UNITED NATIONS

EP

UNEP/MED WG.492/4

UNEP

**UNITED NATIONS**
**ENVIRONMENT PROGRAMME**
**MEDITERRANEAN ACTION PLAN**

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 Lysosomal membrane stability (LMS)



UNEP/MAP
Athens, 2021

## 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 |
| **EC** | European Commission |
| **EEA** | European Environmental Agency |
| **EFSA** | European Food Safety Authority |
| **EO** | Ecological Objective |
| **EPA** | United States Environmental Protection Agency |
| **EU** | European Union |
| **FAO** | Food and Agriculture Organization of the United Nation |
| **GES** | Good Environmental Status |
| **HELCOM** | Baltic Marine Environment Protection Commission - Helsinki Commission |
| **HPLC** | High Performance Liquid Chromatograph |
| **IAEA** | International Atomic Energy Agency |
| **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 |
| **MED POL** | Programme for the Assessment and Control of Marine Pollution in the Mediterranean Sea |
| **MED QSR** | Mediterranean Quality Status Report |
| **MSFD** | Marine Strategy Framework Directive |
| **OECD** | Organisation for Economic Co-operation and Development |
| **OSPAR** | Convention for the Protection of the Marine Environment for the North-East Atlantic |
| **OSW** | Oligotrophic Sea Water |
| **QA/QC** | Quality Assurance/Quality Control |
| **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

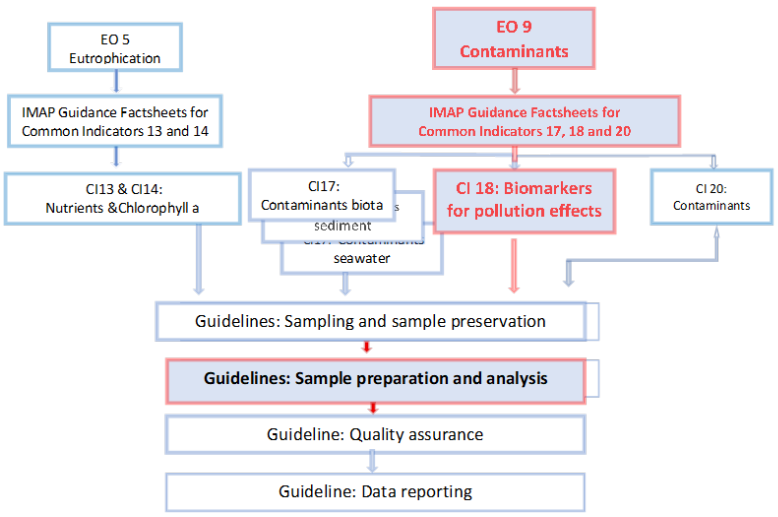
1. A fundamental aspect related to IMAP Ecological Objective 9 concerns the monitoring of the concentrations of different classes of harmful chemicals evaluated in relevant matrices i.e. sediment, sea water, and biota (CI17). These data need to be associated to the results concerning the level of the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

2. 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.

3. The Monitoring Guidelines/ Protocols related to the biomarker analysis 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.

4. The Monitoring Guidelines/ Protocols related to the biomarker analysis in marine molluscs (such as *Mytilus* sp.) and fish (such as *Mullus barbatus*) follow on UNEP/MAP Manual for biomarker analysis (UNEP/RAMOGE, 1999). They are also aligned with the Guidelines for biomarker analysis, which were developed by other Regional Organisations, such as OSPAR (2013[1]) and ICES (Davies & Vethaak, 2012[2]).

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




[1] OSPAR Commission, 2013. Background document and technical annexes for biological effects monitoring, Update 2013. 239 pp.
[2] 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.

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

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

6. 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[3]). 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 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.

7. 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[4]; Cuervo, 2004[5]; Moore, 2008[6]; Moore et al., 2015[7]). The lysosomal vascular system can accumulate both organic lipophilic xenobiotics, and inorganic hydrophilic chemicals (Viarengo, 1989[8]; Moore et al., 2007[9]; Sforzini et al., 2018a[10]). 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.

8. 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[11]; Moore et al., 2008[12]). 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

[3] Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., 2002. Molecular Biology of the Cell, 4th edition. New York: Garland Science.
[4] Klionsky, D.J., Emr, S.D., 2000. Autophagy as a regulated pathway of cellular degradation. Science 290, 1717-1721
[5] Cuervo, A.M., 2004. Autophagy: in sickness and in health. Trends Cell Biol. 14, 70-77.
[6] Moore, M.N., 2008. Autophagy as a second level protective process in conferring resistance to environmentally-induced oxidative stress. Autophagy 4, 254-256
[7] 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.
[8] Viarengo, A., 1989. Heavy metals in marine invertebrates: mechanisms of regulation and toxicity at the cellular level. Aquat. Sci. Review 1, 295-317.
[9] 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.
[10] 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.
[11] 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.
[12] 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.

are very simple and robust (Moore et al., 2008). Confounding factors usually do not represent a serious problem (Svendsen et al., 2004[13]); 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).

9. 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[14]). In fact, Allen and Moore (2004[15]) 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[16]), a decrease of this parameter reflects possible changes at the population level. The data reported in Fig. 1 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.

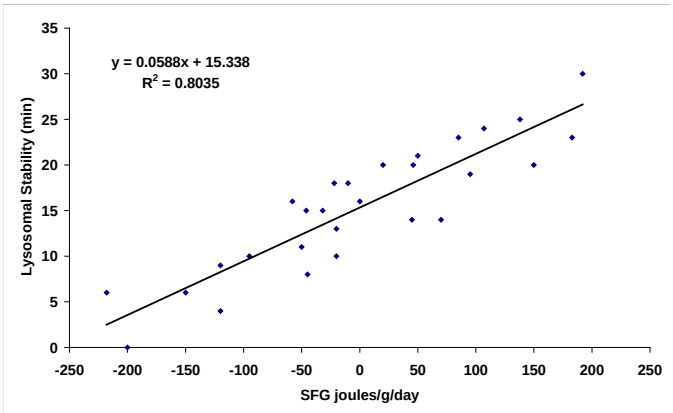



**Fig. 1**. Lysosomal Stability (LMS) as an indicator of whole organism health and possible effects at population level. LMS shows a significant linear relationship with Scope of Grows in the marine mussel *Mytilus edulis*. Data is a compositive of several field and laboratory experiments (Allen & Moore, 2004)

10. 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[17]; Laplante and Sabatini, 2012[18]; 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

[13] 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.
[14] 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.
[15] 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.
[16] 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.
[17] Soulard, A., Cohen, A., Hall, M.N., 2009. TOR signaling in invertebrates. Curr. Opin. Cell Biol. 21, 825-836.
[18] Laplante, M., Sabatini, D.M., 2012. mTOR signaling in growth control and disease. Cell 149, 274-293.

al. (2018b[19]), 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.

11. 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.

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

13. Under this Technical Note, the Monitoring Guidelines for Biomarker Analysis 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.

### 2.1 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

14. 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

15. 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

16. 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

[19] 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.

microscope (10×, 20×, 40× objectives) equipped with a linear colour video camera; pH meter; Magnetic stirrer.

d. Chemicals and solutions

17. 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.

18. 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 (Sigma Aldrich, N4006) are dissolved in 2.5 ml of 2-methoxyethanol and made up to 50 ml with solution A, containing also 3.5 g Polypep (Sigma Aldrich, P5115; 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).

19. 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).

20. Aqueous Mounting Medium (e.g. glycerol gelatin) to mount the sections[20].

e. Tissue section preparation

21. 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 acclimate to room temperature (at least 30 min at 4 °C and 30 min at room temperature).

f. Enzymatic determination of LMS

22. The application of the following procedure is essential according to Moore (1976[21]), UNEP/RAMOGE (1999[22]), Moore et al. (2004[23]) and Martínez-Gómez et al. (2015[24]). 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

[20] 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.
[21] Moore, M.N., 1976. Cytochemical demonstration of latency of lysosomal hydrolases in digestive gland cells of the common mussel *Mytilus edulis*, and changes induced by thermal stress. Cell Tissue Res. 175, 279-287.
[22]UNEP/RAMOGE (1999). Manual on the Biomarkers Recommended for the UNEP/MAP MED POL Biomonitoring Programme. UNEP, Athens.
[23] 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.
[24] Martínez-Gómez, C., Bignell, J. and Lowe, D., 2015. Lysosomal membrane stability in mussels. ICES Techniques in Marine Environmental Sciences No. 56. 41 pp

23. 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. 2).

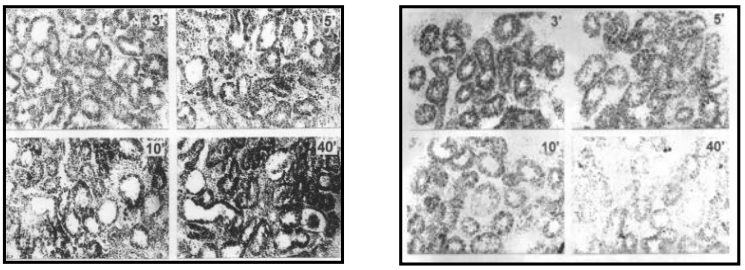



**Fig. 2**. LMS cryostat section of *Mytilus* sp digestive gland, left panel: Unpolluted site, right panel: Polluted site.

24. Evaluation of LP is done as shown in Fig. 3. 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.

25. 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.

26. 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[25], 1991; Köhler and Pluta, 1995[26]). The data can then be statistically analysed using the non-parametric Mann-Whitney U-test (Speigel, 1961[27]) and compared with reference data.

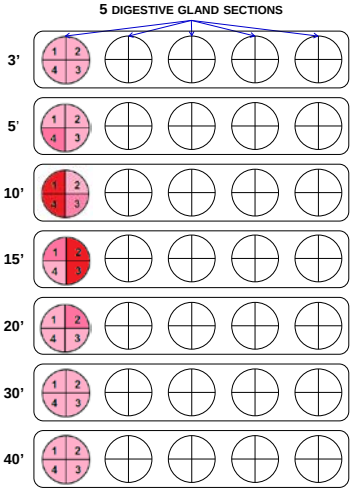



**Fig. 3**. Evaluation of LP: for each section one quarter is analysed and the incubation time in the acid buffer is determined which produces the maximal staining reactivity. The analysis is repeated for the remaining three quarters and the data averaged. This value represents the LP of the first digestive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

[25] Köhler, A., 1991. Lysosomal perturbations in fish liver as indicators for toxic effects of environmental pollution. Comp. Biochem. Physiol. 100C, 123-127.
[26] 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.
[27] Speigel, M.R., 1961. Statistics. Schaum's Outline Series. Mc Graw-Hill Book Company, 359 p.

= 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

27. 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).

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

h. Interpretation of the results

29. 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[28] on 2017 Mediterranean Quality Status Report 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

30. 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.[29], 1980, 2007; ICES, 2011[30]; OSPAR Commission, 2013).

31. In addition, it is important to know that that 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

[28] Decision IG.23/6 2017 Mediterranean Quality Status Report.
[29] 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.
[30] 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.

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

33. 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[31]). 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[32]). 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[33]; Moore et al., 2008).

34. 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

35. 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

36. 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

37. 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).

38. 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.

[31] Seglen. P.o. 1983. Inhibitors of Lysosomal functions. Meth. Emzymol. 96, 737-765.
[32] Lowe, D., Moore M.N., Evans B.M., 1992. Contaminant impact on interactions of molecular probes with lysosomes in living hepatocytes from dab Limanda limanda. Mar. Ecol. Progr. Ser. 91, 135-140.
[33] Lowe, D.M., 1988. Alteration in the cellular structure of *Mytilus edulis* resulting from exposure to environmental contaminants under field and experimental conditions. Mar. Ecol., Prog. Ser. 46, 91-100.

39. 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.

40. 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

41. The Neutral Red (NR) method is recommended according to Lowe et al. (1995[34]). 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.

42. 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 are 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 were 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.

e. Result evaluation

43. 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 4

44. 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.

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

| **Samples** | **0’** | **15’** | **30’** | **45’** | **60’** | **90’** | **120’** |
|---|---|---|---|---|---|---|---|
| **control** | + | + | + | + | + | + | + |
| **treated** | + | + | ± | - | - | - | - |

46. 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

[34] 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.

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.

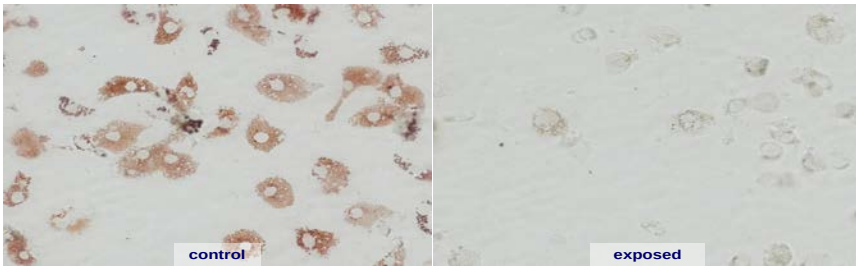



**Fig. 4**: Images of neutral red retention time (NRRT) assay to show lysosomal membrane stability of mussel haemocytes

47. 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.

48. 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.

49. 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

50. 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

51. 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).

52. In addition, it is important to know that that 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

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

# Annex I
# References

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

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.

CAS-Chemical Abstracts Service, 2020. Specialized substance collections count: CHEMLIST®. Chemical Abstracts Service. American Chemical Society. (Available at https://www.cas.org/support/documentation/regulated-chemicals, accessed 31 August 2020).

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

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.

Decision IG.23/6 2017 Mediterranean Quality Status Report.

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.

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

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

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

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

Lowe, D.M., 1988. Alteration in the cellular structure of *Mytilus edulis* resulting from exposure to environmental contaminants under field and experimental conditions. Mar. Ecol., Prog. Ser. 46, 91-100.

Lowe, D., Moore M.N., Evans B.M., 1992. Contaminant impact on interactions of molecular probes with lysosomes in living hepatocytes from dab *Limanda limanda*. Mar. Ecol. Progr. Ser. 91, 135-140.

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.

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

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

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

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.

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.

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.

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.

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.

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.

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

Seglen, P.O., 1983. Inhibitors of lysosomal function. Meth. Enzymol. 96, 737-765.

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.

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.

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

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

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.

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

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

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.

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.