Process towards the definition of the assessment criteria for the biological effects of contaminants on fish in the Italian seawaters national monitoring.

Ginevra Moltedo 1, *,
Giacomo Martuccio 1,
Barbara Catalano 1,
Luca Stellati 1,
Claudia Sebbio 1,
Paola Guarracino 1,
Maria Teresa Berducci 1,
Andrea Tornambè 1,
Chiara Maggi 1

1, ISPRA (Ist. Superiore per la Protezione e la Ricerca Ambientale), Via del Fosso di Fiorano 64, 00143 Roma, Italia
* Corresponding author: ginevra.moltedo@isprambiente.it

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Abstract: The need to define assessment criteria for the biological effects of contaminants on marine organisms has become increasingly pressing in recent years (Barcellona Convention; Marine Strategy Framework Directive). In the Mediterranean region, while threshold values for biomarkers in mussels have been defined under the UNEP-MAP Monitoring Programme, similar criteria for fish are currently lacking. To fill this knowledge gap, Italy has initiated a process under its Marine Strategy National Monitoring Programme, coordinated by ISPRA, to define national thresholds for biomarkers in *Mullus barbatus*. Between 2019 and 2023, ISPRA conducted biomarker analyses on specimens collected across three Italian Mediterranean subregions: Adriatic Sea (MAD), Ionian and Central Mediterranean Sea (MIC), and Western Mediterranean Sea (MWE). The analyses focused on key biomarkers—micronuclei frequency, acetylcholinesterase activity, lysosomal membrane stability, and cytochrome P450 activity—to assess genotoxicity, neurotoxicity, physiological alterations, and alterations of the biotransformation system. Threshold values were calculated using data from reference sites characterized by low chemical contamination in sediments. A biomarker-based index (CBEI index) was developed to evaluate Good Environmental Status (GES) under Descriptor 8 (Criterion 2), taking into account calculated thresholds for *M. barbatus*. The CBEI assessment across the three subregions (MAD, MIC, MWE) revealed distinct quality statuses.

Keywords: biomarker; fish; biological index; assessment criteria; *Mullus barbatus.*

1. Introduction

The Marine Strategy Framework Directive (2008/56/EU Dir.), named MSFD, is an important governance tool for the marine environment, promoting the adoption of complex strategies aimed at achieving Good Environmental Status (GES) for the protection of the marine ecosystems. GES is defined by 11 environmental descriptors, among which Descriptor 8 (D8) concerns the concentration of contaminants, which should be at levels not giving rise to pollution effects. For this purpose, the Commission Decision (EU) 2017/848 established several criteria, among which the secondary one (D8C2) states: "*the health of species and the condition of habitats [...] are not adversely affected due to contaminants including cumulative and synergetic effects*" and "*Member States shall establish those adverse effects and their threshold values through regional or subregional cooperation*".

The MSFD has been transposed into the Italian Legislative Decree, according to which a first National Monitoring Programme (NMP) was established in 2010 and updated in 2020 (art. 11, Legislative Decree 190/2010). In the updated NMP, in addition to the concentration of contaminants in the environmental matrices (D8C1), the effects of contaminants on marine organisms were included (D8C2). Since 2019, the Italian National Institute for Environmental Protection and Research (ISPRA), on behalf of the Italian Ministry of Environment and Energy Security (MASE), has been analyzing several biological responses in marine organisms of national seawaters. The NMP related to D8C2 was defined by ISPRA taking into consideration the recommendations provided in the Integrated Monitoring and Assessment Programme (IMAP) under the Barcelona Convention (1976) and its Protocols, adopted in the framework of the Mediterranean Action Plan of the United Nations Environment Programme (UNEP-MAP, 2017; UNEP-MAP, 2019), besides those of the Italian legislation (Legislative Decree 190/2010). The target species chosen for D8C2 of NMP was the red mullet (*Mullus barbatus*). This species is a territorial benthic fish, widely distributed along all the Mediterranean coasts with well-known ecological and physiological characteristics. The red mullet tends to accumulate pollutants to a greater extent than other species and shows pronounced sensitivity to different kinds of pollutants (Della Torre et al., 2010, and references therein). These characteristics render *M. barbatus* a suitable sentinel organism in Mediterranean marine coastal areas (UNEP-MAP, 2021). Furthermore, the use of a benthic species allows to relate the biological effects (D8C2) with the concentration of contaminants in sediment (D8C1). The NMP included a set of biomarker analyses: three "mandatory" analyses, namely micronuclei frequency, acetylcholinesterase activity and lysosomal membrane stability, and an "additional" biomarker the cytochrome p450 activity. These analyses permitted the assessment of genotoxicity, neurotoxicity, and eventual alterations of the physiological status and of the organic compounds biotransformation system, respectively. According to the GES definition (art.9 of Legislative Decree 190/2010), in order to assess the effects of contaminants on biota (D8C2), "*significant changes with respect to controls or threshold values*" need to be measured. The threshold values correspond to the "baseline levels" of biological responses (Davies and Vethaak, 2012). To date, in the Mediterranean Sea the threshold values for biomarkers have been defined only for mussels (UNEP-MAP, 2016, and UNEP-MAP, 2017), while for fish UNEP suggests referring to the ICES-OSPAR (Internation Council for the Exploration of the Sea - Convention for the Protection of the Marine Environment of the North-East Atlantic) benchmarks (Davies and Vethaak, 2012; ICES, 2013). The ICES-OSPAR threshold values for biomarkers in *M. barbatus* have been defined in 2012 (updated on 2013) using data until 2010 related to the North-East Atlantic and the French and Spanish seawaters of the Mediterranean Sea. Moreover, the Commission Decision (EU) 2017/848 (art.4, par.2) indicates that "*until Member States have established threshold values through Union, regional or subregional cooperation as required under this Decision, they may use any of the following to express the extent to which good environmental status is being achieved: (a) national threshold values, provided the obligation of regional cooperation laid down in Articles 5 and 6 of Directive 2008/56/EC is complied with; (b) directional trends of the values; (c) pressure-based threshold values as proxies*". Therefore, updated threshold values for biomarkers in fish of the Mediterranean Sea need to be defined, considering also the specific condition of the Italian seawaters. To date, few studies on biomarkers in *M. barbatus* caught in the Italian seawaters are available in the scientific literature (Burgeot et al., 1996; Corsi et al, 2002; Lionetto et al., 2003; Bolognesi et al., 2006; Viarengo et al., 2007; Zorita et al., 2008; Morroni et al., 2020). Most of these studies are outdated, and their data are frequently non-comparable as they are derived neither from identical target tissues nor through standardized methodological protocols. Moreover, being related to specific case studies, they do not permit to have a sufficient spatial coverage of Italian seawaters.

The aim of this paper is to fill this knowledge gap presenting some preliminary threshold values calculated for biomarkers in *M. barbatus* from Italian seawaters and a suitable, robust integrated and calibrated index of biological effects of contaminants, which can be used for the GES assessment under MSFD D8C2. This index is necessary for processing data acquired in the national monitoring of the biological effects of environmental contaminants (i.e., PAH, metals, etc.) using an integrated approach that reflects short-term and long-term effects of marine pollution.

The Italian threshold values were calculated using data obtained on red mullet (*M. barbatus*) caught in the Italian seawaters since 2019 during the National MSFD monitoring activities and analyzed according to harmonized methodological protocols (UNEP-MAP, 2021). The MSFD monitoring activity provides a large amount of data, acquired on the same species and target tissue using a consistent methodological protocol, making it highly suitable for threshold calculations.

The Italian threshold values were used to define the good status of the organisms in the Italian Mediterranean Sea and to assess the GES achievement for D8C2, as requested by MSFD.

2. Materials and Methods

2.1 Sampling

From 2019 to 2023 ISPRA, on behalf of the MASE, collected 560 specimens of adult *Mullus barbatus* (both gender, size range: 12-18 cm) during their post-spawning period (from September to November), from 28 sites located in the three subregions of the Italian Mediterranean Sea (temperature range of 14-21°C, salinity range of 38-39 psu, and dissolved oxygen range of 73-124 µmol O_2/l): the Adriatic Sea (MAD), the Ionian Sea and Central Mediterranean Sea (MIC) and the Western Mediterranean Sea (MWE). After being caught by a trawling net, fish have been collected and immediately stored in ice, sacrificed and dissected in the field (approximately after 1.5 hours). For each specimen the biometric parameters such as total length and eviscerated weight, gender, reproductive state, together with gonad and liver weight were scored; the target tissues for the biomarker analyses were collected and stored in agreement to the specific methodological protocol until analysis (UNEP-MAP, 2021). Environmental conditions at the sampling sites (temperature, salinity and dissolved oxygen of bottom water) were obtained through the Copernicus Data Service (https://data.marine.copernicus.eu/viewer) and/or Italian SIC database of the Marine Strategy NMP (http://www.db-strategiamarina.isprambiente.it/app/#/).

2.2 Analyses of biomarkers

Biomarker analyses were performed according to the UNEP or ICES harmonized methodological protocols: UNEP-MAP (2021) for micronuclei frequency (MN), acetylcholinesterase activity (AChE) and lysosomal membrane stability (LMS-HEXO); ICES TIMES (2016) for CYP1A activity (EROD). The method used for protein quantification was Bradford (1976) for AChE, and Lowry et al. (1951) for EROD. LMS-HEXO was measured in liver using a cryostatic method; MN was measured in erythrocytes using an optical method; AChE was measured in brain and muscle using a spectrophotometric method; the CYP1A activity was measured in liver by evaluating the ethoxy-resorufin-O-deethylase activity with a spectrofluorimetric method, using microsomal fraction, which is generally preferred as there are fewer interferences from cytosolic and lysosomal components than S9 fraction (ICES TIMES, 2016). Each analysis was performed in 7-10 specimens (replicates) at each site. The biometric parameters were used to calculate the gonadosomatic index (GSI). Data of biomarker analyses performed on *M. barbatus* by ISPRA in the MSFD National monitoring activity can be found in the Italian SIC (Sistema Informativo Centralizzato) database (http://www.db-strategiamarina.isprambiente.it/app/#/).

2.3 Reference Sites (RSs) selection

To calculate threshold values, UNEP-IMAP and ICES-OSPAR recommend using data obtained from Reference Sites (Davies and Vethaak, 2012; UNEP-MAP, 2015). Therefore, a number of Italian Reference Sites (RSs) were selected from those monitored for MSFD, considering only the sites presenting a low contamination level in sediment (corresponding to chemical quality judgment = GOOD). Contamination was assessed according to the Chemical Quality Index (CQI), integrated for all classes of contaminants (Maggi et al., 2022) in sediments (data from 2016 to 2021).
Italian sediment chemical data related to D8C1 MSFD monitoring activity are reported both in the Italian SIC database and in the MEDPOL (Mediterranean Pollution and Research Programme) of UNEP-MAP Infosystem database (http://imapinfosystem.info-rac.org/app/#/).

2.4 Analysis of data for threshold values

The background/threshold values (BAC, background assessment criteria) have been calculated for each parameter using the precautionary method recommended by UNEP-MAP and ICES-OSPAR (Davies and Vethaak, 2012; UNEP-MAP, 2015; UNEP-MAP, 2016; UNEP-MAP, 2021). This method consists in calculating the empirical 90th percentile of the mean value obtained for organisms from RSs, for parameters with increasing trend (i.e. MN, EROD), and the 10th percentile for biomarkers with a decreasing trend (i.e. LMS-HEXO, AChE). The reason for this decision was that elevated responses would lie above 90th percentile, whereas most of values below 90th percentile belong to unexposed, weakly to medium exposed, or non-responding adapted individuals. Therefore, IT-BAC was defined as a typical biomarker value in fish from a "healthy" Italian population, above which (or below which, in the case of biomarker with a decreasing trend) the presence of a biological alteration can be attested.
Moreover, to identify the threshold level that gives rise to concern "above" the background assessment criteria (value > BAC) (or "below" it, value < BAC, in the case of biomarker with a decreasing trend), a second threshold, called Italian Threshold of Biological Relevance (IT-TBR), was defined for each biomarker. IT-TBR represents a benchmark of biological significance, specifically the value "above which" (or "below which", in the case of biomarker with a decreasing trend) a biologically relevant alteration is likely to occur. It was calculated as reported below:

$$\text{IT-TBR}_i = \text{IT-BAC}_i \pm \left(\text{IT-BAC}_i \times \%\text{dev}_i\right)$$

where "i" corresponds to each biomarker, and "% dev." is the percentage of deviation from BAC considered biologically relevant according to scientific literature (Piva et al., 2011).

2.5 Data cleaning and screening

To define the threshold value for each biochemical parameter, only data from RSs, monitored from 2019 to 2023, were used. Before calculating threshold values any statistical difference between genders was verified. Since no statistical differences between sexes were observed (Kruskal–Wallis test, $p > 0.05$ for each biomarker), data from both genders were pooled. Moreover, to exclude the potential influence of reproductive status on biomarker results (Dalzochio and Gehlen, 2016), only data from individuals with GSI >1.3 were considered. The mean biomarker value was then calculated at each site, excluding any potential aberrant data. Italian biochemical data for *M. barbatus* sampled in the D8C2 MSFD monitoring activity are reported in the Italian SIC database and in the MEDPOL (UNEP-MAP) Infosystem database.

2.6 Calibrated Biological Effect Index (CBEI) and judgment

To assess the status of red mullet at each sampled site, an integrated biological index, called the Calibrated Biological Effect Index (CBEI) was developed.

$$\mathrm{CBEI}_j = \sum_{i=1}^{n} \mathrm{class}_i \times \mathrm{weight}_i$$

The CBEI value at each site (j) corresponds to the sum of the product of the CLASS and the WEIGHT values, defined for each biomarker (i). The quality CLASS gives an indication of the level of biological alteration, and it was assigned according to the comparison of the mean value of each biomarker to its corresponding threshold values, IT-BAC and IT-TBR (Table 1). Moreover, a different WEIGHT factor was assigned to each biomarker according to their corresponding effect significance: a score of “2” for biomarkers “of effect”, considered as “CORE” biomarkers (being mandatory in NMP), such as LMS-HEXO, MN, and AChE, and a score of “1” for biomarkers “of exposure”, considered as “Non-CORE” biomarkers (being additional in NMP), such as EROD (Table 2).

Table 1. Quality classification rules. X = biomarker mean value at each site. In brackets = the case of biomarker with a decreasing trend

	Class value	**Effect**	
X ≤ IT-BAC (or X ≥ IT-BAC)	**0**	absent/low	absence of low biological alteration
IT-BAC < X ≤ IT-TBR (or IT-BAC > X ≥ IT-TBR)	**1**	moderate	presence of biological alteration not particularly relevant
X > IT-TBR (or X < IT-TBR)	**2**	high	presence of biologically relevant alteration

Table 2. Weight assigned to each biomarker type. CORE = mandatory biomarker in NMP; Non-CORE = additional biomarker in NMP

	Class value	Effect	Weight
LMS-HEXO	effect	CORE	2
MN	effect	CORE	2
AChE	effect	CORE	2
EROD	exposure	Non-CORE	1

The CBEI value of each site was translated into a "biological quality judgment" (BQJ) according to the CBEI threshold values reported in Table 3. These CBEI thresholds derived from expert judgment and should be considered as preliminary. A requirement to express the BQJ is that at least three CORE biomarkers must be analyzed.
The Index suitability was tested using data from MSFD monitoring activity related to biomarkers measured in *M. barbatus* (2019-2021) collected at 17 stations distributed across the three Italian subregions: 5 stations in MAD, 5 in MIC, and 7 in MWE.

Table 3. Biological quality judgment (BQJ) related to CBEI threshold values (C = CORE biomarker; NC = Non-CORE biomarker)

Judgment	GOOD	MODERATE	POOR	BAD
3 C + 1 NC	<3	≥3	≥5	≥10
3 C	<2	≥2	≥4	≥8

2.7 GIS elaboration and cartographic representation

Results of the biological effects assessment (D8C2) related to the MSFD monitoring activities (2019-2021) were georeferenced in GIS (Software QGis 3.36; WGS84, EPSG 4258 - ETRS89). The area of each subregion was subdivided using a reference grid with 1:1 cell size ratio and 90-km side for biota, coherently with Maggi et al. (2022).
The CBEI of each site was averaged within each cell and rounded to the nearest whole number to express a BQJ according to Table 3. Finally, each monitored cell was color-coded according to its BQJ (green for GOOD, orange for MODERATE, red for POOR, and violet for BAD).

2.8 GES achievement

To evaluate the Good Environmental Status (GES) in each subregion, a pragmatic approach was established. GES was considered achieved when ≥ 60% of the sampled area was classified as GOOD, and the NO-GOOD (POOR+BAD) area was ≤ 25%. Additionally, to assess the GES achievement, spatial coverage of ≥ 50% of the sampleable area was required.
In the case of *M. barbatus*, consistently with its ecology (Tserpes et al., 2002), the sampleable area was defined within 500 m of depth.

3. Results

3.1 RSs and threshold value for biomarkers in M. barbatus

Based on the CQI assessment of sediment contamination, 18 out of a total of 28 stations were selected as RSs for *M. barbatus* biomarker data (dataset 2019-2023). These stations are located across the three subregions: 8 in MAD, 7 in MIC, and 3 in MWE (Table 4).
To calculate the threshold value of LMS-HEXO, AChE and EROD, data from all RSs were used (N = 126 records for each biomarker). However, for MN, data from only 15 sites were considered (N = 150 records), as in the remaining 3 stations, MN mean values were above the critical limit of 0.9‰ established by Piva et al. (2011) for the “class of hazard C” (corresponding to a moderate hazard).

Table 4. Threshold values of biomarkers in *M. barbatus* in the RSs of the Italian Mediterranean Sea (IT-BAC/TBR). B: brain; M: muscle; mic: microsomal cell fraction

Biomarker	Tissue	u.m.	IT-BAC	IT-TBR
LMS-HEXO	liver	min	22.61	16.96
MN	erythrocyte	‰	0.79	1.77
AChE-B	brain	nmol/min/mg prot	67.40	50.55
AChE-M	muscle	nmol/min/mg prot	33.35	25.16
EROD-mic.	liver	pmol/min/mg prot	81.11	234.32

3.2 Assessment using the CBEI Index

The Italian threshold values and the CBEI index were used as Assessment Criteria (AC) to evaluate the biological responses of red mullet monitored for D8C2 from 2019 to 2021.

During this period 17 sites were sampled. The mean biomarker values and the CBEI index calculated at each site are reported in Table 5. The comparison of mean biomarker values at each site against their specific IT-BACs showed some critical biological conditions in four sites of the MAD subregion (AS_B01, AS_B02, AS_B03, and AS_B04), underlined by high values of MN and/or EROD, and/or low values of LMS-HEXO; in only one site of the MIC subregion, high values of EROD and low values of both AChE-B and LMS-HEXO were found. In none of the MWE sites the mean values of analyzed biomarkers deviated from their specific IT-BACs.

The CBEI was calculated using data from LMS-HEXO, MN, AChE (only data coming from brain tissue) and EROD (3 CORE biomarkers and 1 Non-CORE).

Table 5. MSFD Italian monitoring activity (2019-2021). Mean value of biomarkers in *M. barbatus,* CBEI values and BQJ. SD: standard deviation; sem: standard error. Bolded numbers: values deviating from IT-BACs. B: brain; M: muscle; mic: microsomal cell fraction

Site	Subregion		LMS-HEXO	MN	AChE-B	AChE-M	EROD-mic	CBEI by site	BQJ by grid cell
			min	‰	nmol/min/mg prot	nmol/min/mg prot	pmol/min/mg prot		
19-AS_B01	MAD	mean	24.24	**1.72**	99.32	n.d.	76.14	2	MODERATE
		SD/sem	5.00	0.61	18.11	n.d.	32.54		
19-AS_B02	MAD	mean	**21.88**	**0.90**	80.64	n.d.	78.97	4	
		SD/sem	5.46	0.11	10.84	n.d.	37.66		
19-AS_B03	MAD	mean	27.57	**1.26**	94.06	n.d.	**123.00**	3	MODERATE
		SD/sem	2.33	0.18	17.25	n.d.	31.41		
19-AS_B04	MAD	mean	33.25	**1.06**	97.79	n.d.	75.71	2	
		SD/sem	6.22	0.15	29.67	n.d.	61.75		
20-AS_B09	MAD	mean	28.46	0.38	71.17	n.d.	43.07	0	GOOD
		SD/sem	10.97	0.13	14.28	n.d.	12.05		
20-ISCMS_B02	MIC	mean	**20.88**	0.43	**65.52**	n.d.	**94.35**	5	POOR
		SD/sem	7.37	0.14	11.54	n.d.	25.85		
20-ISCMS_B03	MIC	mean	29.31	0.15	68.06	n.d.	54.31	0	GOOD
		SD/sem	7.50	0.04	18.01	n.d.	19.49		
20-ISCMS_B04	MIC	mean	26.46	0.33	68.86	n.d.	57.19	0	GOOD
		SD/sem	7.88	0.11	18.34	n.d.	26.70		
20-ISCMS_B05	MIC	mean	22.92	0.38	82.96	n.d.	37.36	0	GOOD
		SD/sem	9.28	0.10	23.10	n.d.	18.92		
20-ISCMS_B06	MIC	mean	23.75	0.78	83.33	n.d.	n.d.	0	GOOD
		SD/sem	5.99	0.22	30.27	n.d.	n.d.		
21-WMS_B04	MWE	mean	29.13	0.27	88.86	44.19	19.14	0	GOOD
		SD/sem	12.38	0.06	35.08	14.86	9.76		
21-WMS_B05	MWE	mean	30.90	0.34	106.53	38.81	42.99	0	GOOD
		SD/sem	7.23	0.12	48.38	13.41	12.89		
21-WMS_B07	MWE	mean	28.79	0.14	79.22	32.70	26.58	0	GOOD
		SD/sem	11.85	0.05	14.88	12.37	15.47		
21-WMS_B10	MWE	mean	32.90	0.16	**65.86**	33.25	28.85	2	GOOD
		SD/sem	7.07	0.04	11.68	9.39	11.40		
21-WMS_B11	MWE	mean	29.75	0.22	97.55	37.99	12.08	0	GOOD
		SD/sem	10.46	0.06	42.80	12.79	5.35		
21-WMS_B12	MWE	mean	29.17	0.32	73.48	30.91	30.37	0	GOOD
		SD/sem	11.25	0.09	21.63	18.40	4.32		
21-WMS_B13	MWE	mean	27.29	0.14	85.51	31.03	31.95	0	GOOD
		SD/sem	9.43	0.05	9.35	9.68	20.10		

3.3 GIS elaboration and cartographic representation

Results of the BQJ assigned to each grid cell assessed for D8C2 (dataset 2019-2021) are reported in Table 5 and Figure 1.

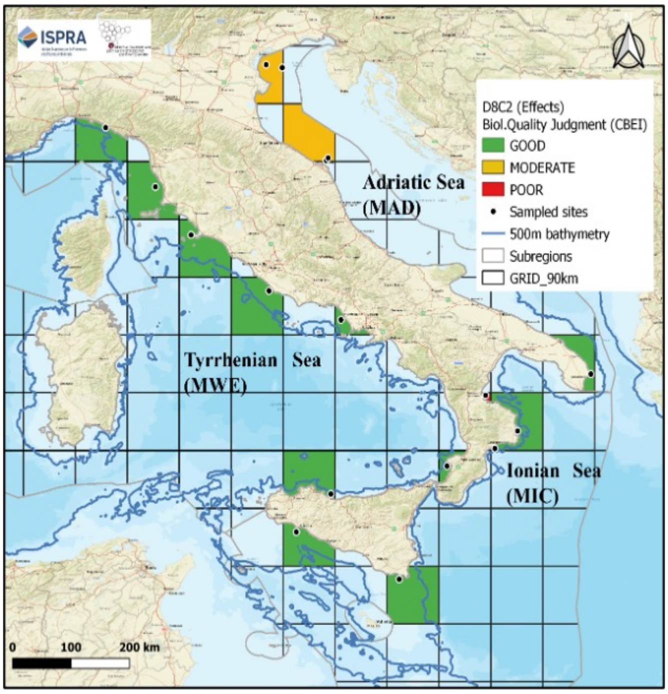

Figure 1. Map of the biological quality judgment (BQJ) assigned according to CBEI to areas (90-km side cells) monitored for MSFD-D8C2 (2019-2021) in the Italian Mediterranean Sea.

The spatial coverage of sampled areas in each subregion and the percentage of sampled cells with equal BQJ are reported in Table 6.

Table 6. Percentage coverage and percentage distribution of Biological Quality Judgment (BQJ) categories in sampled cells for each subregion

Subregion	Coverage (%)	GOOD BQJ (%)	MODERATE BQJ (%)	POOR BQJ (%)	BAD BQI (%)
MAD	17	33	67	0	0
MIC	25	75	0	25	0
MWE	25	100	0	0	0

4. Discussion

The aims of this study were to calculate the national thresholds for LMS-HEXO, MN, AChE, and EROD in the fish species *M. barbatus,* and to develop a robust and calibrated index for the biological effects, to be used as assessment criteria for evaluating the GES in the Italian seawaters, as required by MSFD.
Data from MSFD-D8 monitoring activities 2016-2023 were used to define threshold values for fish biomarkers, while biomarker data from MSFD-D8C2 monitoring activity 2019-2021 were employed to evaluate the suitability of the assessment criteria proposed in this study.
It should be emphasized that threshold values calculated in this study are preliminary and specific for *M. barbatus* individuals of 12-18 cm in length, of both genders, and in post-spawning condition, sampled in the Italian seawaters of the Mediterranean Sea. Specimens of *M. barbatus* with different range sizes, sampled in different reproductive status, and under varying environmental seawater conditions, may exhibit different reference values. Therefore, these thresholds should only be used to assess the health status of fish having the specific characteristics mentioned in Materials and Methods. The thresholds will be updated as additional biological data from Italy and other Mediterranean Countries become available.
The Italian threshold values (IT-BAC), calculated for each biomarker analyzed in the NMP, were compared with threshold values (BAC) established by ICES-OSPAR for *M. barbatus*: 20 min for LMS-HEXO in liver, 0.4‰ for MN in erythrocytes, 118 and 155 nmol/min/mg for AChE in brain and muscle respectively, 115 pmol/min/mg proteins for EROD-mic. This comparison revealed that BAC for LMS-HEXO was similar between the two different assessment criteria, IT-BAC for MN was slightly higher than ICES-OSPAR, and IT-BAC for AChE-C/M and EROD-mic were lower than ICES-OSPAR. These discrepancies underscore the need to define region-specific thresholds for *M. barbatus* of the Italian seawater which take into account local environmental conditions and fish biological traits, emphasizing once again the importance of benchmarks tailored to regional populations.
The Italian threshold values calculated for biomarker in *M. barbatus*, the robustness of CBEI, and its related BQJ were also evaluated by comparing our results with biomarker data from scientific literature related to sites considered as controls, along with chemical sediment data (MSFD-D8) near those sites, and the potential source of contaminants in their proximity.
Regarding the CBEI index and BQJ, the proportion of areas (cells) with the same BQJ suggested a “GOOD” biological status for MWE and MIC subregions (GOOD ≥ 60%,

POOR + BAD ≤ 25%), and a “NON-GOOD” status for MAD (GOOD < 60%). Indeed, in the MIC subregion, most cells have a good judgment and only a single cell has a critical judgment such as “POOR”. The “POOR” BQJ scored in the MIC area was mainly related to neurotoxicity effects, physiological state alterations, and activation of the detoxification system for organic compounds detected in ISCMS_B02. Unfortunately, no sediment chemical data monitored under D8C1 was available from this area at the time of the study to correlate to these biological responses. Future monitoring activities will help identify which contaminant may be responsible for these effects. Anyway, these results could be explained by the proximity of the sampled area to the Crati river mouth, interested by spills of civil, industrial and agricultural wastewater, potentially releasing organic and inorganic compounds into the marine environment. A recent study evidenced high levels, “exceeding the legal limit”, of heavy metals in sediment at the mouth of the Crati river and along its valley “probably due to their proximity to urban areas” (Ioele et al., 2020). Therefore, the high levels of metals that have been found in Ioele’s study could explain the inhibition of AChE measured in fish sampled for the MSFD monitoring in this area. Indeed, although AChE activity is one of the most widely used biomarkers of neurotoxicity caused by organophosphorus and carbamate, several studies attested the inhibition of AChE activity also by metals in many marine species, including fish (Frasco et al., 2005; Lopes et al., 2019).

Similarly, the "MODERATE" BQJ assigned to some cells of the MAD area was mainly ascribed to physiological state alterations, genotoxic effects, and activation of the detoxification system for organic compounds, found in four sites (AS_B01, AS_B02, AS_B03, and AS_B04) of this subregion. Notably, the mean frequency of MN measured in the AS_B01 site was particularly high, approaching the IT-TBR threshold, and comparable to the values measured in *M. barbatus* from impacted areas (Bolognesi et al., 2006; Viarengo et al., 2007). These results could be probably related to the high level of metals and PAH found in the sediments of these areas, as attested by data from the MSFD monitoring activity (2016-2021) in the northern MAD subregion (see ISPRA, 2024). At the AS_B02 site, the overall BQJ was mainly due to the LMS-HEXO results. However, the mean values of this biological response measured in fish from this site were only slightly lower than the IT-BAC value established for this biomarker and comparable to those measured in *M. barbatus* from control areas (Zorita et al., 2008). Therefore, in this case the biological condition of the fish was considered to be only mildly compromised. At the AS_B03 site, the mean value of EROD activity and of MN exceeded the IT-BAC, while at AS_B04 this was observed only for MN. These results suggested an early warning condition for these sites, probably due to high levels of organic compounds. Indeed, the cytochrome P450 (CYP) enzymes constitute a superfamily of heme-containing proteins catalyzing biotransformation reactions of numerous xenobiotics, including polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls, and other halogenated compounds (Benedetti et al., 2009). The induction of EROD activity and the high value of MN frequency are likely related to the high concentrations of halogenated organic compounds (HOCs) found in the sediments of this area (see ISPRA, 2024).

In the other sampled sites, the mean values of the analyzed biomarkers (LMS-HEXO, MN, AChE and EROD) were comparable to those found in fish of the same species sampled in reference sites (Corsi et al., 2002; Porte et al., 2002; Bolognesi et al., 2006; Viarengo et al., 2007; Zorita et al., 2008; Morroni et al., 2020), suggesting a good health condition of fish in those areas, and a good correspondence between the values used to calculate the index and those reported in the literature.

Finally, although a substantial number of cells were sampled for *M. barbatus*, the spatial coverage of the sampleable area in each of the three subregions was insufficient to allow the assessment of GES achievement for D8C2 as none of the three subregions reached the required 50% coverage threshold.

5. Conclusions

The large amount of data for MSFD monitoring activities allowed us to fill the knowledge gap regarding threshold values for biomarkers in *M. barbatus* from Italian seawaters and to develop a preliminary assessment criterion for evaluating the biological quality status of marine organisms from these waters.

During this study preliminary national threshold values for LMS-HEXO, MN, AChE, and EROD were calculated and a calibrated biological index for the assessment of the biological effects of contaminants in fish was developed. These thresholds, in combination with the CBEI Index and its related BQJ, provide a suitable and robust tool for the assessment of the biological effects of contaminants in the target species, *M. barbatus*. Their suitability and robustness were attested by the assessment of biological data from MSFD monitoring activity during the 2019-2021 period, as reported in the Italian Summary Report for D8, submitted to the European Commission in October 2024 (ISPRA, 2024).

The developed CBEI and its related BQJs, considering the set of biomarkers selected for the NMP, can be considered a reliable index representing the health status of red mullet in the Italian seawaters. It provides a sufficiently realistic interpretation of the biological effects on marine organisms measured by biomarker analysis in the target fish species, likely related to the environmental contamination measured in the sediment. Indeed, marine organisms from MIC and MWE resulted to be in good biological conditions while those from MAD resulted to be in the worst biological conditions consistent with findings from the chemical monitoring activities (ISPRA, 2024). The assessment criteria used in this study, however, present some limitations: 1) the threshold values are preliminary and need to be updated with additional data; 2) specific thresholds for each subregion could be more appropriate to account for their unique environmental characteristics; 3) these thresholds are specific for *M. barbatus* of the Italian seawaters and not harmonized at Mediterranean region level. Therefore, in the future, we aim to update the threshold values using upcoming biomarker data and harmonize them with other Mediterranean Countries and/or to attempt to calculate threshold values specific for the condition of each subregion. In addition, we plan to identify potential fixed Reference sites for long-term use, and to test alternative assessment criteria based on comparison with data from control sites.

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REFERENCES

BENEDETTI, M., FATTORINI, D., MARTUCCIO, G., NIGRO, M., & REGOLI, F. (2009). Interactions between trace metals (Cu, Hg, Ni, Pb) and 2,3,7,8-tetrachlorodibenzo-p-dioxin in the Antarctic fish *Trematomus bernacchii*: oxidative effects on biotransformation pathway. *Environmental Toxicology and Chemistry, 28*(4), 818–825. https://doi.org/10.1897/08-066.1

BOLOGNESI, C., PERRONE, E., ROGGIERI, P., PAMPANIN, D. M., & SCIUTTO, A. (2006). Assessment of micronuclei induction in peripheral erythrocytes of fish exposed to xenobiotics under controlled conditions. *Aquatic Toxicology, 78*(S1), S93–S98. https://doi.org/10.1016/j.aquatox.2006.02.015

BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*(1–2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

BURGEOT, T., BOCQUENE, G., PORTE, C., DIMEET, J., SANTELLA, R. M., PFHOL-LESZKOWICZ, A., & GALGANI, F. (1996). Bioindicators of pollutant exposure in the northwestern Mediterranean Sea. *Marine Ecology Progress Series, 131*, 125–141. https://doi.org/10.3354/meps131125

CORSI, I., MARIOTTINI, M., MENCHI, V., SENSINI, C., BALOCCHI, C., & FOCARDI, S. (2002). Monitoring a Marine Coastal Area: Use of *Mytilus galloprovincialis* and *Mullus barbatus* as Bioindicators. *Marine Ecology, 23*(Suppl. 1), 138–153. https://doi.org/10.1111/j.1439-0485.2002.tb00014.x

DALZOCHIO, T., & GEHLEN, G. (2016). Confounding factors in biomonitoring using fish. *Ecotoxicology and Environmental Contamination*, *11*, 53–61. https://doi.org/10.5132/eec.2016.01.08

DAVIES, I. M., & VETHAAK, A. D. (2012). *Integrated marine environmental monitoring of chemicals and their effects*. ICES Cooperative Research Report, 315, 277 pp.

DELLA TORRE, C., CORSI, I., NARDI, F., PERRA, G., TOMASINO, M. P., & FOCARDI, S. (2010). Transcriptional and post transcriptional response of drug-metabolizing enzymes to PAHs contamination in red mullet (*Mullus barbatus*, Linnaeus, 1758): A field study. *Marine Environmental Research*, *70*(2), 95–101. https://doi.org/10.1016/j.marenvres.2010.03.009

DIRECTIVE 2008/56/EC. *Marine Strategy Framework Directive (MSFD)*.

FRASCO, M., FOURNIER, D., CARVALHO, F., & GUILHERMINO, L. (2005). Do metals inhibit acetylcholinesterase (AChE)? Implementation of assay conditions for the use of AChE activity as a biomarker of metal toxicity. *Biomarkers*, *10*(5), 360–375. https://doi.org/10.1080/13547500500264660

ICES. (2013). *Report of the Working Group on the Biological Effects of Contaminants (WGBEC)*. In *Proceedings of the Working Group Meeting on Biological Effects of Contaminants*, San Pedro del Pinatar, Spain, 10–15 March 2013 (ICES CM 2013/SSGHIE:04, 37 pp.).

ICES-TIMES N.57. (2016). *Determination of CYP1A-dependent mono-oxygenase activity in dab by fluorimetric measurement of EROD activity in S9 or microsomal liver fractions*. (Stagg, R., McIntosh, A., & Gubbins, M. J., Eds.).

IOELE, G., DE LUCA, M., GRANDE, F., DURANTE, G., TROZZO, R., CRUPI, C., & RAGNO, G. (2020). Assessment of surface water quality using multivariate analysis: Case study of the Crati River, Italy. *Water*, 12(8), 2214. https://doi.org/10.3390/w12082214

ISPRA. (2024). *Report MSFD Art.8 - D8* (68 pp.).

LEGISLATIVE DECREE 190/2010. Attuazione della direttiva 2008/56/CE che istituisce un quadro per l'azione comunitaria nel campo della politica per l'ambiente marino (G.U. 18 novembre 2010, n. 270).

LIONETTO, M. G., CARICATO, R., GIORDANO, M. E., & SCHETTINO, T. (2004). Biomarker application for the study of chemical contamination risk on marine organisms in the Taranto marine coastal area. *Chemistry and Ecology*, *20*(S), S333–S345. https://doi.org/10.1080/02757540310001629215

LOPES, D. F. C., ASSIS, C. R. D., SANT'ANNA, M. C. S., SILVA, J. F. D., BEZERRA, R. S., & FRÉDOU, F. L. (2019). Brain acetylcholinesterase of three perciformes: From the characterization to the in vitro effect of metal ions and pesticides. *Ecotoxicology and Environmental Safety*, *173*, 494–503. https://doi.org/10.1016/j.ecoenv.2019.02.047

LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L., & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, *193*, 266–275.

MAGGI, C., BERDUCCI, M. T., DI LORENZO, B., LOMIRI, S., & VENTI, F. (2022). Integrated Chemical Status of the Italian Marine Waters sensu Descriptor 8 of the Marine Strategy Framework Directive. *Frontiers in Marine Science*, *9*. https://doi.org/10.3389/fmars.2022.1006595

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., & 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. https://doi.org/10.1016/j.marenvres.2020.104984

PIVA, F., CIAPRINI, F., ONORATI, F., BENEDETTI, M., FATTORINI, D., AUSILI, A., & REGOLI, F. (2011). Assessing sediment hazard through a weight of evidence approach with bioindicator organisms: A practical model to elaborate data from sediment chemistry, bioavailability, biomarkers and ecotoxicological bioassays. *Chemosphere*, *83*(4), 475–485. https://doi.org/10.1016/j.chemosphere.2010.12.064

PORTE, C., ESCARTÍN, E., DE LA PARRA, L. M. G., BIOSCA, X., & ALBAIGÉS, J. (2002). Assessment of coastal pollution by combined determination of chemical and biochemical markers in *Mullus barbatus*. *Marine Ecology Progress Series*, *235*, 205–216. https://doi.org/10.3354/meps235205

TSERPES, G., FIORENTINO, F., LEVI, D., CAU, A., MURENU, M., ZAMBONI, A. D. A., & PAPACONSTANTINOU, C. (2002). Distribution of *Mullus barbatus* and *M. surmuletus* (Osteichthyes: Perciformes) in the Mediterranean continental shelf: Implications for management. *Scientia Marina*, *66*(S2), 39–54. https://doi.org/10.3989/scimar.2002.66s239

UNEP-MAP. (2015). *1st Report of the Informal Online Working Group on Contaminants* (WG411/inf8). United Nations Environment Programme/Mediterranean Action Plan.

UNEP-MAP. (2016). *Decision IG.22/7. Integrated Monitoring and Assessment Programme of the Mediterranean Sea and Coast and Related Assessment Criteria*. United Nations Environment Programme/Mediterranean Action Plan.

UNEP-MAP. (2017). *Decision IG.23/6. Mediterranean Quality Status Report*. United Nations Environment Programme/Mediterranean Action Plan.

UNEP-MAP. (2019). *IMAP Guidance Factsheets: Update for Comm. Indic. 13, 14, 17, 18, 20 and 21; New proposal for Candidate Indicators 26 and 27 (WG.467/5)*. United Nations Environment Programme/Mediterranean Action Plan.

UNEP-MAP. (2021). *Report of the Meeting of the MED POL Focal Points, 27–28 May and 6–7 Oct. 2021. Annex III, App. 22–24 (WG509/43, 2514 pp.)*. United Nations Environment Programme/Mediterranean Action Plan.

UNEP-MAP. (2023). *2023 Mediterranean Quality Status Report (QSR) - Pollution Ecological Objectives (EO5, EO9): The Proposal of the IMAP Pollution Cluster Chapters of the 2023 MED QSR* (WG.556/3, Agenda item 3). United Nations Environment Programme/Mediterranean Action Plan.

VIARENGO, A., DONDERO, F., PAMPANIN, D. M., FABBRI, R., POGGI, E., MALIZIA, M., BOLOGNESI, C., PERRONE, E., GOLLO, E., & COSSA, G. P. (2007). A biomonitoring study assessing the residual biological effects of pollution caused by the HAVEN wreck on marine organisms in the Ligurian Sea (Italy). *Archives of Environmental Contamination and Toxicology*, *53*(4), 607–616. https://doi.org/10.1007/s00244-005-0209-2

VIARENGO, A., LOWE, D., BOLOGNESI, C., FABBRI, E., & KOEHLER, A. (2007). The use of biomarkers in biomonitoring: A 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *146*(1–2), 281–300. https://doi.org/10.1016/j.cbpc.2007.04.011

ZORITA, I., ORTIZ-ZARRAGOITIA, M., APRAIZ, I., CANCIO, I., ORBEA, A., SOTO, M., MARIGÓMEZ, I., & CAJARAVILLE, M. P. (2008). Assessment of biological effects of environmental pollution along the NW Mediterranean Sea using red mullets as sentinel organisms. *Environmental Pollution*, *153*(1), 157–168. https://doi.org/10.1016/j.envpol.2007.07.028