UNITED NATIONS

UNEP

EP

UNEP/MED WG.556/Inf.11

United Nations Environment Programme Mediterranean Action Plan

Distr.: General
17 February 2023

Original: English only

Meeting of the Ecosystem Approach Correspondence Group on Pollution Monitoring

Athens, Greece, 1-2 March 2023

Agenda item 3: 2023 Mediterranean Quality Status Report (QSR) - Pollution Ecological Objectives (EO5, EO9)

The Marine Environment Assessment in the Areas with Insufficient Data: The Results of Assessment for IMAP Common Indicator 18 in the Mediterranean

UNEP/MAP
Athens, 2023

Table of Contents

List of Abbreviations / Acronyms

AChE	Acetylcholinesterase
ADR	Adriatic Sea sub-region
AEL	Aegean and Levantine Seas sub-region
BAC	Background Assessment Concentrations
BC	Background Concentration
BChE	Butyrylcholinesterase
BFCOD	7-benzyloxy-4-[trifluoromethyl]-coumarin-O-debenzyloxylase
CAT	Catalase
CE	Carboxylesterase
CEN	Central Mediterranean Sea sub-region
CI	Common Indicator
COP	Conference of the Parties
CORMON	Correspondence Group on Monitoring
CS	Citrate synthase
DD	Data Dictionarie
DS	Data Standard
EAC	Environmental Assessment Criteria
EC	European Commission
EO	Ecological Objective
EROD	Ethoxyresorufin-O21 deethylase
ETS	Electron Transport System
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
GES	Good Environmental Status
GLY	Glycogen
GPx	Glutathione peroxidase
GRd	Glutathione reductase
GSH	Glutathione
GST	Glutathione-S-transferase
IMAP	Integrated Monitoring and Assessment Programme of the Mediterranean Sea and Coast and Related Assessment Criteria
LDH	Lactate dehydrogenase
LMS	Lysosomal Membrane Stability
LPO	Lipid peroxidation
MAP	Mediterranean Action Plan
MDA	Malondialdehyde
MED	Mediterranean
MB	*Mullus barbatus*
MED POL	Programme for the Assessment and Control of Marine Pollution in the Mediterranean Sea
MG	*Mytilus galloprovincialis*
MN	Micronucleus Assay
MT	Metallothionein
NRTT	Neutral red retention time
PAHs	Polycyclic Aromatic Hydrocarbons
PCB	Polychlorinated Biphenyl

QSR	Quality Status Report
SOD	Superoxide dismutase
SoS	Stress on Stress
TM	Trace metals
UNEP	United Nations Environmental Program
VTG	Vitellogenin
WHO	World Health Organization
WMS	Western Mediterranean Sea sub-region

1. Background Information

Common Indicator 18 (CI-18). Level of pollution effects of key contaminants where a cause and effect relationship has been established (EO9)

1. Biomarkers are used to infer on a cause-effect relationship between a stressor and a biotic effect. The biotic effects can be biochemical, physiological, toxicological and/or be detected at the ecological/community level. The World Health Organization (WHO, 1993) further defined biomarkers as: (1) a biomarker of exposure (effect measured within an organism) and (2) a biomarker of effect (effect measured as a health impairment or disease) (Lomartire et al. 2021).

2. Moreover, biomarkers are classified as per the expected protection/defense response as follows below, including the most used as collected from the recent literature for the Mediterranean Sea. (See Annex I and Section 4). In bold, there are biomarkers that appear in Data Dictionaries (DDs) and Data Standards (DSs) (WG 533/10, Appendix V and Table 1). Their status (mandatory or additional (non-mandatory), and organism and tissue to apply test appear in Table 1.

- **Lysosomal Membrane Stability (LMS)** as a method for general physiological status screening
- **Lysosomal Membrane Stability (LMS)- Neutral red retention time (NRRT) assay**
- **Micronucleus Assay test** (MN frequency) - A tool for assessing cytogenetic/DNA damage in marine organisms
- **Acetylcholinesterase (AChE)** enzymatic activity - enzyme involved in impulse transmission. A method for assessing neurotoxic effects in aquatic organisms
- **Metallothionein (MTs).** An increase of the cellular MT-protein level is considered as a protective effect against an excess of harmful metals
- **Stress on Stress (SoS),** the survival on air is a general method to determine the physiological condition in bivalves
- Antioxidant defense enzymes: Superoxide dismutase (SOD) that eliminates superoxide anion, catalase (CAT) and glutathione peroxidase (GPx) which remove hydrogen peroxide and glutathione reductase (GRd) which regenerates glutathione (GSH), an important non-enzymatic antioxidant against ROS (reactive oxygen species)
- Detoxification enzymes: CYP1A1 (frequently measured as ethoxyresorufin-O21 deethylase (EROD) activity and glutathione-S-transferase (GST)
- Aldehyde, formed from oxidative damage to lipids- malondialdehyde accumulation levels (MDA)
- Butyrylcholinesterase enzymatic activity (BChE)
- Detoxifying enzyme Carboxylesterase (CE) activity - potential biomarkers of chemical exposure, as they are an important family of enzymes involved in the metabolism of xenobiotic and endogenous compounds.
- Expression of vitellogenin (VTG), a lipoprotein produced in female fish and used as a biomarker for endocrine disruption
- Metabolic biomarkers : Electron Transport System activity (ETS) and glycogen reserves (GLY)
- Lactate dehydrogenase (LDH), and citrate synthase (CS): biomarkers for anaerobic and aerobic metabolism.
- Oxidative damage markers: lipid peroxidation (LPO) and protein carbonyl derived levels (CARB)
- Phase I detoxifying enzyme - 7-benzyloxy-4-[trifluoromethyl]-coumarin-O-debenzyloxylase (BFCOD)
- Histopathological alteration

- DNA damage

3. More background information about the mandatory and additional biomarkers, along with how to sample biota and measure biomarkers, is presented in UNEP/MED WG. 509/27.

4. Biomarkers measuring catalytic activity can be quantified as katal or enzyme units. The katal (kat) is the unit of catalytic activity in the International System of Units (SI). One katal refers to an enzyme catalysing the reaction of one mol of substrate per second (katal=mole/sec). The enzyme unit, or international unit for enzyme (symbol U, sometimes also IU) is defined as the amount of enzyme that catalyzes the conversion of one micromole of substrate per minute under the specified conditions (U= μmol/min). One kat = $6x10^7$ U and One U=16.67 nkatal.

1.1. The biomarkers monitoring as defined within implementation of the Integrated Monitoring and Assessment Programme (IMAP) of UNEP/MAP

5. Updated Guidance Fact sheet for IMAP CI 18[1] from 2019, provided the GES definition as "Concentrations of contaminants are not giving rise to acute pollution events" while the related operational objective was "Effects of released contaminants are minimized". The two proposed targets under this CI were:" 1) Contaminants effects below threshold and 2) Decreasing trend in the operational releases of oil and other contaminants from coastal, maritime and off-shore activities".

6. The Updated Guidance Fact sheet also defines that the targets of GES under CI 18 "will be based upon data of a selected biological effects parameters and biomarkers (reflecting the scope of current programmes and research, see Indicator Justification above) and the availability of suitable agreed assessment criteria". Biomarkers will be measured in marine bivalves (such as *Mytilus galloprovincialis*) and/or fish (such as *Mullus barbatus*). The Updated Guidance Fact sheet for IMAP CI 18 also provides the following indicators: Lysosomal Membrane Stability (LMS) (units: (retention) minutes) as a method for general status screening; Acetylcholinesterase (AChE)(nmol/min mg protein in gills (bivalves)) assay as a method for assessing neurotoxic effects in aquatic organisms and Micronucleus assay (Number of cases, ‰ in haemocytes) as a tool for assessing cytogenetic/DNA damage in marine organisms. Complementary (additional, non-mandatory) biomarkers, bioassays and histology techniques and methods are also recommended to be carried out on a country basis (such as, hepatic pathologies assessment, reduction of survival in air by Stress on Stress (SoS), larval embryotoxicity assay, Comet assay, etc.). Metallothionein in mussels and Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposures.

7. These indicators served as the basis for the development and decision on the biomarkers that were included in the IMAP Info System Data Dictionaries (DDs) and Data Standards (DSs) as presented in Table 1, taken from WG 533/10, Appendix V (CI-18 Data Dictionaries (DDs) and Data Standards (DSs)). Those are also presented in IMAP Monitoring Guidelines for CI-18[2].

Table 1: List of mandatory Biomarkers as presented in document UNEP/MED WG. 533/10, Appendix V

Biomarker	Description (EN)	Organism	Tissue	Mandatory	Additional (Not-mandatory)

[1] UNEP/MED WG473/7 Annex I "IMAP Guidance Factsheets: Update for Common Indicators 13, 14, 17, 18, 20 and 21; New proposal for Candidate Indicators 26 and 27

[2] UNEP/MED WG. 509/27, Monitoring Guideline/Protocols for Sampling and Sample Preservation of Marine Molluscs (such as *Mytilus* sp.) and Fish (such as *Mullus barbatus*) for IMAP Common Indicator 18
UNEP/MED WG. 509/27, 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)

LMS-HEXO	Lysosomal membrane stability on cryostat sections - enzymatic determination	Fish/Mussel	Liver/Digestive gland	Y	
LMS-NRRT	Lysosomal membrane stability in mussel haemocytes - in vivo determination (neutral red retention time (NRRT) assay)	Mussel	Haemocytes (in vivo)	Y	
MN_F	Micronuclei frequency in fish blood cells	Fish	Erythrocytes	Y	
MN_MH	Micronuclei (MNi) frequency in mussel gill cells and haemocytes	Mussel	Gill cells, Haemocytes	Y	
AChE	Acetylcholinesterase activity - enzymatic determination	Mussel / Fish	Gills / Muscle	Y	
% LMS	% LMS Mean percentage of Lysosomal membrane stability in mussel	Mussel	Haemocytes		Y
MT	Metallothioneins	Fish	Digestive gland		Y
SoS	Stress on stress	Mussel			Y
NM	Other: not mandatory biomarker	Specify	Specify	-	Y

8. By Decisions IG.22/7 and IG. 23/6, the Contracting Parties endorsed BAC and EAC values for biomarkers for the mussel (*Mytilus galloprovincialis*): Acetylcholinesterase activity (AChE), Metallothionein (MT), Micronuclei frequency (MN), Lysosomal membrane stability (LMS-NRR and LMS-LP methods) and Stress on Stress (SoS). These values, presented in Table 2, are indicative and serve as the initial assessment criteria.

Table 2. Mediterranean BACs and EACs levels for biomarkers in mussel (*M. galloprovincialis*) as agreed upon during COP19 and COP 20. The values calculated in 2016 BCs and BACs from the Mediterranean Sea, and BC for two sub regions are given as well.

Biomarker/Bioassay		Decisions IG.22/7 and IG.23/6 (COP 19 and COP 20)				UNEP/MAP (2016)			
		BAC	EAC	BAC	EAC	BC	BAC	BC	BC
	Units	IG.22/7	IG.22/7	IG.23/6	IG.23/6	Med	Med	WMS	ADR
Stress on Stress (SoS)	days	10	5	11	5	11	11		
Metallothioneins	ug/g (digestive gland)			247		192	247	191.3	200.5
Lysosomal membrane stability Neutral Red	minutes	120	50	120	50	45	120	45	47.4

Biomarker/Bioassay		Decisions IG.22/7 and IG.23/6 (COP 19 and COP 20)				UNEP/MAP (2016)			
		BAC	EAC	BAC	EAC	BC	BAC	BC	BC
	Units	IG.22/7	IG.22/7	IG.23/6	IG.23/6	Med	Med	WMS	ADR
Retention Assay (LNS-NRR)									
Lysosomal membrane stability Cytochemical method (LNS-LP)	minute	20	10	20	10	13	20		16.8
Acetylcholinesterase (AChE) activity in gills (French Mediterranean waters	nmol/min/mg protein	29	20	29	20				
Acetylcholinesterase (AChE) activity in gills (Spanish Mediterranean waters)	nmol/min/mg protein	15	10	15	10				
Acetylcholinesterase (AChE) activity in gills	nmol/min/mg protein					21	15	20.86	12.2
Micronuclei frequency	number of cases /1000 cells (‰) ~~0/00*~~ in haemocytes	3.9		1		0	1	0	0.5

~~* MN in 1000 blood cells~~

2. The assessment methodology related to IMAP CI 18 provided in the MED QSR 2017 (https://www.medqsr.org/assessment-methods-CI18)

9. The previous assessment of CI-18, performed during the preparation of the 2017 Mediterranean Quality Status Report (2017 MED QSR), was based on bibliographic studies and scientific documents in the Mediterranean Sea. There were no data sets available for CI-18 in MEDPOL.

10. The assessment was based on the integrated evaluation of the biomarkers selected for their monitoring in the Mediterranean Sea, namely, Acetylcholinesterase activity (AChE), Lysosomal membrane stability (LMS) and Micronuclei frequencies (MN) and in addition, the enzyme 7-ethoxy-resorufin-O-deethylase (EROD) for fish and Metallothionenin (MT) for mussel.

11. The key messages and knowledge gaps, as reported in the 2017 QSR assessment, were as presented here-below.

12. Key messages:

- Biological effects monitoring tools still in a research phase for biomarker techniques (i.e. method uncertainty assessments and confounding factors evaluations) which limits the implementation of these tools in the long-term marine monitoring networks.
- Lysosomal Membrane Stability (LMS) as a method for general status screening, Acetylcholinesterase (AChE) assay as a method for assessing neurotoxic effects and Micronucleus assay (MN) as a tool for assessing cytogenetic/DNA damage in marine organisms have been selected as primary biomarkers.

13. Knowledge gaps:

- Important development areas in the Mediterranean Sea over the next few years should include: confirmation of the added value of these batteries of biomarkers in long-term marine monitoring as ‘early warning’ systems; test of new research-proved tools such as ‘omics’, analytical quality harmonization, development of suites of assessment criteria for the integrated chemical and biological assessment methods, and review of the scope of the biological effects monitoring programmes.
- Through these and other actions, it will be possible to develop targeted and effective monitoring programmes tailored to meet the needs of CI18 within the IMAP implementation and GES assessments.

14. Further to these recommendations, progress was made by proposing the integration of CI-18 (Biomarkers) and CI-17 (Chemical monitoring) data in the assessment process. The proposed methodology is detailed in UNEP/MED WG.492/6[3]. Briefly, it was proposed to combine the effects of single chemical pollutants, to take into consideration possible additive or synergistic environmental effects among themand then evaluate the risk to marine organisms exposed to the pollutants in water and sediments. The integration procedure consists of three modules: (i) a chemical module for integration of the data concerning the concentration of the pollutants; (ii) an ecotoxicological module that integrates data of the biological effects in marine organisms; and (iii) an integration module that combines the two lines of evidence in an Environmental Risk Index.

15. Evaluation of the environmental risk is usually obtained by an “expert judgement of a panel of qualified environmental scientists. Applying the Environmental Risk Index will result in objective risk values which allow national and regional policy makers and environmental managers to decide on required actions to decrease marine contamination, or to remediate a polluted area. The effectiveness of these actions can subsequently be monitored and quantified until an acceptable Environmental Risk Index is achieved.

16. However, this approach still cannot be applied given lack of data reporting and capacities at the national level.

3. The assessment methodology applied for present assessment of IMAP CI 18

17. Given complete lack of data reportinginto IMAP IS by the CPs prevents implementation of the recommendations of COP 19, the input for the 2023 Mediterranean Quality Status Report (2023 MED QSR) related to the status assessment of CI-18 was performed based on bibliographic studies, as also applied within the preparation of the 2017 MED QSR, using newer available scientific literature. The studies used in the discussion below are presented in Annex I.

18. The studies surveyed were chosen based on the following criteria:

[3] Ecosystem Approach Correspondence Group on Pollution Monitoring (26-28 April 2021) (Agenda item 3: Monitoring Guidelines/Protocols for IMAP Common Indicator 18, Implementation of IMAP Common Indicator 18 on Biomonitoring)

- Containing data only from the Mediterranean Sea;
- Containing data from studies conducted since 2016[4] and published since 2018. It should also be mentioned that there are papers that were published in 2020-2022, however they present data collected prior to 2016. Those were not considered.

19. From the literature search results it is clear that comparison among the studies is hard or mostly impossible. This is due to the use of different biomarkers, with different biota species, using different tissues, and different methodologies. Moreover, as found in the 2017 QSR, there are confounding factors that hinders environmental status assessment such as species, gender, maturation status, season and temperature. In addition, an inherent bias exists in publications towards studies showing an effect. Authors and journals do not usually publish studies showing lack of effect or response. Italy submitted national data for CI 18 following the Meeting of CorMon Pollution that took place in Athens, 1-2 March 2023.[5]

3.1. Assessment findings

20. A short summary of the findings is presented below while the extended summary is given in Annex I. Table 3 summarizes the studies reviewed in this document and Figures 1 and 2 depict the sampling areas. Figure 1 shows the whole Mediterranean Sea, while Figure 2 shows in details the study areas off eastern Algeria and Tunisia, where many of the reviewed studies were performed.

Table 3. Studies on biomarkers in the Mediterranean Sea since 2016 reviewed in present assessment of CI 18. The list is sorted alphabetically by country.

Reference	Country	Sub-region	Sampling year	Taxa	Species	Organ/tissue	Stressor	Biomarker
Kaddour et al. 2021	Algeria	WMS	2019-2020	Fish	*Mullus barbatus*	blood	non specific	MN, NRRT
Amamra et al. 2019	Algeria	WMS	2016	mollusc	*Donax trunculus*	gonad, mantle, digestive gland	non specific	AChE, GST, MDA
Benaissa et al. 2020	Algeria	WMS	2016	mollusc	*Patella rustica*	Soft tissue	desalination brine	AChE, CAT, SOD, GR, GPx, GST, LPO, Genotox
Laouati et al. 2021	Algeria	WMS	2017	mollusc	*Perna perna*	digestive gland and gills	non specific, TM	AChE, CAT, GSH, GST, MDA
Gabr et al. 2020	Egypt	AEL	2018-2019	mollusc	*Ruditapes decussatus*	soft tissue	TM	AChE, SOD, GPx, MDA
Salvaggio et al. 2019	Italy	FAO Area 37	not reported	Fish	*Lepidopus caudatus*	liver, gonads	Microplastic, TM	VTG, MT

[4] Except for one study conducted in Turkeye due to the lack of data in the area and the very relevant biomarkers measured.

[5] The data included biomarkers (Acetylcholinesterase activity, Lysosomal membrane stability on cryostat sections, Micronuclei frequency, Metallothioneins, EROD-microsomal, EROD-S9, Fulton's Condition Factor, Gonadosomatic Index and Hepatosomatic Index) were measured in the fish M. barbatus sampled in 2019 and 2020. The data were not uploaded in the IMAP-Info System because they were found not compliant given the lack of data related to the 'maturation key' and of the 'tissue weight', which are considered mandatory. The national data could not be integrated into the CI 18 assessment as the 2023 MED QSR for CI18 was based on the use of regional scientific literature sources, using the evaluation provided by the authors. The newly submitted data of Italy were all for M. barbatus, to which no criteria were adopted yet, by the CPs. The assessment criteria for the biological effects on *M. barbatus* might be set in the future conditional to optimal data reporting by the CPs. Moreover, no conclusions were set, as in the scientific literature.

Reference	Country	Sub-region	Sampling year	Taxa	Species	Organ/tissue	Stressor	Biomarker
Frapiccini et al. 2021	Italy	ADR	2019	Fish	*Mullus barbatus*	muscle	PAH	CAT,SOD,GST,LPO
Chenet et al. 2021	Italy	CEN	2018	fish	*Trachurus trachurus*	liver	plastic	VTG, MT
Morroni et al. 2020	Italy	WMS	2017	Fish	*Diplodus vulgaris*	various	PAH, TM	AChE, MT, MN, LMS, EROD
Morroni et al. 2020	Italy	WMS	2017	Fish	*Mullus barbatus*	various	PAH, TM	AChE, MT, MN, LMS, EROD
Morroni et al. 2020	Italy	WMS	2017	Fish	*Pagellus erythrinus*	various	PAH, TM	AChE, MT, MN, LMS, EROD
Parrino et al. 2020	Italy	WMS	not reported	Fish	*Parablennius Sanguinolentus*	Brain and blood	pesticides	AChE, BChE
Morroni et al. 2020	Italy	WMS	2017	mollusc	*Mytilus galloprovincialis*	various	PAH, TM	AChE, MT, MN, LMS, EROD
Capo et al. 2022	Spain	WMS	2019	Fish	*Sparus aurata*	blood, plasma, liver	microplastic, plasticizers	CAT,SOD,GRd,GPx, MPO, GST, MDA, EROD, BFCOD, CE
Solomando et al. 2022	Spain	WMS	2020	Fish	*S. dumerili*	liver	microplastic	CAT,SOD,GST, EROD, MDA
Rios-Fuster et al. 2022	Spain	WMS	2019	mollusc	*Mytilus galloprovincialis*	Soft tissue	Anthrop. Particles, bisphenols , phthalate	CAT,SOD,GRd,GPx, GST, TES, GLY, CE, LPO, CARB, GSH
Capo et al 2021	Spain	WMS	not reported	mollusc	*Mytilus galloprovincialis*	gills	microplastic	CAT,SOD,GRd,GPx, GST,MDA, ROS
Rodríguez-Romeu et al., 2022	Spain	WMS	2019	Fish	*Engraulis encrasicolus*	Muscle and liver	Anthopogenic items ingestion	AChE, LDH, CS, CE, CAT, GST, EROD
Mansour et al. 2021	Tunisia	CEN	2016	mollusc	*Ruditapes decussatus*	Soft tissue	hydrocarbons	CAT,SOD,GRd,MDA, AChE
Zaidi et al. 2022	Tunisia	CEN	2018	mollusc	*Patella caerulea*	soft tissue	TM	CAT,SOD,GPx,GST,MDA
Ghribi et al. 2020	Tunisia	CEN	2017 mesocosm	mollusc	*Mytillus spp*	hemolymph, gills, and digestive gland	non specific PAH, TM	CAT, GPx, GST, AChE
Missawi et al. 2020	Tunisia #	CEN	2018	Seaworm	*Hediste diversicolor*	whole (gut cleaned)	Microplastic	CAT,GST,MDA, AChE
Zitouni et al. 2020	Tunisia *	WMS	2018	Fish	*Serranus scriba*	gastrointestinal tract	Microplastic	CAT,GST,MDA, AChE,MT

Reference	**Country**	**Sub-region**	**Sampling year**	**Taxa**	**Species**	**Organ/tissue**	**Stressor**	**Biomarker**
Telahigue et al. 2022	Tunisia	WMS	2020-2021	mollusc	*Flexopecten glaber*	gills, digestive gland	TM	CAT,SOD,GPx,GSH, MT, MDA
Bouhedi et al 2021	Tunisia	WMS	not reported	polychaete	*Perinereis cultrifera*	whole body	TM	CAT,GST, AChE, MT, GSH, TBARS
Uluturhan et al. 2019	Turkeye	AEL	2015	mollusc	*Mytilus galloprovincialis*	Hepatopancreas	TM, Pesticides	CAT,SOD,GPx, AChE
Uluturhan et al. 2019	Turkeye	AEL	2015	mollusc	*Tapes decussatus*	Hepatopancreas	TM, Pesticides	CAT,SOD,GPx,AChE
Dogan et al, 2022	Turkeye	AEL	2021	Fish	*Mullus barbatus*	muscle, liver	TM	CAT, MDA
Dogan et al, 2022	Turkeye	AEL	2021	Fish	*Boops boops*	muscle, liver	TM	CAT, MDA
Dogan et al, 2022	Turkeye	AEL	2021	Fish	*Trachurus trachurus*	muscle, liver	TM	CAT, MDA

#data related to the WMS as well; * data related to the CEN as well.

Figure 1. Areas of study for biomarkers, reviewed in the recent (since 2016) scientific literature for the Mediterranean Sea. When no coordinates were presented in the papers, the general area was marked in the map.

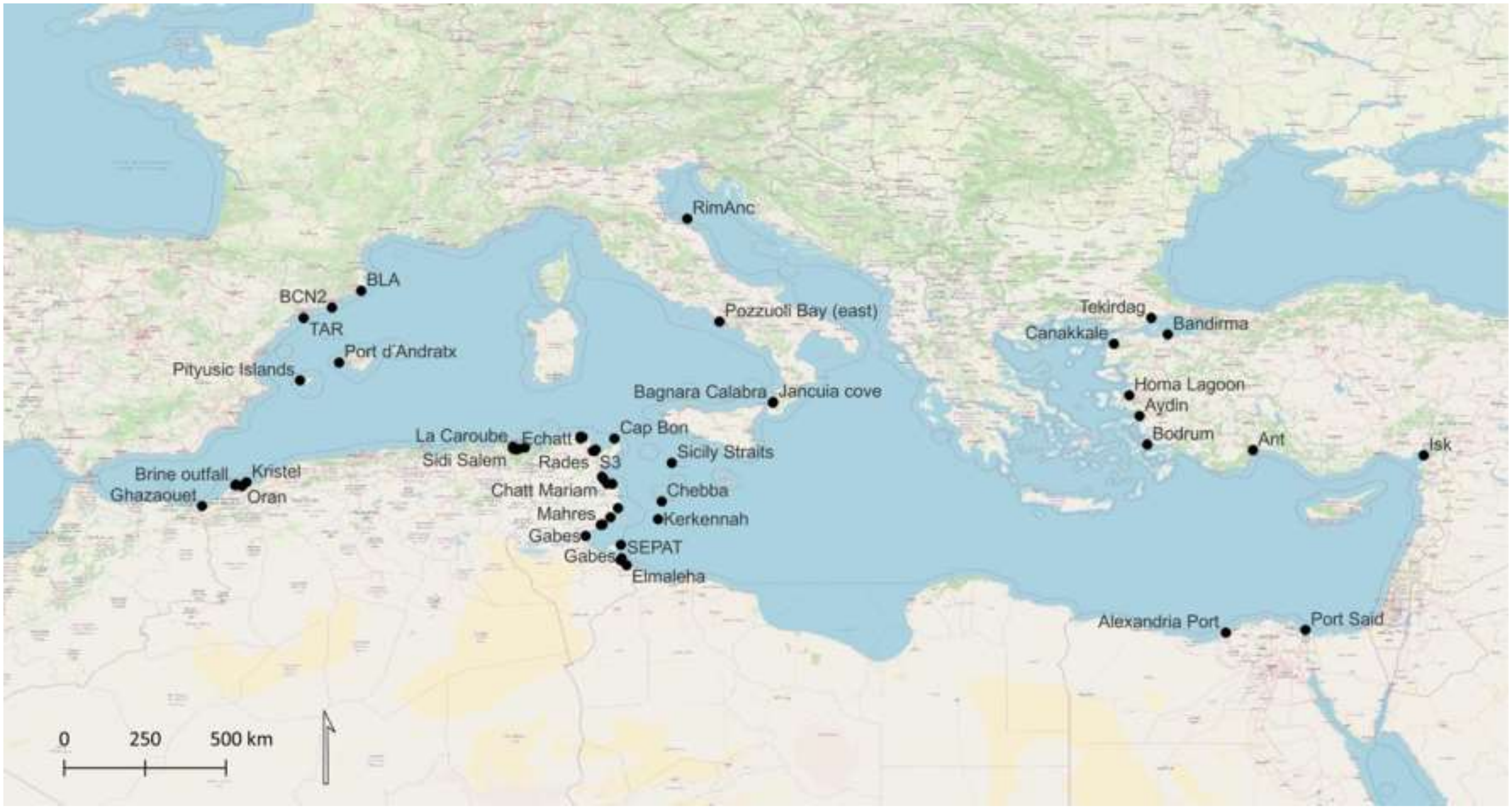
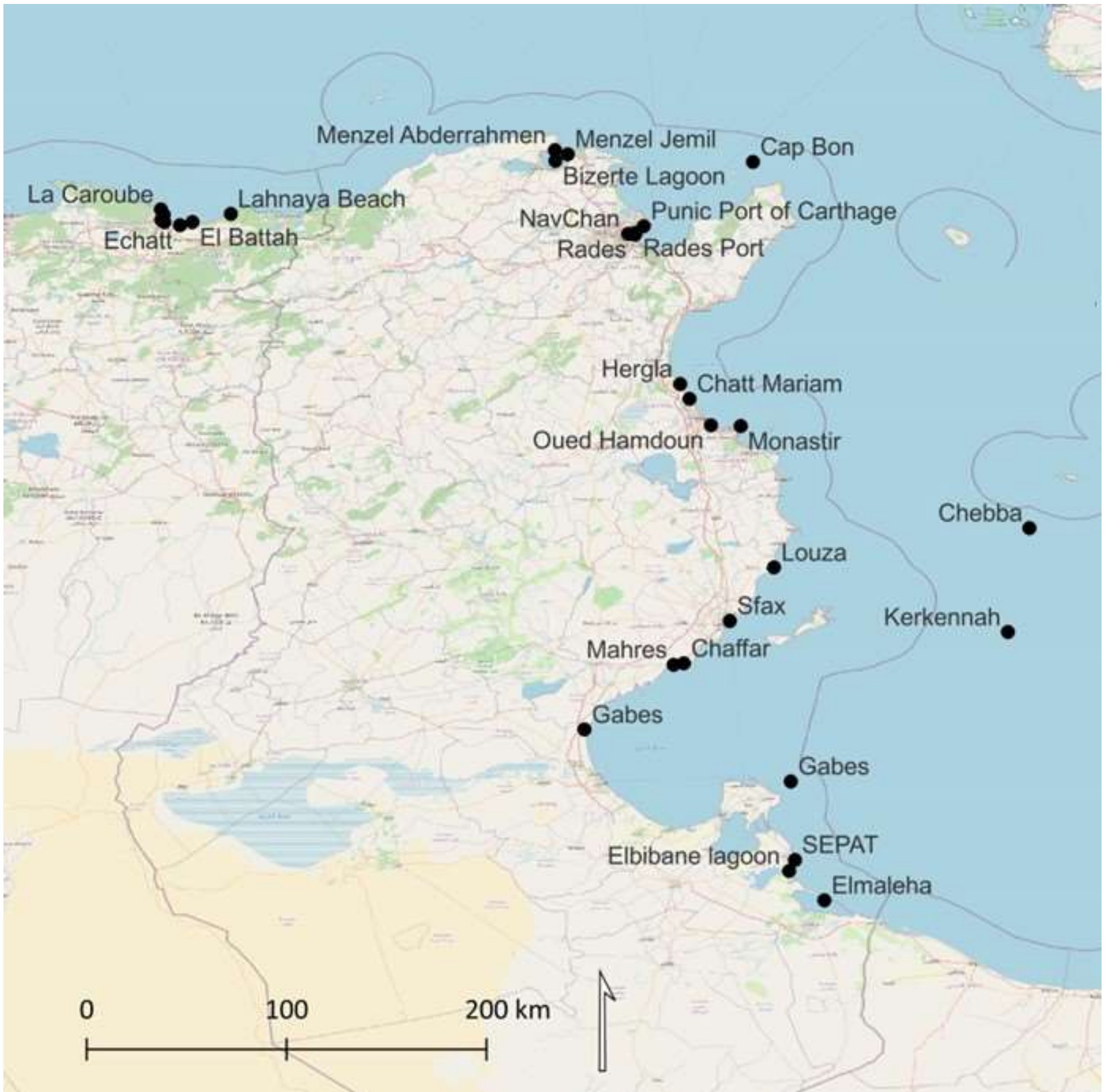


Figure 2. Detailed map of the study areas for biomarkers reviewed in the recent (since 2016) scientific literature for eastern Algeria and Tunisia coasts. A large number of stations were occupied in this area of the Mediterranean Sea.



21. Below is a short summary of reviewed studies (See Annex I), sorted by sub-regions and countries. The biomarkers that were affected by contamination are marked in red, those that were not affected are marked in green, while inconclusive results are marked in blue. Moreover, the biomarkers that are listed in the DDs and DSs are highlighted in yellow, but with no differentiation among species or tissues studied. The below section shows further analysis of the assessment findings available per country (Table 3) grouped in the in Mediterranean sub-regions.

3.1.1 WMS sub-region (Algeria, Spain, Tunisia, Italy)

22. **Algeria.** Four studies reviewed for Algeria studied the effects of non specific stressor in the mollusc *Donax trunculus* from Annaba Bay (Amamra et al. 2019) , in the fish *Mullus barbatus* along the

Algerian west coast (Kristel, Oran, Ghazaouet) (Kaddour et al. 2021), on the mollusc *Perna perna* transplanted to the Gulf of Annaba initianorth-eastern coast) (Laouati et al. 2021) and on the mollusc *Patella rustica* affected by the brine of the Bousfer desalination plant in Oran Bay (Benaissa et al. 2020).

23. *Donax trunculus* specimens showed a significant inhibition of AChE and induction of GST and MDA in individuals of Sidi Salem and Echatt as compared to El Battah with significant effects of both site and season. The effects were more pronounced during summer and spring compared to the other seasons. In addition, the comparison between tissues revealed a more marked response in gonad than mantle and digestive gland.

24. In *M. barbatus*, a significant increase in the frequency of micronuclei (MN) occurrence in the summer period correlated with significantly shorter NRRT. In addition, the erythrocytes of *M. barbatus populations* from polluted areas presented statistically higher MN frequencies and shorter NRRT than those of the reference site.

25. GSH decreased in the gills and digestive glands of *P. perna* specimens transplanted to two of the sites affected by anthropogenic input while GST and CAT activities showed no significant variation. The MDA content in the mussel digestive glands, but not in the gills, increased significantly after the deployment period in the three caging sites, and were significantly different among the 3 sites. AChE activity was significantly inhibited registered in the gills of mussels from the 3 sites and in the digestive glands from one site.

26. A multibiomarker approach (oxidative stress, biotransformation enzyme, lipid peroxidation, neurotoxicity and genotoxicity) were applied in the soft tissue of *P. rustica* . This biomonitoring confirmed the negative impact of brine discharges of the desalination plant, with samples collected close to the outfall more affected. by all the environmental disturbances than ones from the other sites. CAT, TGPx, GR, GST, CSP-3like activities were increased in samples from the outfall. AChE was lower however not significantly different from samples collected from the reference site. Genotoxic effect revealed by ADN and lipid damages.

27. **Spain.** Five studies were reviewed for Spain: four studies studied the effect of microplastic ingestion and of plasticizers on the biomarker responses, while one studied the effect of anthropogenic items ingestion. Three studies were conducted in the Integrated Multi-Trophic Aquaculture cages in Palma de Majorca, where specimens of the mussel *Mytilus galloprovincialis* and of the fish *Sparus aurata* were transplanted to and analyzed at time 0, after 60 days (T_{60}) and after 120 days (T_{120}) of exposure (Capó et al. 2022, Capo et al. 2021, Rios-Fuster et al. 2022). One study was performed with *S. dumerili* collected around the Balearic Islands (Solomando et al. 2022). Anthropogenic items ingestion was studied in *E. encrasicolus* collected off Catalunia (Rodríguez-Romeu et al. 2022).

28. No effects of time were observed in CAT, SOD, and GRd activities *M. galloprovincialis*, but they were significantly higher in specimens sampled from the cages than in specimens from the controls. GST activity did not change with time, and it increased significantly only in samples for the cages at T_{60}. In T_{120} activity was higher in the cages only if compared to one of the control sites. GPx activity was modulated by both sampling site and time: higher activities in specimens from the cages at T_{120}. MDA was higher in samples from the cages compared to the controls at T60. In a different study with *M. galloprovincialis* higher expressions were observed in the biomarkers CAT, SOD, GPx and LPO in specimens from the aquaculture cages. Those could be triggered by the presence of bisphenol but also by other possible contaminant inputs from the aquaculture.

29. MDA increased throughout the study both in liver and blood cells of *S. aurata* but with a progressive decrease in plasma. EROD, BFCOD and CE, showed a comparable decrease at T_{60} with a slight recovery at T_{120}. In contrast, GST activity was significantly enhanced at T_{60} compared to the other sampling stages.

30. SOD, CAT, and GST activity were significantly higher in *S. dumerili* with higher microplastic (MP) load, while no significant differences were observed for MDA, and EROD enzyme activity

AChE, CAT and GST were lower in *E. encrasicolus* collected off Barcelona, compared to specimens collected Blanes and Tarragona; Terragona LDH, CE and EROD were higher in Terragona than in the other two locations; Blanes CS was higher than in Tarragona. These differences could not be correlated with any potential stressors nor with fish size Catalunia (Rodríguez-Romeu et al. 2022).

31. **Italy.** Five studies were reviewed for Italy: 2 from the WMS, 1 from FAO zone 37 (not further specified), 1 from the CEN (Section 3.1.2), 1 from the ADR (Section 3.1.3). In the WMS, the effect of pesticides were studied in the fish *Parablennius sanguinolentus* from the port of Bagnara (western Calabria) (Parrino et al. 2020), and the effect of TM and PAHs on mollusc (*Mytilus galloprovincialis*) and fish (*Mullus barbatus*, *Pagellus erythrinus* and *Diplodus vulgaris)*from the bay of Pozzuoli (Naples)(Morroni et al. 2020). Microplastics and TM effects were studied on the fish *Lepidopus caudatus* collected from FAO area 37 (area not further specified) (Salvaggio et al. 2019).

32. AChE activity in the brain and BChE activity in blood were significantly inhibited in specimens of *P. sanguinolentus* from the affected port area, by 23.5 and 72.0%, respectively. The esterase inhibition was primarily due to carbamate and organophosphorus insecticides presence.

33. In the Bay of Pozzuoli, the effect of pollution varied by species and biomarkers. In *M. galloprovincialis*, there was a decreased LMS and increased MN at two sites compared to organisms from other areas while no variations were observed for the AChE in haemolymph, nor for MT in digestive gland of mussels from various sites. AChE activity was not affected in *M. barbatus* sampled in the industrial area while a decrease of this biomarker AChE was observed in *P. erythrinus* and *D. vulgaris*. The EROD enzymatic activity was significantly induced in *M. barbatus* and *P. erythrinus* sampled in the industrial area compared to specimens from the reference site, while the cytochrome P450 biotransformation pathway was unaffected in *D. vulgaris*. At the same time, all the fish species exhibited higher levels of aromatic metabolites, particularly B[a]P-like and pyrene-like, in organisms sampled in the industrial compared to reference area. MN increased in gills of *M. barbatus* from the industrial area.

34. Immunohistochemical analysis for anti-metallothionein 1 antibody in *L. caudatus* showed a strong positivity of liver cells, both in females and males, showing a strong stress that activated a cell detoxification system. The immunohistochemical analysis for the anti-vitellogenin antibody showed in females a strong positivity both in the liver cells, and in the gonads, as expected. The analysis of the liver and gonadal preparations of the male specimens was found to be always negative except for one specimen.

35. **Tunisia.** Seven studies were reviewed for Tunisia: 2 from the WMS, 3 from the CEN (Section 3.1.2) and 2 with data from both the WMS and the CEN. In the WMS, the effect of TM was studied in the mollusc *Flexopecten glaber* collected from the Bizerte Lagoon (Telahigue et al. 2022) and on the polychaete *Perinereis cultrifera* collected from the port of Tades and the Punic port of Carthage (Bouhedi et al. 2021). The following 2 studies have data from the two sub-regions: WMS and CEN. The effect of microplastic ingestion was studied in the fish *Serranus scriba* collected from 6 sites along the Tunisian coast (Zitouni et al. 2020) and on the seaworm *Hediste diversicolor* collected from 8 sites along the Tunisian coast (Missawi et al. 2020).

36. The distribution of most analyzed metals in *F. glaber* tissues varied significantly between sites, seasons, and organs. The highest levels were recorded at the polluted site during the warm period. Moreover, the digestive gland was found to accumulate greater concentrations of TM than the gills. The biomarkers (MDA, GSH, GPx, SOD, CAT) in gills were higher in the polluted site while MT was not affected. In the digestive gland, only CAT and MDA showed an increase activity in the polluted site.

37. Higher level of thiobarbituric acid were found in *P. cultrifera specimens* from polluted site. In addition, CAT, GST, SOD, glutathione and MT were enhanced and AChE activities decreased in *specimens from* the contaminated site compared to those from the reference (or less contaminated site).

38. Biomarkers of oxidative stress (MT, CAT, GST, MDA) and neurotoxicity (AChE) responses in *S. scriba* were dependent on site and on the size of the microplastic. High content of microplastic in the

gastrointestinal track increased MT levels and GST activity. CAT activity and MDA accumulation were positively related with the medium size class MP A significant negative correlation was found between AChE activity and the small size class of microplastic (MP). The study could not rule out some influence of other pollutants that may be present in some of the sites on biomarker response.

39. In the seaworm *Hediste diversicolor*, responses increased with increased microplastic tissue concentration, in particular CAT but also MDA. A decrease of GST activity was reported in the same sites. AChE was significantly inhibited indicating neurotoxicity.

3.1.2 CEN sub-region (Tunisia, Italy)

40. **Tunisia.** Seven studies were reviewed for Tunisia: 2 from the WMS (Section 3.1.1), 3 from the CEN (Section 3.1.2) and 2 with data from both the WMS and the CEN (Section 3.1.1). In the CEN, one mesocosm experiment was performed in *Mytilus spp*. exposed to sediment contaminated by PAH and TM collected from the Zarzis area (Ghribi et al. 2020), while the effects of hydrocarbons were studied in the mollusc *Ruditapes decussatus* collected from the southern Lagoon of Tunis (Mansour et al. 2021). The effect of TM on the mollusc *Patella caerulea* was studied in specimens collected from 4 sites in the CEN (Zaidi et al. 2022). Two studies with data from the two sub-regions: WMS and CEN were summarized in Section 3.1.1.

41. *Mytilus spp* exposed to contaminated sediments in a mesocosm experiment presented the highest values of the tested oxidative stress biomarkers (CAT, GST, GPx) and a significant inhibition of AChE activity in comparison with the unpolluted reference site.

42. Hydrocarbons were found to affect the biomarkers CAT, GR, SOD, MDA and AChE activities in *Ruditapes decussatus.*

43. SOD and GPx activities measured in *P. caerulea* were different among sites (higher in more affected stations), while CAT was similar on all four stations. MDA was inducted but no differences were found among the sites.

44. **Italy**. In the CEN, the effect of plastic ingestion was studies in the fish *Trachurus trachurus* collected for the Sicily straits (Chenet et al. 2021).

45. Vitellogenin was highly expressed in *T. trachurus* females as expected, there is also a significant expression of the VTG gene in 60% of the males analyzed, from both sampling sites. Moreover, females in Lampedusa island showed a lower expression of vitellogenin than in Mazara del Vallo (with one female sample, TT54, not expressing VTG at all). The endocrine disruption represented by the alteration of VTG expression in specimens observed in this work can be caused by microplastic ingestion, as well as by the interactions between the marine organisms and the wide variety of endocrine-disrupting chemicals possibly present in seawater.

3.1.3 ADR sub-region (Italy)

46. **Italy**. One study reported the effect of PAHs in the fish *Mullus barbatus* collected in the northern Adriatic (Frapiccini et al. 2020). The expressions of CAT and GST in *M. barbatus* were dependent on the season, lower in the winter and higher in the summer. SOD expression did not depend on the season. LPO was higher in the winter. CAT showed a significant negative correlation with total PAH concentrations, especially total LMW-PAH, in individuals collected during winter. Both GST and SOD did not show any significant correlation with PAH levels.

3.1.4 AEL sub-region (Egypt, Turkeye)

47. **Egypt.** One study was reviewed. The effect of TM was studied in the mussel *Ruditapes decussatus* collected from Alexandrian Port and Port Said (Gabr et al. 2020).The concentrations of metals

were higher in samples from the Alexandrian Port (Site I). Malondialdehyde (MDA) and SOD were higher in samples from Site I while GPx, Total protein and AChE were lower. The reported values in this study are considered as basic data to monitor of the anthropogenic influence on the coastal environment.

48. **Turkeye.** Two studies were reviewed for Turkeye: one from 2015 and one from 2022[6]. The effect of TM and pesticides was studied on the molluscs *Mytilus galloprovincialis* and *T. decussatus* collected from Homa Lagoon (Aegean Sea). The study showed marked differences on the biomarkers (CAT, SOD, GPx, and AChE) but the differences were mainly attributed to seasonal variations and to differences among the two species (Uluturhan et al. 2019). The effect of TM was also studied in the fish *M. barbatus, B. boops and T. trachurus* collected along the Turkish coast in the Levantine and the Aegean Seas. Correlations were found between CAT and MDA and some of the trace metals measured in the fish specimens.

3.2 Comparison to the 2017 MED QSR

49. In the 2017 MED QSR, the results were visualized in 3 figures, that included also the adopted Mediterranean BACs and EACs (Table 2). The figures depicted LMS-NRR (Neutral red retention) in mussel, AChE in mussel gills and digestive gland and MN in mussel haemocytes.

50. Due to lack of data reporting by the CPs, data were retrieved from scientific literature as explained above; however, for the present assessment of CI 18 the studies do not include the parameters assessed in the 2017 MED QSR in mussel. The only exception is Morroni et al., 2020 that measured LMS, AChE and MN in *M. galloprovincialis* but not in the same organs except for MN that was measured in haemocytes with a value of 0.3 permil in reference area and a maximal value of 1.3 permil. The maximal value is slightly higher than 1 permil, the MED BAC adopted in decision IG.23/6 (Table 2). Ghribi et al., 2020 and Uluturhan et al, 2019 reported AChE in haemolymph and hepatopancreas, respectively and not in gills.

4. Key findings

51. The following key points may be summarized as presented here-below.

52. Twenty four studies were retrieved from the scientific literature as follows: 4 studies from Algeria (WMS), 1 from Egypt (AEL), 5 from Italy (2 from WMS, 1 from ADR, 1 from CEN and one from FAO zone 37), 5 from Spain (WMS), 7 from Tunisia (2 from WMS, 2 from CEN and 3 with data from both the WMS and CEN), and 2 from Turkeye (AEL).

53. The sub-region most represented is the WMS, followed by the CEN. In the CEN all studies except one were performed in Tunisia. There was one study from the ADR and three in the AEL.

54. The monitoring species, *M. galloprovincialis and M. barbatus*, appeared in 5 and 4 studies, respectively. In addition, 10 fish species, 6 mollusc species and 2 polychaeta species were also studied.

55. Of the mandatory biomarkers as defined in in the DDs and DSs for IMAP CI-18, AChE appeared in 13 studies, MT in 5 studies (2 with molluscs, 2 with fish and one with a polychaete species), MN in 2 and LMS-NRTT in 1 study.

56. Data from studies cannot be compared to BAC and EACs values as agreed in Decisions IG.22/7 and IG.23/6 (COP 19 and COP 20) (Table 2) because they were not measured in the specific tissue of *M. galloprovincialis*.

57. The most common additional biomarkers measured in the reviewed studies were: CAT (15 studies), MDA (12 studies), GST (11 studies), SOD (9 studies), and GPx (8 studies).

58. The anthropogenic stressors identified were: Trace metals (10), Plastic/microplastic (8), non-specific (4), PAHs (3), Pesticides (2), hydrocarbons (1), anthropogenic items, and one study with desalination brine as a source.

---

[6] Submitted to Research Square, not peer reviewed by a scientific journal

59. Drivers and pressures reported in the studies, encompassed the whole range of them: domestic and industrial discharges, agricultural and riverine runoff, fisheries, harbor and marina utilization, maritime activities, tourism. Most of the studies described the environmental conditions at the sampling areas. The exemption was for microplastics, where the source was not determined, and microplastics were considered ubiquitous in the environment.

60. Most biomarkers studied showed a response to anthropogenic stressor. In the case of microplastics, the size of the microplastic also influenced the response.

61. Studies demonstrated that, in addition to anthropogenic stressors, biomarker responses were influenced also by seasonality, tissue analyzed, spawning status, and on species identity.

5. Measures towards achieving GES regardin IMAP CI 18

62. Based on the reviewed literature studies it is not possible to assess the environmental status of the Mediterranean Sea concerning CI-18 for the following reasons:

i) The biomarkers that were studied in the reviewed literature are not listed by IMAP and have no BACs or EACs, criteria needed to assess and classify areas as GES or non-GES. Three studies included the parameters listed by IMAP but not in the same organ or tissue.

ii) With no criteria, it is impossible to state that even if the study showed an effect on the biomarker, then the station is non-GES or at risk

iii) Most of the studies measured various biomarkers in the same station, with some showing an effect and others not. So, again, it is impossible to assess the station as in GES or non-GES

63. The following steps should be taken in order to enable the process of GES assessment for CI-18.

a) Data of relevant parameters (Table 1), following the DDs and DSs, should be reported by the CPs by the 31st of October 2022.

b) Once **sufficient** data are reported it will be possible to:

   i) Compare the reported data to adopted MedBACs and MedEACs (Table 2).
   ii) Apply the CHASE+7 methodology to classify each station into GES (high and good) or non-GES (moderate, poor and bad)
   iii) Aggregate stations to sub-areas, where possible, for their assessment as GES or non-GES.
   iv) Map the assessment results.

64. Therefore, for the preparation of the 2023 QSR, it is imperative that countries report current data on CI-18 based on DDs and DSs as specified in UNEP/MED WG. 533/10, Appendix V.

[7] UNEP/MED WG 533/Inf.3/Rev.1 Adjusted Background (Assessment) Concentrations (BC/BAC) for Common Indicator 17 and Upgraded Approach for Environmental Assessment Criteria (EAC) for IMAP Common Indicators 17, 18 and 20, and UNEP/MED WG 533/10, Appendix IV The pilot example for Marine Environment Assessment in the Areas with Insufficient Data: The Results of GES Assessment for IMAP Common Indicator 17 in the Levantine Sea Basin

Annex I. Extended summary of the studies reviewed for the present assessment of CI-18 (biomarkers) in the Mediterranean Sea

Twenty four studies were retrieved from the recent (since 2016) scientific literature for biomarkers in the Mediterranean Sea as follows: 4 studies from Algeria (WMS), 1 from Egypt (AEL), 5 from Italy (2 from WMS, 1 from ADR, 1 from CEN and one from FAO zone 37), 5 from Spain (WMS), 7 from Tunisia (2 from WMS, 2 from CEN and 3 with data from both the WMS and CEN), and 2 from Turkeye (AEL).

The reviewed studies were sorted by sub-regions and countries. WMS and CEN were grouped together because 3 studies from Tunisia addressed both sub-regions.

WMS (Algeria, Spain, Tunisia, Italy) and CEN sub-regions (Tunisia, Italy)

Algeria (WMS), mollusc (*Donax trunculus*), gonad, mantle, digestive gland, stressor (nonspecific), Biomarker: AChE, GST, MDA

Samples of *Donax trunculus* were collected in 4 seasons in 2016 from 2 impacted and one reference site in the Annaba Bay. The results showed a significant inhibition of AChE and induction of GST and MDA in individuals of Sidi Salem and Echatt as compared to El Battah with significant effects of both site and season. Indeed, the season effect was showed by an inhibition of AChE and an induction of GST and MDA more pronounced during summer and spring compared to the other seasons. In addition, the comparison between tissues revealed a more marked response in gonad than mantle and digestive gland (Amamra et al. 2019).

AChE- 0.1-1.5 mM/min /mg prot; - 0.05-0.9 mM/min /mg prot; MDA 1.0-15 mM/mg prot.

Algeria (WMS), fish (*Mullus barbatus*), blood, stressor (nonspecific), Biomarker: MNT, NRRT

Temporal and spatial variability of the biological responses of *Mullus barbatus* collected between autumn 2019 and summer 2020 from two impacted sites (Oran, Ghazaouet) and a control site (Kristel), along the Algerian west coast. Besides the condition indexes (gonadosomatic index GSI, hepatoso-matic index HSI and Fulton's condition factor K), the micronucleus test (MNT) and the neutral red retention time (NRRT) assay were measured in erythrocytes. The results revealed a significant correlation between the different fish condition indexes, in link with their reproduction cycle at the three sampling sites. Also, GSI, HSI and K showed significant differences between the sites ($p<0.05$), highlighting the fact that the worst environmental conditions occur at the polluted sites in comparison to the reference one. The study of the two biomarkers showed a significant increase ($p<0.05$) in the frequency of micronuclei (MN) occurrence in the summer period correlated with significantly ($p<0.05$) shorter NRRT. In addition, the erythrocytes of M. barbatus populations from the so-called polluted areas presented statistically ($p<0.05$) higher MN frequencies and shorter NRRT than those of the reference site (Kaddour et al. 2021)

MN in erythrocytes‰ Summer – Ref 16.4±1.6 ‰; Affected 25.2±1.6 ‰ and 24.3 ±1.6 ‰, winter, Ref 3.7 ±0.9 ‰; Affected 8.3 ±0.7 ‰ and 9.0±0.5 ‰
NRRT (min) in erythrocytes Summer – Ref 40.5±2.3 min; Affected 27.8±2.7 min and 29.1 ±0.6 min. winter -Ref 77.2 ±5.3 min; Affected 57.8 ±2.8 min and 57.6±2. 9 min

Algeria (WMS), mollusc (*Perna perna*), digestive glands and gills, stressor (nonspecific, TM), Biomarker: GST, CAT, MDA, AchE, GSH

Specimens of the brown mussel *Perna perna* were collected from a reference site in 2017 and transplanted to three sites in the Gulf of Annaba (north-eastern Algeria) for 12 weeks. The concentrations of Cu, Zn, Pb and Cd were measured sediments of all sites and in the mussels before and after the transplantation period. The biomarkers: GST, CAT, MDA, AchE, GSH were also measured in the digestive glands and gills of the moluscs before and after the transplantation. Cu and Pb increased in the mussels after 12 weeks, in accordance with the concentrations in the sediments. Cd and Zn concentrations did not reach significant levels of bioaccumulation at any of the three study sites. GSH decreased in the gills and digestive glands of the mussels transplanted to two of the sites affected by anthropogenic input while GST and CAT activities showed no significant variation. The MDA content in the mussel digestive glands, but not in the gills, increased significantly after the deployment period in the three caging sites, and were significantly

different among the 3 sites. AChE activity was significantly inhibited registered in the gills of mussels from the 3 sites and in the digestive glands from one site. The biomarker response results were associated with the metal accumulation index (Laouati et al. 2021).
CAT 60-80 umol/min mg.prot; GST 80-100 umol/min mg.prot; GSH 20-40 nmol/mg.prot; MDA 0.5-6 nmol/mg.prot; AChE 65-150 nmol/min mg.prot

Algeria (WMS), mollusc (*Patella rustica*), soft tissue, stressor (desalination brine discharge) Biomarkers: AChE , CAT, SOD, GR, GPx ,GST, LPO, genotoxicity.
The effect of desalination brine on the mollusc *Patella rustica* were examined in February 2016. *P. rustica* was sampled from four sites (3 affected and one reference) off the Bousfer desalination plant (Oran Bay, Algeria). A multibiomarker approach have been chosen for assessing of oxidative stress (GR, T-GPx, Se-GPx, CAT, SOD activities) biotransformation enzyme (GST activity), lipid peroxidation (LPO revealed by the MDA/TBARS rates), neurotoxicity (AChE activity) and genotoxicity (through CSP3-like activity) in the soft tissue. This ecotoxicological biomonitoring confirmed the negative impact of brine discharges of the desalination plant, with samples close to the discharge (site H) more affected by all the environmental disturbances than ones from the other sites. The responses of the antioxidant defense and biotransformation enzymes reached the highest levels in population of site H. This oxidative stress is also accompanied by genotoxic effect revealed by ADN and lipid damages (Benaissa et al. 2020).
Ranges of averages:
SOD 70-100 U/mg.prot; CAT 20-70 umol/min mg.prot; GPx 30-75 umol/min mg.prot; GST 1-1.4 umol/min mg.prot; GRd 25-80 nmol/ mg.prot; AChE 15-22 nmol/ mg.prot; MDA 0.25-0.4 nmol MDA/ mg.prot; CSP3 2-7 umol/min mg.prot;

Spain (WMS), mollusc (*Mytilus galloprovincialis*), gills, stressor (microplastic ingestion), Biomarkers: CAT, SOD, GRd, GPx, GST, MDA, ROS production
Microplastics were determined in the soft tissue of 84 specimens of *Mytilus galloprovincialis* that were transplanted into Integrated Multi-Trophic Aquaculture cages in Palma de Majorca and two control sites. Year of study not mentioned. Samples were analyzed at time 0, after 60 days and after 120 days of exposure. Biomarkers were determined in the gills of 84 additional speciemens. No effects of time were observed in CAT, SOD, and GRd activities, but they were significantly higher in specimens sampled from the cages than in specimens from the controls. GST activity did not change with time, and it increased significantly only in samples for the cages at T60. In T120 activity was higher in the cages only if compared to one of the control sites. GPx activity was modulated by both sampling site and time: higher activities in specimens from the cages at T120. MDA was higher in samples from the cages compared to the controls at T60 (Capo et al. 2021).

ROS production in the control at T60 was 4819 ± 1174 RLU min/mg of prot and 9912 ± 1728 RLU min/mg of prot in samples from aquaculture cages at T60. ROS production remain the same at T120 at the controls and decreased in the aquaculture cage.

SOD- 2.19-3.53 pkat/mg prot; CAT 99.5-203 mK/mg prot; GPx 31.0-60.4 nkat/mg prot, GRd 147-258 nkat/mg prot, GST 192-417 nkat/mg prot, MDA 15-25 mM/mg prot

Spain (WMS), mollusc (*Mytilus galloprovincialis*), soft tissue, stressor (anthropogenic particles, bisphenols and phthalates), Biomarkers: CAT, SOD, GRd, GPx, GST, ETS, GLY, CE, LPO, CARB, GSH
Same set up as Capo et al., 2021. Experiment conducted from May to September 2019. In order to evaluate the biochemical response of mussels towards anthropogenic particle ingestion and associated contaminants, detoxification (CEs, GST), metabolism (ETS, GLY), antioxidant (CAT, SOD, GRd, GPx, GSH), oxidative stress damage (LPO, CARB) related biomarkers were measured in the soft tissue of the mussels. Concentrations of phthalates and bisphenols in mussels' soft tissues did not show a sampling location trend. However, higher expressions were observed in the biomarkers CAT, SOD, GPx and LPO

in specimens from the aquaculture cages. Those could be triggered by the presence of bisphenol but also by other possible contaminant inputs from the aquaculture (Rios-Fuster et al. 2022).
Starting values
CAT 0.5 ukat/g FW; SOD 0.12 ukat/g FW; GRd 0.0045 ukat/g FW; GPx 0.0022 ukat/g FW; GSH 1400 umol/g FW; ETS 1.0 ukat/g FW; CEs 16 ukat/g FW; GSTs 0.0038 ukat/g FW.

Spain (WMS), Fish (*Sparus aurata*), blood, plasma and liver, stressor (microplastic ingestion, plasticizers), Biomarkers: CAT, SOD, GPx, GRd, MPO, GST, MDA, EROD, BFCOD, CE
Same set up as Capo et al, 2021 and Rios-Fuster et al., 2022. However, fish were kept only in the aquaculture site and not on the reference sites. Sampling in May, July and September 2019. 45 specimens in total.
Fish sampled at T_{60} presented the highest MPs intake and plasticizers accumulated in muscle over time, but with a different pattern according to type: bisphenols and phthalates. This indicates MPs ingestion induces a differential tissue response in *S. aurata*. Similarly, stress biomarkers presented a differential response throughout the study, depending on the analysed tissue. In the case of oxidative damage markers, for malondialdehyde (MDA) an increase throughout the study was observed both in liver and blood cells but with a progressive decrease in plasma. In the case of phase I detoxifying enzyme activities in liver, 7-ethoxyresorufin O-deethylase (EROD), 7-benzyloxy-4-[trifluoromethyl]-coumarin-O-debenzyloxylase (BFCOD) and carboxylesterases (CE), showed a comparable decrease at T_{60} with a slight recovery at T_{120}. In contrast, glutathione-*S*-transferase (GST) activity was significantly enhanced at T_{60} compared to the other sampling stages. In conclusion, MPs ingestion occurs in aquaculture reared seabream where potentially associated plasticizers accumulate in the muscle and both could be responsible for plasma and liver oxidative stress damage and alterations on detoxifying biomarkers responses (Capó et al. 2022).

Table 2
Antioxidant and inflammatory enzymes determined in the three analysed tissues of *Sparus aurata* individuals.

	T0	T60	T120
Liver			
CAT (mK/mg prot)	550 ± 71.8	1015 ± 67.9*	908 ± 103*
SOD (pkat/mg prot)	19.4 ± 1.36	18.4 ± 2.32	22.4 ± 3.14
GPx (nK/mg prot)	38.3 ± 2.94	33.3 ± 2.15	43.2 ± 4.35#
GRd (nkat/mg prot)	23.9 ± 4.27	28.5 ± 3.75	30.7 ± 4.42
MPO (nkat/mg prot	15.8 ± 5.30	13.2 ± 3.85	20.1 ± 4.27
Plasma			
CAT (mK/mg prot)	237 ± 36.5	364 ± 52.4*	283 ± 56.1
SOD (pkat/mg prot)	23.4 ± 3.15	33.7 ± 4.24	32.9 ± 4.23
MPO (nkat/mg prot	14.8 ± 3.3	30.5 ± 10.4	75.9 ± 18.5*#
Blood cells			
CAT (mK/mg prot)	15.4 ± 1.56	18.2 ± 2.82	15.5 ± 1.16
SOD (pkat/mg prot)	18.8 ± 2.27	14.5 ± 1.80	7.30 ± 1.26*#
MPO (nkat/mg prot	14.0 ± 1.19	13.6 ± 0.64	13.2 ± 0.42

Statistical analysis: one-way ANOVA. $p < 0.05$. * indicates differences respect to T0, # reveals differences respect to T60.

Table 3
Detoxifying enzymes activity in *Sparus aurata* liver throughout the study period.

	T0	T60	T120
CE (nmol/min/mg protein)	446 ± 20.4	343 ± 15.5*	484 ± 27.4#
GST (nmol/min/mg protein)	371 ± 12.1	633 ± 18.4*	483 ± 22.3*#
EROD (pmol/min/mg protein)	1.48 ± 0.20	0.22 ± 0.02*	0.30 ± 0.04*
BFCOD (pmol/min/mg protein)	0.21 ± 0.03	0.03 ± 0.005*	0.07 ± 0.009*

Statistical analysis: one-way ANOVA. $p < 0.05$. * indicates differences respect to T0, # reveals differences respect to T60.

Table 4
Oxidative damage markers in liver, plasma and erythrocytes measured in *Sparus aurata* from aquaculture facilities.

	T0	T60	T120
MDA liver (nmols/mg of prot)	341 ± 44.5	471 ± 35.7*	681 ± 50.9*#
Carbonyls liver (nmols/mg of prot)	119 ± 8.53	124 ± 11.4	114 ± 12.2
MDA Plasma (nmols/mg of prot)	2994 ± 376	2362 ± 371	1792 ± 285*
MDA erythrocytes (nmols/mg of prot)	464 ± 27.9	515 ± 30.9	541 ± 20.5*

Statistical analysis: one-way ANOVA. $p > 0.05$. * indicates differences respect to T0, # reveals differences respect to T60.

Spain (WMS), fish (*Seriola dumerili*), liver, stressor (microplastic ingestion), Biomarkers: SOD, CAT, GST, EROD, MDA
From September to November 2020, 52 specimens of *S. dumerili* were collected around the Pityusic Islands, (Eivissa and Formentera; Balearic Islands). Microplastics ingestion was measured in the entire digestive tract while the following biomarkers were measured in the liver: SOD and CAT activities, as antioxidant enzymes, GST and EROD activities, as detoxification biomarkers, and MDA levels as marker of oxidative damage to lipids were determined. SOD and CAT were significantly increased (12% and 20.4% respectively) in fish with higher MPs load, while no significant differences were observed for MDA, although they were higher in the group with higher MPs load. GST activity was significantly higher in fish with high MPs load by 44%. The activity of EROD enzyme remained similar between both groups (Solomando et al. 2022).

SOD- 0.4-0.5 pkat/mg prot; CAT 30-40 mK/mg prot; MDA 1.0-2.3 mM/mg prot.

Spain, fish (*Engraulis encrasicolus*), muscle and liver, stressor (anthropogenic items ingestion) Biomarkers: AChE, LDH, CS, CE, CAT, GST, EROD

European anchovy (*Engraulis encrasicolus*) specimens were collected in 2019 at three areas off Catalonia (Spain): Barcelona, Tarragona and Blanes. The biomarkers measured in the muscle tissue were acetylcholinesterase (AChE), lactate dehydrogenase(LDH), and citrate synthase (CS), and the biomarkers measured in the liver were Glutathione-S-transferase (GST), catalase (CAT), carboxylesterase (CE), and ethoxyresorufin-O-deethylase (EROD). Anthropogenic items were counted in the digestive tracts of the specimens.
Significant differences among sites were found in the following cases: Barcelona AChE, CAT and GST were lower than in Blanes and Tarragona; Terragona LDH, CE and EROD were higher than in the other two locations; Blanes CS was higher than in Tarragona. The differences among localities could not be clearly correlated with any potential stressors (AIs, parasites) nor with fish size, which suggests that this variability could be linked to a high levels of variation among individuals (Rodríguez-Romeu et al., 2022).
Average values

		Barcelona	Blanes	Tarragona
AChE	nmol/min mg.prot	21.3	33.18	28.37
LDH	nmol/min mg.prot	5915.18	5917.09	6840.94
CS	nmol/min mg.prot	77.09	81.43	75.74
CbE	nmol/min mg.prot	46.68	50.11	76.37
CAT	umol/min mg.prot	311.5	456.82	451.7
GST	nmol/min mg.prot	163.81	236.95	229.8
EROD	pmol/min mg.prot	0.79	0.86	1.24

Italy (WMS) (western Calabria, Tyrhenian), fish (*Parablennius sanguinolentus*), brain and blood, stressor (pesticides), Biomarker: AChE, BChE
A total of 40 specimens of the fish of *Parablennius Sanguinolentus* collected from the port of Bagnara Calabra on the western Calabrian coast of Italy and from a reference site, Jancuia Cove to investigate the presence of pesticides in the coastal marine environment. No sampling date reported. AChE activity in the brain and BChE activity in blood were significantly inhibited in specimens from the port, by 23.5 and 72.0%, respectively. The esterase inhibition was primarily due to carbamate and organophosphorus insecticides presence (Parrino et al. 2020).

AChE 0.39 vs 0.51 umol substrate/min/g brain and BChE 0.28 vs 1.00 umol substrate/min/ml serum

Italy (WMS), stressor (PAHs, trace metals) mollusc (*Mytilus galloprovincialis*) and fish (*Mullus barbatus, Pagellus erythrinus* and *Diplodus vulgaris)*, Various organs (see below), Biomarkers: LMS, MT, AChE, EROD, MN, Aromatic metabolites
The mussel, *Mytilus galloprovincialis*, and fish, *Mullus barbatus*, *Pagellus erythrinus* and *Diplodus vulgaris*, were sampled in December 2017 from different stations at the Bay of Pozzuoli, within the Gulf of Naples. Bioaccumulation of trace elements and PAHs was measured on the whole soft tissues of mussel and in the liver tissues of fish. Sediments were characterized for OM, metals, hydrocarbons, PAHs, PCBs, organotin compounds, organochlorine pesticides, dioxin (PCDDs) and furan (PCDFs). Lysosomal membrane stability was measured in mussels hemocytes while metallothioneins (MTs) were determined in digestive glands. Acetylcholinesterase enzymatic activity (AChE) was assayed in mussels haemolymph and fish brain. Ethoxyresorufin O-deethylase (EROD) was determined in individual fish livers. Aromatic metabolites were assessed in fish bile. Micronuclei (MN) frequency was microscopically measured in mussels hemocytes and fish gills.

Effect of pollution varied by species and biomarkers. For example, in mussel, there was a decreased lysosomal membrane stability (LMS) and increased MN at two sites compared to organisms from other areas while no variations were observed for the AChE in haemolymph, nor for MT in digestive gland of mussels from various sites. AChE activity was not affected in *M. barbatus* sampled in the industrial area while a decrease of this biomarker was observed in *P. erythrinus* and *D. vulgaris*. The EROD enzymatic activity was significantly induced in *M. barbatus* and *P. erythrinus* sampled in the industrial area compared to specimens from the reference site, while the cytochrome P450 biotransformation pathway was unaffected in *D. vulgaris*. At the same time, all the fish species exhibited higher levels of aromatic metabolites, particularly B[a]P-like and pyrene-like, in organisms sampled in the industrial compared to reference area. MN increased in gills of *M. barbatus* from the industrial area.

This study presents an integrative approach that should be further studied towards the application of biomarkers in GES determination. It takes into account concentrations in sediments and biota, in addition to biomarker responses and analyze all statistically. “The integrative approach of this study showed that toxicity of sediments, measured through standardized batteries of ecotoxicological bioassays, was often not in accordance with chemical characterization. In fact, despite an evident contamination, sediments from the area of Bagnoli inlet as well as those from the southern and the northern stations, showed low levels of acute toxicity; only some samples, particularly those collected close to the industrial plant, revealed evidence of a major acute toxicity, but the overall HQ for ecotoxicological bioassays resulted as “Moderate” in these sub-areas. Despite the lack of an elevated acute toxicity, the bioavailability of contaminants was evident in terms of bioaccumulation of PAHs, and of HMW hydrocarbons, both in mussels and fish from the industrialized area” (Morroni et al. 2020) .

Table S2a. Results of biomarkers analyzed in mussels sampled. Data expressed as mean ± standard deviation (n=5). Asterisks (*) indicate significant differences between areas and reference area ($p < 0.05$), as determined by t-student test.

		PGT (Area 1;9)	PGP (Area 1;7)	P2 (Area 2;10)	BB (Area 6;9;11)	BNF (Area 8)	BRM (Reference area)	*Sig.*
Lysosomal membranes stability	(min)	53.1 ± 8.0		55.2 ± 19.8	76.8 ± 5.0	61.8 ± 15.5	73.5 ± 28.9	*n.s.*
Acetylcholinesterase enzyme activity	(nmol/min/mg prt)	88.1 ± 10.1	94.5 ± 21.7	99.9 ± 23.8	82.7 ± 19.4	99.1 ± 30.5	105.1 ± 25.1	*n.s.*
Metallothioneins	(nmol eq.(G)SH/mg prt)	3.2 ± 1.7	1.7 ± 0.5	2.4 ± 0.6	2.00 ± 1.2	2.8 ± 0.5	2.3 ± 0.7	*n.s.*
Micronuclei frequency	(‰)	1.3 ± 0.3	0.8 ± 0.1	0.5 ± 0.3	0.4 ± 0.2	0.3 ± 0.3	0.3 ± 0.1	*n.s.*

n.s. = not significant

Table S2b. Results of biomarkers analyzed in fish species. Data expressed as mean ± standard deviation (n=5). Asterisks (*) indicate significant differences between areas and reference area ($p < 0.05$), as determined by t-student test.

		Mullus barbatus			*Diplodus vulgaris*			*Pagellus sp.*		
		OUTSIN (Reference area)	INSIN (Area 3;4;5;8)	*Sig.*	OUTSIN (Reference area)	INSIN (Area 3;4;5;8)	*Sig.*	OUTSIN (Reference area)	INSIN (Area 3;4;5;8)	*S*
Acetylcholinesterase enzyme activity	(nmol/min/mg prt)	63.3 ± 14.3	64.9 ± 13.1	*n.s.*	85.5 ± 10.6	61.2 ± 12.9	*n.s.*	91.4 ± 16.3	66.5 ± 11.8	
EROD enzyme activity	(pmol/min/mg prt)	85.3 ± 23.4	162.6 ± 47	$p < 0.05$	7.3 ± 4.1	4.3 ± 2.6	*n.s.*	27.2 ± 20.1	67.7 ± 15.3	*p*
Pyrene-like metabolites	(μg/μmol biliverdina)	0.4 ± 0.3	6.7 ± 2.3	$p < 0.01$	1.3 ± 1.0	5.4 ± 5.9	*n.s.*	0.1 ± 0.1	3.8 ± 2.7	*p*
B[a]P-like metabolites	(μg/μmol biliverdina)	3.6 ± 1.7	20.2 ± 4.7	$p < 0.01$	7.1 ± 3.9	10.5 ± 7.8	*n.s.*	0.6 ± 0.5	11.8 ± 3.6	*p*
Naphtalene-like metabolites	(mg/μmol biliverdina)	9.0 ± 5.9	8.6 ± 1.8	*n.s.*	3.9 ± 2.0	8.2 ± 3.4	*n.s.*	1.1 ± 0.6	6.5 ± 4.0	*p*
Micronuclei frequency	(‰)	6.3 ± 0.5	10.6 ± 1.8	$p < 0.05$	4.8 ± 0.6	5.3 ± 1.1	*n.s.*	5.5 ± 0.8	6.3 ± 0.8	

n.s. = not significant

Italy (CEN) (Sicily straits), fish (*Trachurus trachurus*), liver, stressor (plastic ingestion), Biomarker: VTG

A total of 92 specimens of *Trachurus trachurus* (TT) were collected from two sampling sites in the Strait of Sicily between April and May 2018. Plastic debris in the gastrointestinal tract was quantified and characterized. A potential link between the occurrence of plastics and adverse effects on TT health status

was evaluated by investigating the expression of vitellogenin (VTG) in liver, a lipoprotein produced in female fish and used as a biomarker for endocrine disruption. Vitellogenin was highly expressed in TT females as expected, there is also a significant expression of the VTG gene in 60% of the TT males analysed, from both sampling sites. Moreover, females in LMP showed a lower expression of vitellogenin than in MDV (with one female sample, TT54, not expressing VTG at all). The endocrine disruption represented by the alteration of VTG expression in TT specimens observed in this work can be caused by microplastic ingestion, as well as by the interactions between the marine organisms and the wide variety of endocrine-disrupting chemicals possibly present in seawater (Chenet et al. 2021).
Males 9-17, females 25-650 units of fold expression

Italy (Area 37 – did not specify sub-area), Fish (*Lepidopus caudatus*), Liver and gonads, stressor (microplastic and TM), Biomarkers: VGT, MT (immunohistochemical examination)
Impact of microplastic, plastic additives and heavy metals contamination was examined on the commercial fish species Lepidopus caudatus. No sampling date was given and the area (FAO area 37) includes the whole Mediterranean Sea.
Immunohistochemical analysis for anti-metallothionein 1 antibody showed a strong positivity of liver cells, both in females and males in all 20 the samples analyzed, showing a strong stress that activated a cell detoxification system. The immunohistochemical analysis for the anti-vitellogenin antibody showed in females a strong positivity both in the liver cells, and in the gonads, as expected. The analysis of the liver and gonadal preparations of the male specimens was found to be always negative except for one specimen (Salvaggio et al. 2019).

Tunisia (WMS), mollusc (*Flexopecten glaber*), Gills and digestive glands, stressor (nonspecific, TM), Biomarkers: MDA, MT, GSH, GPx, SOD, CAT

Specimens of the smooth scallop *Flexopecten glaber* were collected in Sept 2020 (warm season) and Feb 2021 (cold season) from two sites in the Bizerte Lagoon (Tunisia): Site S1, less contaminated located at an exchange point between the lagoon of Bizerte and the Mediterranean Sea, and site S2 situated near Menzel Abderrahmen locality, contaminated by inputs from the surrounded industrial manufactories and urban agglomerations.

TM and biomarkers (malondialdehyde (MDA), metallothioneins (MT), reduced glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT)) were determined in the gills and digestive gland across seasons (warm and cold) and sites (S1 and S2). The distribution of almost all analyzed metals in *F. glaber* tissues varied significantly between sites, seasons, and organs. The highest levels were recorded at S2 during the warm period. Moreover, the digestive gland was found to accumulate greater concentrations of HM than the gills. Marked spatio-temporal variations were also observed for oxidative stress biomarkers, mainly in the gills, while the digestive gland seems to be rather sensitive to seasonal variability. Particularly, we noticed that among the used biomarkers, MT did not show significant variations in the two tested organs across seasons and sites. From the obtained results, *F. glaber* appears as a sensitive organism to anthropogenic metal contamination and can be proposed as a promising bioindicator species for marine pollution (Telahigue et al. 2022).

Gills (average values warm season) MDA 12.5 vs 20 nmol/ml mg; GSH 33 vs 40 umol/mg.prot; MT about 6.8 umol GSH/g tissue; GPx 40 vs 45 nmol GSH/min mg.prot, SOD 38 vs 41 U/ mg.prot, CAT 32 vs 37 umol/min mg.prot

Digestive gland (average values warm season) MDA 35 vs 42 nmol/ml mg; GSH 45 vs 52 umol/mg.prot; MT about 8.8 umol GSH/g tissue; GPx 85 vs 95 nmol GSH/min mg.prot, SOD 52 vs 57 U/ mg.prot, CAT 55 vs 65 umol/min mg.prot

Tunisia (WMS), polychaete (*Perinereis cultrifera*), whole body, stressor (TM), Biomarkers: TBARS, AChE, GST, CAT, MT, RedGSH

Biomarker response to TM pollution was examined on the polychaete *Perinereis cultrifera* collected from two Tunisian ports (Rades, S1 (higher metal concentrations); and the Punic port of Carthage, S2). No sampling date given. The results showed a higher level of thiobarbituric acid in *specimens* from S1 than from S2. In addition, catalase, glutathione-S-transferase, superoxide dismutase, glutathione and metallothionein) was enhanced and AChE activities decreased in *specimens from* in S1 compared to those from S2 (Bouhedi et al. 2021)

Table 3. – Seasonal variation in the oxidative stress biomarkers in *P. cultrifera* tissues collected from the port of Rades (S1) and the Punic port of Carthage (S2).

Parameters	Sites	Spring	Summer	Autumn	Winter
TBARS	S1	2.39±0.12	5.01±0.14###	1.71±0.26	1.79±0.41
	S2	1.58±0.31*	3.63±0.32*	1.02±0.13	1.33±0.46
AChE	S1	29.39±2.90	17.55±1.91	18.58±1.99	52.78±2.53###
	S2	36.19±1.16	23.56±3.1*	19.32±1.97	57.50±2.77
MTs	S1	0.07±0.00	0.14±0.01##	0.07±0.00	0.04±0.00
	S2	0.05±0.00***	0.11±0.00***	0.06±0.00*	0.04±0.00
GSH	S1	0.40±0.05	0.55±0.09###	0.32±0.08	0.17±0.042
	S2	0.24±0.02***	0.44±0.06*	0.28±0.03	0.15±0.015
SOD	S1	5.12±0.87	6.93±0.56	3.27±0.46	1.90±0.352###
	S2	2.61±0.57***	6.14±0.64*	2.76±0.35*	1.67±0.184
CAT	S1	53.73±2.66	101.90±3.78###	46.80±9.49	41.49±1.05
	S2	49.10±9.02*	83.17±6.78**	33.71±8.60*	30.16±2.43*
GST	S1	57.58±2.82	190.30±6.09###	33.49±1.45	41.41±1.69
	S2	48.98±3.85	138.93±4.58***	29.12±8.92	21.83±9.95

The results are represented as mean ± sd (n=20 individuals).
The statistical differences between sampling sites are represented at 5%: *p<0.05; **p<0.01; ***p<0.001 using one-way ANOVA.
The statistical differences between seasons are represented at 5%: #p<0.05; ##p<0.01; ###p<0.001 using one-way ANOVA.
TBARS, thiobarbituric acid; AChE, acetylcholinesterase; MTs, metallothionein; GSH, glutathione; SOD, superoxide dismutase; CAT, catalase; GST, glutathione-S-transferase.

Tunisia (CEN), mollusc (Mytilus spp), hemolymph, gills, and digestive gland, stressor (nonspecific (PAH and metals) Biomarkers: CAT, GPx, GST, AChE

This is actually a mesocosm experiment where *Mytilus spp.* collected in Portugal in October 2017 were exposed for 28 days to sediments from 3 sites in Tunisia (Zarzis area) and from a reference site in Portugal. The study was conducted to assess the chronic biological effects of potentially contaminated sediments on the marine mussel species (*Mytilus* spp.). Biomarkers involving oxidative stress and neurotoxicity were quantified in hemolymph, gills, and digestive gland tissues of *Mytilus* spp. following the chronic exposure to the distinct sediments. Organisms exposed to sediments collected at the Zarzis area presented the highest values of the tested oxidative stress biomarkers and a significant inhibition of AChE activity in comparison with the unpolluted reference site(Ghribi et al. 2020).

Reference Digestive gland- CAT 20 umol/min mg.prot, GST 0.3 mmol/min mg.prot, TGPx 20 nmol/min mg.prot; Gills TGPx 25 nmol/min mg.prot, Hemolymph AChE 140 nmol/min mg.prot

Affected Digestive gland- CAT 40-60 umol/min mg.prot, GST 0.6-0.8 mmol/min mg.prot, TGPx 30-35 nmol/min mg.prot; Gills TGPx 60-70 nmol/min mg.prot, Hemolymph AChE 100-120 nmol/min mg.prot

Tunisia (CEN), mollusc (*Ruditapes decussatus*), soft tissue, stressor (hydrocarbons), Biomarkers: CAT, SOD, GR, MDA and AChE.
Carpet shellclams (*Ruditapes decussatus*) and sediments were collected in February 2016 from the Southern Lagoon of Tunis, possible affected by hydrocarbons. The biomarkers CAT, GR, SOD, MDA and AChE activities were measured in the soft Tissue. The study found that hydrocarbons affected the biomarkers activities (Mansour et al. 2021)

The ranges found were:
CAT- 50-130 umol/ min mg.prot; SOD- 70-120 U/ mg.prot, GR- 70-120 nmol/ min mg.prot;
MDA- 0.9-1.2 nmol / mg.prot ; AchE 8-14 nmol/ min mg.prot

Tunisia (2 stations WMS, 4 stations CEN), fish (*Serranus scriba*). gastrointestinal tract, stressor (microplastic ingestion), Biomarkers: CAT, GST, MDA, AChE, MT
From July to September 2018, 240 adult specimens of *Serranus scriba* were sampled from 6 sites along the Tunisian coast. 20 pools (made of 2 fishes each) were analyzed. Biomarkers of oxidative stress (MT, CAT, GST, MDA) and neurotoxicity (AChE) were measured in the gastrointestinal tract. Biomarker responses of oxidative stress were dependent on site and on the size of the microplastic. Specimens from areas with high microplastic (MP) content in the gastrointestinal tract exhibited high MT levels and GST activity. CAT activity and MDA accumulation were positively related with the medium size class MP in fish from BC. The results support the hypothesis that MPs considered a potential vector for the transmission of adsorbed environmental chemicals to marine organisms. A significant negative correlation was found between AChE activity and the small size class of microplastic (MP), suggesting a possible contamination of MP by ACHE inhibitors. The study could not ruled out some influence of other pollutants that may be present in some of the sites on biomarker response (Zitouni et al. 2020).

CAT- 87-535 umol/ min mg.prot; GST 150-262 umol/ min mg., MDA- 0.5-2.4 umol MDA/ mg.prot ;
AchE 50-228 umol/ min mg.; MT ug/ mg.prot

Tunisia (2 stations WMS, 6 stations CEN), seaworm (*Hediste diversicolor*), Gut-cleaned seaworms, stressor (microplastic) Biomarkers: CAT, GST, MDA, AChE.
Microplastics were determined in sediment and in the seaworm (*Hediste diversicolor)* from eight sites from the Tunisian coasts, affected by different anthropogenic stresses in June 2018. Biomarkers (Catalase, Glutathione-S-Transferase, Malondialdehyde and Acetylcholinesterase) were measured in gut cleaned specimens collected at the same time as the sediments. Results revealed a significant variation among sites in the parameters associated with oxidative stress. Thus, size abundance of microplastics in seaworms was mainly correlated with oxidative stress biomarkers. Biomarkers responses were site-dependent and low in sites less impacted by plastic particles. Responses increased with increased microplastic tissue concentration, in particular CAT but also MDA. However, a adecrease of GST activity was reported in the same sites. AChE was significantly inhibited indicating neurotoxicity (Missawi et al. 2020).
Although no reference site was sampled, laboratory studies provided the mean data for reference as : 72 umol/ min mg protein, 25 nmol / min mg protein, 20 nmol / min mg protein and 0.4 nmol / min mg protein respectively for CAT, GST, AChE and MDA. In the study – CAT 100-500 umol/ min mg protein, GST 20-160 nmol / min mg protein, MDA 0.3-0.9 nmol/ g tissue and AChE 10-90 nmol / min mg protein.

Tunisia (4 stations CEN), mollusc (*Patella caerulea*), soft tissue, stressor (trace metals) Biomarkers: CAT, SOD, GPx, GST, MDA
Concentrations of six trace metals (cadmium, copper, iron, lead, nickel, and zinc) were measured in surface seawater and in *Patella caerulea* individuals collected in 2018 from four coastal stations on the Tunisian coast with different levels of metal contamination. Trace metal contamination was identified in particular in the Gulf of Gabes. SOD and GPx activities were different among sites, while CAT was similar on all four stations. MDA was inducted but no differences were found among the sites (Zaidi et al. 2022).

ADR sub-region (Italy)

Italy (ADR), fish (*Mullus barbatus*), muscle, stressor (PAHs), Biomarker: CAT, SOD, GST, LPO
A total of 36 sexually mature but inactive red mullet females were sampled in the Northern Adriatic Sea during the winter and summer 2019, before and after the spawning period, respectively. Sum of High MW PAHs were higher in the winter in the muscle tissue. The biomarkers were not measured directly but by proxy, using gene expression profiles of antioxidant enzymes. The expressions of CAT and GST were dependent on the season, lower in the winter and higher in the summer. SOD expression did not depend on the season.LPO was higher in the winter. CAT showed a significant negative correlation with total PAH concentrations, especially total LMW-PAH, in individuals collected during winter. Both GST and SOD did not show any significant correlation with PAH levels (Frapiccini et al. 2021) .

AEL sub-region (Egypt, Turkeye)

Egypt (AEL), mollusc (*Ruditapes decussatus*), soft tissue, stressor (heavy metals) Biomarkers: MDA, SOD, GPx, AChE.

Heavy metals (Cd, Cu, Pb, Mn, and Zn) were measured in the soft tissues of *Ruditapes decussatus* collected from December 2018 to February 2019 from Alexandrian Port and Port Said, Egypt. The biomarkers: MDA, SOD, GPx, AChE were measured as well in the soft tissues of the specimens. The concentrations of metals were higher in samples from the Alexandrian Port (Site I). Malondialdehyde (MDA) and SOD were higher in samples from Site I while GPx, Total protein and AChE were lower. The reported values in this study are considered as basic data to monitor of the anthropogenic influence on the coastal environment (Gabr et al. 2020).
Averages in site I and II, respectively for MDA 12.9 vs 9.7 U/g (umol/ min g); GPx 10.6 vs 11.6 U/g (umol/ min g), SOD 1.8 vs 1.1(umol/ min g) ; Total protein 5.5 vs 6.5 mg/g and AChE 446 vs 468 U/l (umol/ min l).

Turkeye (AEL) (Aegean), mollusc (*Mytilus galloprovincialis* and *T. decussatus*), Hepatopancreas and soft tissue, stressor (metal, pesticides), Biomarkers: CAT, SOD, GPx and AChE.
Biomarkers (CAT, SOD, GPx, and AChE) were investigated in hepatopancreas and soft tissues of mussels (Mytilus galloprovincialis) and clams (Tapes decussatus) in response to metal and pesticide pollutions in Homa Lagoon. Mussel and clam samples were seasonally collected in January, May, August and November 2015. The study showed marked differences on the biomarkers (CAT, SOD, GPx, and AChE) but the differences were mainly attributed to seasonal variations and to differences among the two species. (Uluturhan et al. 2019).

M. galloprovincialis CAT 0.1-0.7 U/mg.prot; AChE 1-2.7 mU/ mg.prot; SOD 20-70 mU/ mg.prot, GPx 5-16 mU/ mg.prot
T. decussatus CAT 0.1-0.3 U/mg.prot; AChE 1-4 mU/ mg.prot; SOD 20-40 mU/ mg.prot, GPx 8-10 mU/ mg.prot

Turkeye, fish (*Mullus barbatus, Boops boops, Trachurus trachurus*), muscle and liver, stressor (TM) Biomarkers: CAT, MDA

Mullus barbatus, Boops boops and Trachurus trachurus specimens were collected in January-February 2021 along the Turkish coastal including Aegean Sea, Marmara Sea, and the Levantine Basin. The oxidative stress biomarkers (catalase-CAT, Malondialdehyde-MDA) were measured in muscle and liver tissues to check for possible impact of trace metals toxicity. A significant positive correlation between Fe, Cu, Cr and MDA levels and negative correlation between MDA and CAT in the muscle. Similarly, MDA levels were found to be positively correlated with Al, Cr, Cd and CAT levels were found to be negatively correlated with Cd, Al and MDA (Dogan et al., 2022).

Mean MDA and CAT activity of all studies fish varied between 1.40–13.70 nmol/ml, 2.05–10.10 U/g protein in muscle and 3.90-88.95 nmol/ml, 2.05-5.00-18.20 U/g protein in liver, respectively.

Annex II.
References

Amamra, F., Sifi, K., Kaouachi, N. and Soltani, N. (2019) Evaluation of the impact of pollution in the gulf of Annaba (Algeria) by measurement of environmental stress biomarkers in an edible mollusk bivalve Donax trunculus. Fresenius Environmental Bulletin 28(2), 908-915.

Benaissa, M., Rouane-Hacene, O., Boutiba, Z., Habib, D., Guibbolini-Sabatier, M.E. and Risso-De Faverney, C. (2020) Ecotoxicological effects assessment of brine discharge from desalination reverse osmosis plant in Algeria (South Western Mediterranean). Regional Studies in Marine Science 39, 101407.

Bouhedi, M., Antit, M., Chaibi, M., Perrein-Ettajani, H., Gillet, P. and Azzouna, A. (2021) Assessment of trace element accumulation on the Tunisian coasts using biochemical biomarkers in Perinereis cultrifera. Scientia Marina 85(2), 91-102.

Capó, X., Alomar, C., Compa, M., Sole, M., Sanahuja, I., Soliz Rojas, D.L., González, G.P., Garcinuño Martínez, R.M. and Deudero, S. (2022) Quantification of differential tissue biomarker responses to microplastic ingestion and plasticizer bioaccumulation in aquaculture reared sea bream Sparus aurata. Environmental Research 211, 113063.

Capo, X., Rubio, M., Solomando, A., Alomar, C., Compa, M., Sureda, A. and Deudero, S. (2021) Microplastic intake and enzymatic responses in Mytilus galloprovincialis reared at the vicinities of an aquaculture station. Chemosphere 280, 130575.

Chenet, T., Mancia, A., Bono, G., Falsone, F., Scannella, D., Vaccaro, C., Baldi, A., Catani, M., Cavazzini, A. and Pasti, L. (2021) Plastic ingestion by Atlantic horse mackerel (Trachurus trachurus) from central Mediterranean Sea: A potential cause for endocrine disruption. Environmental Pollution 284, 117449.

Doğan, S., E. Kiliç, E. Uğurlu and Ö. Duysak (2022). "Investigation of Metal Toxicity Response and Health Risk Assessment of Commonly Consumed Marine Fish Species along the Turkish coast. Submitted to Research Square, not peer reviewed by a scientific journal.

Frapiccini, E., Panfili, M., Guicciardi, S., Santojanni, A., Marini, M., Truzzi, C. and Annibaldi, A. (2020) Effects of biological factors and seasonality on the level of polycyclic aromatic hydrocarbons in red mullet (Mullus barbatus). Environmental Pollution 258, 113742.

Gabr, G.A.E.-F., Masood, M.F., Radwan, E.H., Radwan, K.H. and Ghoenim, A.Z. (2020) Potential Effects of Heavy Metals Bioaccumulation on Oxidative stress Enzymes of Mediterranean clam Ruditapes decussatus. Catrina: The International Journal of Environmental Sciences 21(1), 75-82.

Ghribi, R., Correia, A.T., Elleuch, B. and Nunes, B. (2020) Effects of chronic exposure to sediments from the Zarzis area, Gulf of Gabes, measured in the mussel (Mytilus spp.): a multi-biomarker approach involving oxidative stress and neurotoxicity. Soil and Sediment Contamination: An International Journal 29(7), 744-769.

Kaddour, A., Belhoucine, F. and Alioua, A. (2021) Integrated use of condition indexes, genotoxic and cytotoxic biomarkers for assessing pollution effects in fish (Mullus barbatus L., 1758) on the West coast of Algeria. South Asian Journal of Experimental Biology 11(3), 287-299.

Laouati, I., Rouane-Hacene, O., Derbal, F. and Ouali, K. (2021) The mussel caging approach in the assessment of trace metal contamination in southern Mediterranean coastal waters: a multi-biomarker study. Environmental Science and Pollution Research 28(44), 63032-63044.

Lomartire, S., J. C. Marques and A. M. M. Gonçalves (2021). Biomarkers based tools to assess environmental and chemical stressors in aquatic systems. Ecological Indicators 122: 107207.

Mansour, C., Ben Taheur, F., Mzoughi, R. and Mosbahi, D.S. (2021) Hydrocarbon levels and biochemical biomarkers in the clam Ruditapes decussatus collected from Tunis lagoon (Tunisia), Basel, Switzerland.

Missawi, O., Bousserrhine, N., Belbekhouche, S., Zitouni, N., Alphonse, V., Boughattas, I. and Banni, M. (2020) Abundance and distribution of small microplastics (≤ 3 μm) in sediments and seaworms from the

Southern Mediterranean coasts and characterisation of their potential harmful effects. Environmental Pollution 263, 114634.

Morroni, L., d'Errico, G., Sacchi, M., Molisso, F., Armiento, G., Chiavarini, S., Rimauro, J., Guida, M., Siciliano, A., Ceparano, M., Aliberti, F., Tosti, E., Gallo, A., Libralato, G., Patti, F.P., Gorbi, S., Fattorini, D., Nardi, A., Di Carlo, M., Mezzelani, M., Benedetti, M., Pellegrini, D., Musco, L., Danovaro, R., Dell'Anno, A. and Regoli, F. (2020) Integrated characterization and risk management of marine sediments: The case study of the industrialized Bagnoli area (Naples, Italy). Marine Environmental Research 160, 104984.

Parrino, V., Minutoli, R., Lo Paro, G., Surfaro, D. and Fazio, F. (2020) Environmental assessment of the pesticides in Parablennius sanguinolentus along the Western Calabrian coast (Italy). Regional Studies in Marine Science 36, 101297.

Rios-Fuster, B., Alomar, C., Capó, X., Paniagua González, G., Garcinuño Martínez, R.M., Soliz Rojas, D.L., Silva, M., Fernández Hernando, P., Solé, M., Freitas, R. and Deudero, S. (2022) Assessment of the impact of aquaculture facilities on transplanted mussels (Mytilus galloprovincialis): Integrating plasticizers and physiological analyses as a biomonitoring strategy. Journal of Hazardous Materials 424, 127264.

Rodríguez-Romeu, O., A. Soler-Membrives, F. Padrós, S. Dallarés, E. Carreras-Colom, M. Carrassón and M. Constenla (2022). "Assessment of the health status of the European anchovy (Engraulis encrasicolus) in the NW Mediterranean Sea from an interdisciplinary approach and implications for food safety." Science of The Total Environment 841: 156539.

Salvaggio, A., Tiralongo, F., Krasakopoulou, E., Marmara, D., Giovos, I., Crupi, R., Messina, G., Lombardo, B.M., Marzullo, A., Pecoraro, R., Scalisi, E.M., Copat, C., Zuccarello, P., Ferrante, M. and Brundo, M.V. (2019) Biomarkers of Exposure to Chemical Contamination in the Commercial Fish Species Lepidopus caudatus (Euphrasen, 1788): A Particular Focus on Plastic Additives. Frontiers in Physiology 10.

Solomando, A., Cohen-Sánchez, A., Box, A., Montero, I., Pinya, S. and Sureda, A. (2022) Microplastic presence in the pelagic fish, Seriola dumerili, from Balearic Islands (Western Mediterranean), and assessment of oxidative stress and detoxification biomarkers in liver. Environmental Research, 113369.

Telahigue, K., Rabeh, I., Chouba, L., Mdaini, Z., El Cafsi, M.h., Mhadhbi, L. and Hajji, T. (2022) Assessment of the heavy metal levels and biomarker responses in the smooth scallop Flexopecten glaber from a heavily urbanized Mediterranean lagoon (Bizerte lagoon). Environmental Monitoring and Assessment 194(6), 397.

WHO (1993). World Health Organization & International Programme on Chemical Safety. Biomarkers and risk assessment : concepts and principles / published under the joint sponsorship of the United Nations environment Programme, the International Labour Organisation, and the World Health Organization. World Health Organization.

Zaidi, M., Athmouni, K., Metais, I., Ayadi, H. and Leignel, V. (2022) The Mediterranean limpet Patella caerulea (Gastropoda, Mollusca) to assess marine ecotoxicological risk: a case study of Tunisian coasts contaminated by metals. Environmental Science and Pollution Research 29(19), 28339-28358.

Zitouni, N., Bousserrhine, N., Belbekhouche, S., Missawi, O., Alphonse, V., Boughatass, I. and Banni, M. (2020) First report on the presence of small microplastics (≤ 3 μm) in tissue of the commercial fish Serranus scriba (Linnaeus. 1758) from Tunisian coasts and associated cellular alterations. Environmental Pollution 263, 114576.

UNEP/MAP – MED POL (2019). UNEP/MED WG. 473/7, Annex I "IMAP Guidance Factsheets: Update for Common Indicators 13, 14, 17, 18, 20 and 21; New proposal for Candidate Indicators 26 and 27

UNEP/MAP – MED POL (2021). UNEP/MED WG. 509/27, Monitoring Guideline/Protocols for Sampling and Sample Preservation of Marine Molluscs (such as *Mytilus* sp.) and Fish (such as *Mullus barbatus*) for IMAP Common Indicator 18

UNEP/MAP – MED POL (2022). UNEP/MED WG 533/10, Appendix I. Adjusted Background (Assessment) Concentrations (BC/BAC) for Common Indicator 17 and Upgraded Approach for Environmental Assessment Criteria (EAC) for IMAP Common Indicators 17, 18 and 20. Meeting of the Ecosystem Approach Correspondence Group on Pollution Monitoring, Videoconference, 27 and 30 May 2022

UNEP/MAP – MED POL (2022). UNEP/MED WG 533/10, Appendix IV. The pilot example for Marine Environment Assessment in the Areas with Insufficient Data: The Results of GES Assessment for IMAP Common Indicator 17 in the Levantine Sea Basin.

UNEP/MAP – MED POL (2022). UNEP/MED WG 533/10, Appendix V. Data Standards and Data Dictionaries for IMAP Common Indicator 18.

UNEP/MAP – MED POL (2023). UNEP/MED WG.556/Inf.8. The pilot example for Marine Environment Assessment in the Areas with Insufficient Data: The Updated Results of GES Assessment for IMAP Common Indicator 17 in the Levantine Sea Basin.