UNEP/MED WG.492/5

UNEP

UNITED NATIONS
ENVIRONMENT PROGRAMME
MEDITERRANEAN ACTION PLAN

26 March 2021
Original: English

Meeting of the Ecosystem Approach Correspondence Group on Pollution Monitoring

Videoconference, 26-28 April 2021

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

Monitoring Guideline/Protocols for Biomarker Analysis of Marine Molluscs (such as *Mytilus sp*.) and Fish (such as *Mullus barbatus*) for IMAP Common Indicator 18 – Analysis of and micronuclei (MNi) frequency, Acetylcholinesterase (AChE) activity and Stress on Stress (SoS)

Note by the Secretariat

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

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

The Monitoring Guidelines/Protocols build upon the knowledge and practices obtained over 40 years of MED POL monitoring implementation and recent publications, highlighting the current practices of the Contracting Parties' marine laboratories.as well as other relevant experiences.

The Monitoring Guideline for Biomarker Analysis of Marine Molluscs (such as *Mytilus*) and Fish (such as *Mullus barbatus*) for the analysis of IMAP Common Indicator 18 provides the following four Technical Notes:

1) Technical note for the analysis of Lysosomal membrane stability (LMS) on cryostat sections in mussel digestive gland and fish liver and in vivo evaluation in mollusc haemocytes (UNEP/MED WG.492/4), which includes the two following 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 evaluation and interpretation of the results;
    ii) Protocol for in vivo determination of lysosomal membrane stability (LMS) in mussel haemocytes and evaluation and interpretation of the results;
2) Technical note for the analysis of micronuclei (MNi) frequency in fish blood cells and in mussel gill cells and haemocytes (UNEP/MED WG.492/5), which includes the following two Protocols:
    i) Protocol for the analysis of micronuclei (MNi) frequency in fish blood cells and evaluation and interpretation of the results;
    ii) Protocol for the analysis of micronuclei (MNi) frequency in mussel gill cells and haemocytes and evaluation and interpretation of the results;
3) Technical note for the analysis of Acetylcholinesterase (AChE) activity in mussel gills and fish muscle (UNEP/MED WG.492/5) which includes the Protocol for tissue homogenate preparation and for enzymatic determination of AChE activity, as well as evaluation and interpretation of the results;
4) Technical note for the analysis of Stress on Stress (SoS) in mussels which includes the Protocol for the evaluation of SoS and interpretation of the result.

These Monitoring Guidelines/Protocols, including this one related to the biomarker analysis in marine molluscs (such as *Mytilus* sp.) and fish (such as *Mullus barbatus*), establish a sound ground for further regular update of monitoring practice for the purpose of successful IMAP implementation.

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

Table of Contents

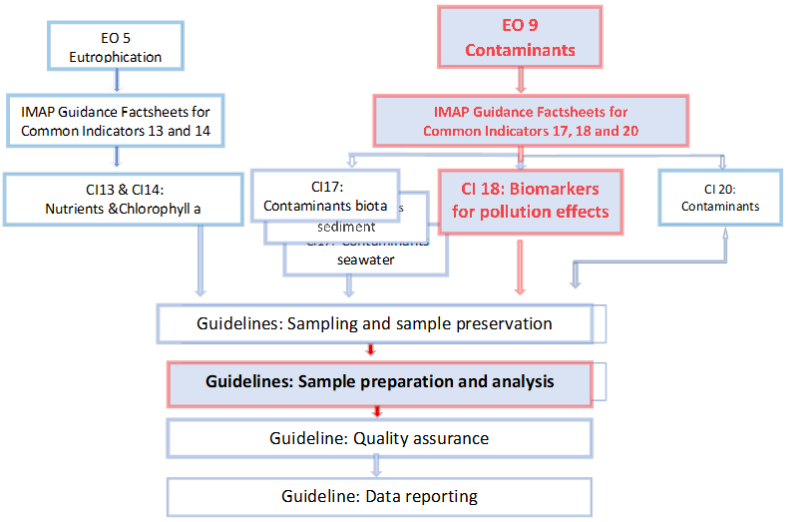
Annexes:

List of Abbreviations / Acronyms

ASTM	American Society for Testing and Materials
BDH	British Drug Houses, a big chemical company that was merged with Merck KGaA
BODC	British Oceanographic Data Centre
CAS	CAS Registry Number, is a unique numerical identifier assigned by the Chemical Abstracts Service (CAS)
CI	Common Indicator
COP	Conference of the Parties
CORMON	Correspondence Group on Monitoring
DDW	Double-*distilled water*
EcAp	Ecosystem Approach
EO	Ecological Objective
EPA	United States Environmental Protection Agency
EU	European Union
GES	Good Environmental Status
HELCOM	Baltic Marine Environment Protection Commission - Helsinki Commission
HPLC	High Performance Liquid Chromatography
IMAP	Integrated Monitoring and Assessment Programme of the Mediterranean Sea and Coast and Related Assessment Criteria
ISO	International Standard Organization
JGOFS	Joint Global Ocean Flux Study
LOD	Limit of Detection
MAP	Mediterranean Action Plan
MEDPOL	Programme for the Assessment and Control of Marine Pollution in the Mediterranean Sea
MSFD	Marine Strategy Framework Directive
OSPAR	Convention for the Protection of the Marine Environment for the North-East Atlantic
OSW	Oligotrophic Sea Water
SI	International System of Units (SI, abbreviated from the French Système international (d'unités))
SCOR	Scientific Committee on Oceanic Research
SFA	Segmented Flow Autoanalyser
UNESCO	United Nation Educational Scientific and Cultural Organization
WOCE	World Ocean Circulation Experiment

1 Introduction

1. 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).



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

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

2. 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[1]; Hayashi et al., 1998[2]; Bolognesi and Fenech, 2012[3]).

3. 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.

4. Studies indicate that the relative occurrence of micronuclei provides an indication of accumulated 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 use 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[4]; Bolognesi and Hayashi, 2011[5]; Bolognesi and Cirillo, 2014[6]). The MN test, due to its potential for application to any proliferating cell population regardless

[1] Al-Sabti, K., Metcalfe, C.D., 1995. Fish micronuclei for assessing genotoxicity in water. Mutat. Res. 343, 121-135.
[2]Hayashi, M., Ueda, T., Uyeno, K. et al., 1998. Development of genotoxicity assay systems that use aquatic organisms. Mutat. Res. 399, 125-133.
[3] Bolognesi, C., Fenech, M., 2012. Mussel micronucleus cytome assay, Nat. Protoc. 17, 1125-1137.
[4] 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.
[5] Bolognesi, C., Hayashi, M., 2011. Micronucleus assay in aquatic animals. Mutagenesis 26, 205-213.
[6] Bolognesi, C., Cirillo, S., 2014. Genotoxicity biomarkers in aquatic bioindicators. Zoology 60, 273-284.

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.

5. 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.

6. 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).

7. 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[7]). 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[8], Bolognesi and Hayashi, 2011; Bolognesi and Fenech, 2012).

8. 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.

9. In line with above elaborated under this 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 following two Protocols: i) Protocol for the analysis of micronuclei (MNi) frequency in fish (*Mullus barbatus*) blood cells and evaluation and interpretation of the results; and ii) Protocol for the analysis of micronuclei (MNi) frequency in mussel gill cells and haemocytes and evaluation and interpretation of the results.

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

a. Materials

10. 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;

[7] 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.
[8] 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.

Coplin jar, glass, for 10 slides; Microscope slide staining container, glass; slide staining rack; Storage microscope slides boxes.

b. Equipment

11. The equipment needed for the analysis 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

12. For the analysis 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.

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

- Sorensen buffer, pH 6.8: Prepare two solution (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.

14. 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[9].

d. Practical evaluation

15. 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 theTechnical note for the collection, sampling and sample preservation of marine fish (*Mullus barbatus*) for biomarker analysis.

16. Slide staining procedure includes the following steps:

- Fixed slides are stained with 10% Giemsa solution for 10 min;

[9] 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 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.

17. 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.

18. 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 that 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

19. 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.

20. 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;
- 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.

21. 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.

22. 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.

23. The data can then be statistically analysed using the non-parametric Mann-Whitney *U*-test (Speigel, 1961[10]) and compared with reference data.

f. Interpretation of the results

24. 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[11]; Viarengo et al., 2007b[12]). 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[13] 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.

25. 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

26. As reported for LMS, the animals show different physiological conditions in the different seasons (UNEP/RAMOGE, 1999; ICES, 2001; Moore et al., 2004[14]; Viarengo et al., 2007a[15]; 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

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

[10] Speigel, M. R., 1961. Statistics. Schaum`s Outline Series. Mc Graw-Hill Book Company, 359 p.
[11] Bolognesi, C., Perrone, E., Roggeri, P., Sciutto, A., 2006. Bioindicators in monitoring long term genotoxic impact of oil spill: Haven case study. Mar. Environ. Res. 62, S287-S291.
[12] Viarengo, A., Dondero, F., Pampanin, D.M., Fabbri, R., Poggi, E., Malizia, M., Bolognesi, C., Perrone, E., Gollo, E., Cossa, G.P., 2007b. 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.
[13] Decision IG.23/6 2017 Mediterranean Quality Status Report.
[14] 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.
[15] 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.

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

a. Materials

28. 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

29. The equipment needed for the analysis 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

30. For the analysis 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.

31. 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.

32. 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.

33. 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[16].

d. Practical evaluation

34. 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. 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.

35. 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.

36. 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.

37. 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;
- 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.

38. 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 that performed the analysis. All the information should be added in the

[16] 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.

Biomarker Analysis Register in which the position of the container in the lab should also clearly reported.

f. Result evaluation

39. 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. 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.

40. 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.

41. 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.

42. 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

43. 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.

44. 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

45. 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.

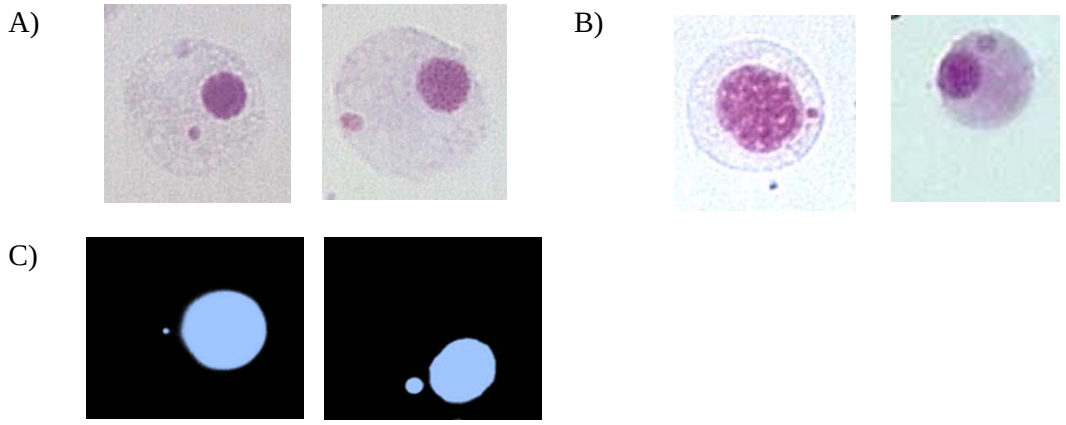


Fig 5: Images of different cell types from mussels (*Mytilus galloprovincialis*) stained using 3% Giemsa. A) Agranular gill aciliated cells with MNi, B) Agranular Hemocytes with MNi, C) Agranular gill cells with MNi, stained using DAPI.

46. 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

47. 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 Technical note for the analysis of Acetylcholinesterase (AChE) activity in mussel gills and fish muscle

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

Acetylcholine $\longrightarrow$ choline + acetic acid.

49. AChE activity was proposed as a biomarker of exposure to anticholinergic compounds such as Carbamates and Organophosphorus Pesticides (OP) (Bocquené et al., 1993[17]; Escartín and Porte,

[17] 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.

1997[18]; Boucquené and Galgani, 1998; Burgeot et al., 2001[19]; Galloway et al., 2002[20]). 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[21]).

50. 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[22]). 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.

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

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

52. As indicated in the IMAP Guidance Factsheets (UNEP/MED WG.467/5, 2019[23]), 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

53. 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[24]). 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[25]) and Galloway et al. (2002).

b. Materials

54. 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

[18] Escartín, E., Porte, C., 1997. The use of cholinesterase and carboxylesterase activities from *Mytilus galloprovincialis* in pollution monitoring. Environ. Toxicol. Chem. 16, 2090-2095.
[19] 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.
[20] 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.
[21] 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.
[22] 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.
[23] UNEP/MED WG.467/5, 2019. IMAP Guidance Factsheets: Update for Common Indicators 13, 14, 17, 18, 20 and 21; New proposal for Candidate Indicators 26 and 27.
[24] 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.
[25] Bocquené, G., Galgani, F. 1998. Biological effects of contaminants: Cholinesterase inhibition by organophosphate and carbamate compounds. ICES Techniques in Marine Environmental Sciences, No. 22.

mL, 200 mL glass beaker; Glass graduated cylinders; 1-3 mL Spectrophotometer Cuvettes (10 mm light path).

a. Equipment

55. 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

56. 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

57. 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

58. 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

59. 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);
- 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 that can change in different animals as well in animals in different physiological states.

f. Protein determination

60. The procedure for protein determination can be summarized as follows:

- Protein concentration is evaluated in accordance with Bradford (1976[26]), following the indications of Sigma-Aldrich producer (B6916). The method consists of mixing 1 part of the

[26] 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.

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 room temperature) 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

61. 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 OD * nmol^{-1} mg protein.[27]

62. 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}.

63. At the end of the analysis, 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

64. 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.

65. 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

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

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

66. 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.

67. 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.

68. 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[29]): 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.

69. 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[30]). 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

70. 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[31]).

71. 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[32]). In *Mytilus edulis* from the Baltic Sea, the AChE values vary

[29] 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.
[30] 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.
[31]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.
[32] 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).

twofold depending on the sampling season, in relation to the temperature changes (Leiniö and Lehtonen, 2005[33]).

72. The AChE activity was found to be affected by algal toxin (Dailianis et al., 2003[34]; Kankaanpää et al., 2007[35]). 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

73. 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.

4 Technical note for the analysis of Stress on Stress (SoS) in mussels

74. 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[36]). 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[37]) 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[38];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.

75. 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[39]; Viarengo et al., 1995[40]; Marcheselli et al., 2011[41]).

76. 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[42]). 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.

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[33] 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.

[34]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.

[35]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.

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

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

[38] 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.

[39] 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.

[40] 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.

[41] 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.

[42]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.

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[43]).

77. 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.

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

a. Equipment

78. 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

79. 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[44]; Mann, 1992[45]), 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.

80. 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

81. Air exposure experiment: In the laboratory at least 10 x 4 animals from each site were 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 were 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

82. The table 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 observed 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).

[43] 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.
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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

83. 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[46]) and the Spearman-Karber test (Hamilton et al., 1977[47]). 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

84. This proposal of the SoS BAC value is also in line with the data reported in ICES Item n. 59 (Thain et al., 2019) where the experimental data reported show SoS control values of about 7.5 days. These discrepancies of the literature data emphasize the importance of an intercalibration activity in the framework of the Q.A. programme in order to establish correct BAC and EAC values for the Mediterranean Sea. The correct strategy is, therefore, to compare the field data with the results of SoS obtained in mussels sampled in a reference coastal area.

85. The indications for data interpretation related to SoS reported in Decision IG. 23/6 on 2017 Mediterranean Quality Status Report based on ICES Item n. 59 (Thain et al., 2019[48]) are: LT50 (days): BAC 11, EAC 5. SoS values higher than 10 days indicate healthy molluscs; values between 5 and 10 days identify stressed animals; values lower than 5 days highlight mussels highly stressed in a pathological situation. However, the analysis of the data reported by different authors such as Smaal et al. 1991[49] and Viarengo et al. (1995) indicate that a more realistic BAC value should be considered as an LT50 of 7.5 days.

f. Confounding factors

86. 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.

87. 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 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).

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Reporting data

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

Annex I
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