



Comparative Analysis of the Oxidative Stress in Bulgarian Black-Sea Bivalves and their Bioindicator Potential

Luchezar P. Yakimov¹, Elina R. Tsvetanova², Almira P. Georgieva², Galina T. Nenkova²,
Nesho H. Chipev¹ & Albena V. Alexandrova^{2*}

¹Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 2 Gagarin Street, 1113 Sofia, Bulgaria

²Institute of Neurobiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Street, Bl. 23, 1113 Sofia, Bulgaria;

E-mail: a_alexandrova_bas@yahoo.com

Abstract: A variety of pressures on the environment of aquatic organisms can increase production of reactive oxygen by species or decrease antioxidant defence resulting in the development of oxidative stress (OS). It is known that xenobiotics induce OS in marine bivalves. Studies on bivalves as bioindicators in the Bulgarian Black Sea are restricted to *Mytilus galloprovincialis* Lam. and there are no data on the eco-toxicological status of other benthic bivalve species and their stress ecology. The aim of the present study was to comparatively assess the OS biomarkers in different bivalves (*Mytilus galloprovincialis*, *Donax trunculus* and *Chamelea gallina*) from representative habitats of the Bulgarian Black Sea aquatory. In conclusion, the OS biomarkers of the studied species can be successfully used in the assessment and monitoring of marine environments. However, OS data should be combined with data from other indicators (e.g. community indices and ecosystem processes) in order to provide a more complete and at a higher biological resolution assessment of marine ecosystem “health” and early warning of impending ecological problems in ecosystems.

Key words: antioxidant enzymes, *Chamelea gallina*, *Donax trunculus*, *Mytilus galloprovincialis*, pro-oxidant indicators

Introduction

Anthropogenic pressures on marine environments are causing increasing concerns, recently being related also to effects on marine ecosystems resilience and potential to deliver vital ecosystem services. The Black Sea makes no exception of the increasing anthropogenic pressures due to its geographical position, limited water exchange, the inflow of large amounts of river waters from a number of countries and the increasing local coastal pollution (MONCHEVA et al. 2016). This urgently requires development of novel integrative tools and methods to assess changes in marine ecosystem status, monitoring and

management using multiple biological, physical and chemical and pollution quality indicators (BORJA et al. 2008).

The benthic environment is a fundamental compartment of the Black Sea coastal ecosystem and different xenobiotics can be bioaccumulated in macrozoobenthic organisms and transmitted through food chains. Benthic Black Sea bivalves, in particular the black mussel *Mytilus galloprovincialis* Lam. and psammophilic clam species of the genera *Chamelea*, *Donax*, etc., have a special place in the ecosystems providing also valuable ecosystem services (UZUNOV et al. 2017).

Recently, biomarkers are becoming an impor-

*Corresponding author: a_alexandrova_bas@yahoo.com

tant and integral part of the assessment of marine ecosystems (VALAVANIDIS et al. 2006) and using sets of different biomarkers is strongly recommended for monitoring and management programs (LAM 2009). The overall adaptive response of marine organisms to environmental stress at the cellular level can be expressed through changes in their pro/antioxidant status. All aerobic organisms, including also marine bivalves, produce reactive oxygen species (ROS) that are neutralised by an evolutionary developed antioxidant system. Under normal physiological conditions, a balance exists between the processes producing ROS (pro-oxidant processes) and eliminating them (antioxidant processes). The disruption of this balance is known as oxidative stress (OS) (SIES 1985). Overproduction of ROS in marine bivalves has been established as a response to xenobiotics, changes in oxygen content, water temperature (heat stress) etc. (WINSTON et al. 1990, ABELE et al. 2002). Environmental conditions across a range of habitat types are associated with OS threats. Marine environments vary widely in characteristics, which can influence the degree of oxidative insult experienced by an individual, and the organisms adjust their antioxidant (AO) systems in response to environmental variability so as to maintain a stable redox state (LESSER 2006). The oxidative status of marine benthic bivalves is known to be a reliable model for monitoring of ecosystem changes (BOLOGNESI & CIRILLO 2014).

So far, studies on bivalves as bioindicators of pollution in the Bulgarian Black Sea aquatory are restricted to *M. galloprovincialis* (GORINSTEIN et al. 2003, MONCHEVA et al. 2004). It should be strongly emphasised that baseline studies on the ecotoxicology and stress ecology of the common psammophilic clams have not been previously conducted in the Bulgarian Black-Sea sector.

The aim of the present study was to carry out an initial comparative study of bivalves from representative habitats of the Bulgarian Black Sea aquatory by assessing oxidative stress biomarkers and their bio-indicative potential.

Materials and Methods

Sampling

Specimens of *Mytilus galloprovincialis* Lam., *Donax trunculus* L. and *Chamelea gallina* L. were collected from representative sites along the Bulgarian Black Sea coast (north and south from Cape Emine) (Fig. 1). The bivalve species were gathered manually from their natural habitats or were obtained from commercial providers. The collected specimens were transferred to the laboratory in containers with sea water.

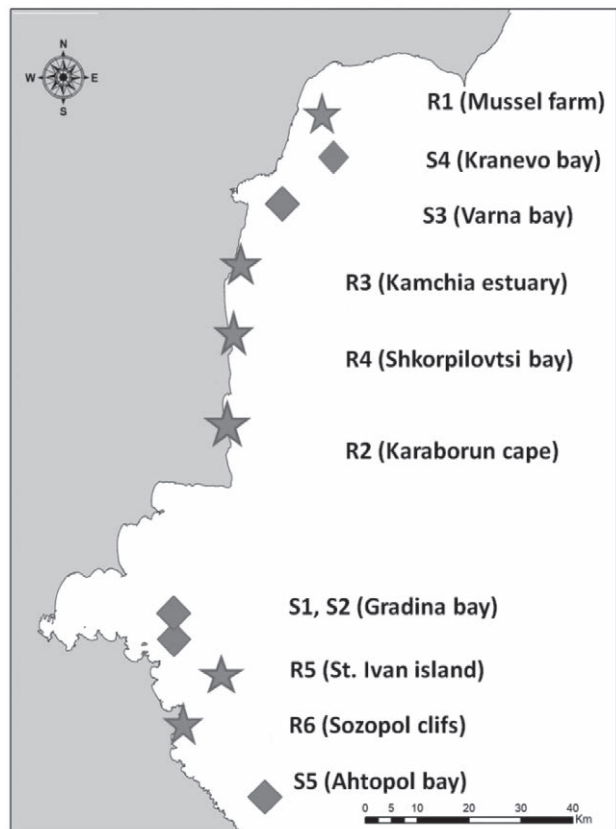


Fig. 1. Distribution of sampling sites among ecosystem types: R (rhombus) – “Infralittoral rocks and other hard substrates”; S (stars) – “Sublittoral sediments”.

Tissue preparation

In the laboratory, ten mussels from each sample were immediately dissected on ice and their soft tissues were frozen in liquid nitrogen and stored at -80°C until analysis. On the day of analysis, the tissues were homogenised in 100 mM potassium phosphate buffer (pH 7.4). The homogenates were centrifuged at 3000 g for 10 min, obtaining a post-nuclear fraction, from which the lipid peroxidation and glutathione levels were determined. A portion of the fraction was re-centrifuged at 12 000 g for 20 min to obtain a post-mitochondrial supernatant used for measurement of the antioxidant enzymes activity. All work was carried out at 4°C .

Measurement of oxidative stress biomarkers

Protein concentrations were measured according to LOWRY et al. (1951) by using a standard curve of bovine serum albumin as a standard.

Lipid peroxidation in tissues was determined according to HUNTER et al. (1963). The colour complex, malondialdehyde (MDA), formed in the reaction of thiobarbituric acid (TBA) with lipid peroxidation end products has a maximum absorption at 532 nm. The amount of TBA-reactive substances

was calculated as nmoles MDA/mg protein using a molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

Protein oxidation was measured according to WHITEKUS et al. (2002). The method is based on the reaction of the protein carbonyl groups with 2,4-dinitrophenylhydrazine (2,4-DNPH). The carbonyl content was expressed in nmoles protein carbonyls (PC)/mg protein, using a molar extinction coefficient of $2.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

Total glutathione concentration was measured according to RAHMAN et al. (2006). The reaction of reduced glutathione with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) gives a colour compound with an absorption peak at 412 nm. The amount of glutathione was calculated from the reference standard and expressed as ng/mg protein.

Superoxide dismutase activity was measured according to PESKIN & WINTERBOURN (2017). The water-soluble tetrazolium (WST-1) is reduced by the superoxide radicals, generated in the xanthine-xanthine oxidase system to formazan. The inhibition of the WST-1 reduction is considered a measure of enzyme activity. One unit is defined as the amount of enzyme needed to inhibit the WAT reduction by 50%.

Catalase activity was determined after AEBI et al. (1984). The absorption decrease at 240 nm corresponds to the decomposition of H_2O_2 and is a measure of the catalase activity. Enzyme activity was expressed as $\Delta A_{240}/\text{min}/\text{mg}$ protein.

Glutathione peroxidase activity was measured using a commercially available kit CGP 1 (Sigma-Aldrich Co. LLC, USA). The enzyme activity was expressed as units/mg protein using $e^{\text{mM}} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ for NADPH.

Glutathione reductase activity was measured using a commercially available kit GRSA (Sigma-Aldrich Co. LLC, USA). The enzyme activity was expressed as unit/mg protein using $e^{\text{mM}} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ for NADPH.

Statistical analyses

Statistical analyses of raw data were carried out using STATISTICA 10 package (StatSoft Inc., USA, 2010). The measured values for every OS biomarker were compared (t-test; $p \leq 0.05$) among the bivalve samples from the different habitats.

Results

The estimated OS biomarkers values in the studied clam species are presented in Table 1. The highest values for lipid peroxidation (LPO) and protein oxidation (PO) were recorded in clams (*D. trunculus* and *C. gallina*) from the Gradina Bay (sites S1 and

S2). There, high activities of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) were also measured. The lowest glutathione (GSH) concentrations, accompanied with low activities of glutathione peroxidase (GPX) and glutathione reductase (GR), were also found in the soft tissues of the clams from these sites.

The OS biomarkers measured in *D. trunculus* did not differ significantly among the sites, with the exception of the high values of GSH found in clams from site S5 (Ahtopol Bay; Table 1).

The measured biomarker values in *M. galloprovincialis* are presented in Table 2. Significantly higher values of LPO (t-test; $p \leq 0.05$) were recorded in the samples from the mussel farm (R1) and the Shkorpilovtsi Bay (R4), compared to all other sites. It is noteworthy to stress the lower GSH concentration and higher activities of SOD, CAT and GPX, measured in the tissues of the mussels from the sites situated south from Cape Emine (R5 and R6).

In order to compare the overall similarities of the OS biomarker values among the studied habitat types, cluster analyses were performed (Figs. 2 and 3). Two well-separated groups of sites were present in the cluster dendrogram: one consisted only of site S5, representing the sublittoral sandy habitats, and a second one, comprising the remaining sites (Fig. 2). Two sites, S1 and S2 (*C. gallina* and *D. trunculus* from the Gradina Bay, respectively) showed high similarity in the values of the OS biomarkers in the clam species and formed a separate group. This strongly indicated the presence of similarity in the marine environmental conditions of this two sites and, hence, similarity in the OS status of the clam species. Sites S3 (Varna Bay) and S4 (Kranevo Bay) had similar values of the OS biomarkers in *D. trunculus* and, hence, also formed a separate group on the cluster dendrogram (Fig. 2). These sites were situated relatively close to each other and both were in the northern part of the Bulgarian Black Sea aquatory, which might explain the similar level of OS in *D. trunculus* sampled from those sites.

The cluster analysis of OS bioindicators in *M. galloprovincialis* sampled from habitats of ecosystem type "Infralittoral rocks and other hard substrates" (Fig. 3) clearly distinguished between the mussel samples from the southern (R5 and R6) and the northern (R1–R4) sites (north and south from Cape Emine, respectively). The northern sites formed two separated groups of sites. The first included two sites, namely R1 (Mussel Farm) and R3 (Kamchia estuary), which were presumably in "good" environmental condition. The second group was formed by sites R2 (Karaborun Cape) and site

Table 1. Oxidative stress indicator values (mean ± SD) in the clam species from the ecosystem type "Sublittoral sediments".

OS indicators	<i>C. gallina</i>		<i>D. trunculus</i>		
	S1 Gradina Bay	S2 Gradina Bay	S3 Varna Bay	S4 Kranevo Bay	S5 Ahtopol Bay
LPO (nmoles MDA/mg protein)	1.17±0.14	0.95±0.14	0.75±0.23	0.45±0.06	0.43±0.1
PO (nmoles PC/mg protein)	20.56±0.2	18.75±1.2	10.01±2.784	8.79±2.12	8.98±1.14
GSH (ng/mg protein)	220.75±21.5	175±55.1	450±104.7	609±53.76	1457±384
SOD (U/mg protein)	14.9±2.95	4.39±1.4	0.48±0.11	1.24±0.25	0.87±0.14
CAT (U/mg protein)	4.17±0.6	2.86±0.87	0.99±0.37	1.62±0.43	1.33±0.32
GPX (U/mg protein)	0.87±0.19	1.77±0.76	4.24±2.76	3.80±1.01	5.29±1.39
GR (U/mg protein)	1.2±0.15	1.57±0.76	4.17±0.99	7.80±2.27	9.44±3.41

Table 2. Oxidative stress indicator values (mean± SD) in black mussels from the ecosystem type "Supralittoral rocks".

OS indicators	<i>M. galloprovincialis</i>					
	R1 Mussel Farm	R2 Karaborun Cape	R3 Kamchia estuary	R4 Shkorpilovtsi Bay	R5 St. Ivan Island	R6 Sozopol cliffs
LPO (nmoles MDA/mg protein)	4.26±1.72	2.41±0.61	2.09±0.13	3.44±0.05	2.12±0.14	1.90±0.32
PO (nmoles PC/mg protein)	12.05±1.12	10.74±1.71	11.38±3.44	11.91±0.57	12.24±1.13	11.42±1.73
GSH (ng/mg protein)	1374±439.1	1671±491	1065±150	1884±841	607.9±162.3	420.64±160.3
SOD (U/mg protein)	0.38±0.02	0.35±0.09	0.53±0.08	0.32±0.07	1.30±0.73	1.34±0.21
CAT (U/mg protein)	0.94±0.15	0.91±0.15	0.81±0.33	1.01±0.48	1.88±0.29	1.53±0.29
GPX (U/mg protein)	4.25±2.76	3.41±1.51	4.25±1.89	8.34±0.81	23.58±2.51	19.02±2.45
GR (U/mg protein)	12.18±1.82	10.52±1.47	10.76±2.02	10.71±3.19	6.50±1.01	10.14±2.13

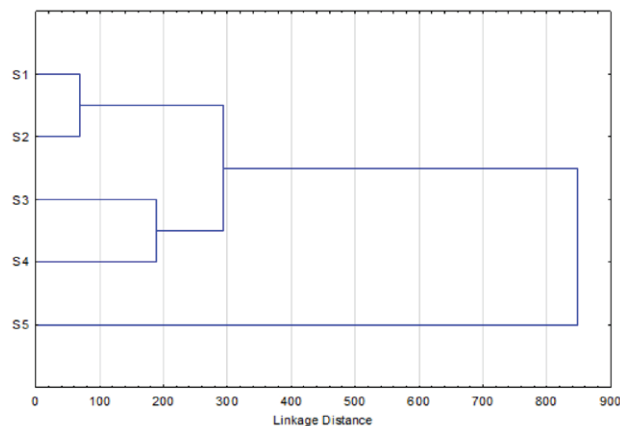


Fig. 2. Cluster dendrogram (Euclidian distance; non-weighted average) of oxidative stress indicators in clam species from sites belonging to the ecosystem type "Sublittoral sediments" (S1 – Gradina Bay; S2 – Gradina Bay; S3 – Varna Bay; S4 – Kranevo Bay; S5 – Ahtopol Bay).

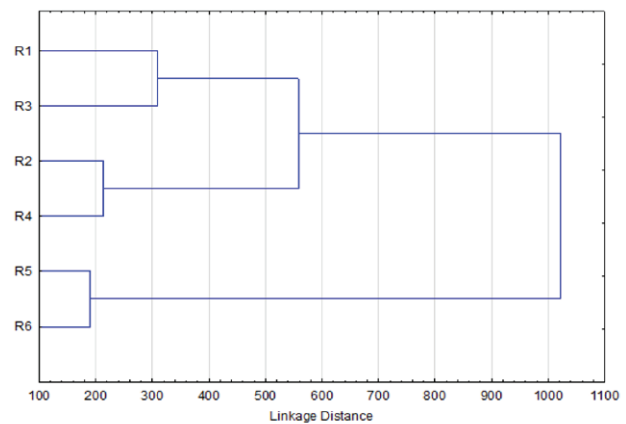


Fig. 3. Cluster dendrogram (Euclidian distance; non-weighted average) of oxidative stress indicators in *M. galloprovincialis* from the ecosystem of type "Infralittoral rocks and other hard substrates" (R1 – Mussel Farm; R2 – Karaborun Cape; R3 – Kamchia estuary; R4 – Shkorpilovtsi Bay; R5 – St. Ivan Island; R6 – Sozopol cliffs).

R4 (Shkorpilovtsi Bay), which were sites situated relative close to each other. Additionally, R2 and R4 had similar marine environmental conditions which however, differed from those of the first group, causing differences in the OS status of the mussels.

Discussion

This study reports results of a preliminary research on the stress ecology of Black Sea bivalve species in Bulgaria. Biomarkers of OS were measured in three bivalve species from representative habitats

of the southern and northern Bulgarian Black Sea coastal zone. These biomarkers are a sub-class of effect biomarkers that recently have been widely used in environmental studies. The changes in the oxidative status of sentinel marine organisms in response to environmental pressures could be used in the assessment and early warning of impending ecological problems (COSTANTINI et al. 2010).

As a whole, the levels of OS biomarkers were found to vary among the studied bivalve species and habitats. One of the most informative OS status biomarker is (LPO), since the polyunsaturated fatty acids (PUFA) with their double bonds are highly susceptibility to oxidative alterations. Changes in LPO have been demonstrated to respond to a wide range of pollutants exposures of marine bivalves, including metals (VLACHOGIANNI & VALAVANIDIS 2007, BELCHEVA et al. 2015), polycyclic aromatic hydrocarbons (PAHs) (SUREDA et al. 2013), herbicides (WENNING et al. 1988) and, more recently, also effects of microplastics (REVEL et al. 2019). In our study, high LPO values were recorded for *M. galloprovincialis* from a mussel farm, which was unexpected. Although the conditions (sea water quality) at the mussel farm met all national requirements for breeding of shellfish, the presence of high LPO values are probably due to the daily servicing of the aquaculture, boats and also the harvesting of the mussels, which could have induced shifts in the redox balance. A similar finding was recorded for the mussels gathered from Shkorpilovtsi Bay, which also had high LPO levels. Here, the mussels were collected from the iron columns of a pier located in the mouth of the Fandakliyska River. According to the national monitoring data (BSBD-VARNA 2017), dissolved manganese and iron in this water body exceeded the average annual quality standard during recent years. The role of transition metal ions, especially manganese, iron and copper in OS reactions (Fenton reaction) is well known (HALLIWELL & GUTTERIDGE, 2015) and their high presence at the site can well explain the induction of oxidative damages to lipids and proteins in the mussel tissues.

Higher values of LPO were found in the mussels (Table 2), as compared with the studied clam species (Table 1), indicating the presence of higher OS levels in these species. However, this could be simply due to the higher content of PUFA in the mussels. Published data have shown that PUFA are the dominant type of lipids in *M. galloprovincialis* (RUDNEVA 1999, DERNEKBAŞI et al. 2015) and several species of clams have been found to contain about 2.5 times less omega fatty acids in comparison to the mussels (FDS 2019). Thus, the higher content of

LPO measured by us in *M. galloprovincialis* cannot be used alone for comparing of OS levels among different bivalve species. The observed patterns of variation in LPO confirmed their suitability as possible bioindicators of environmental stress but should be used together with other OS biomarkers when assessing the oxidative status of bivalve species.

Protein oxidation levels in all samples of *M. galloprovincialis* (Table 2) did not differ significantly from those in *D. trunculus*, collected from the studied sites of the sublittoral sandy substrates. This suggests the presence of a close similarity in PO processes in *M. galloprovincialis* and clams. In one case, however, the PO values measured in *C. gallina* and *D. trunculus* sampled from the Gradina Bay (S1 and S2) were significantly higher in comparison to *M. galloprovincialis* and also higher than in the other samples of *D. trunculus*. Thus, it can be concluded, with a high degree of reliability, that the environmental conditions of the sandy habitats in the Gradina Bay were stressful. Moreover, there the highest values of LPO in *D. trunculus* samples were observed, together with the lowest values of GSH, an OS biomarker well known to respond with depletion in the presence of OS (GOSTYUKHINA & ANDREENKO 2015). The observed activation of the antioxidant enzymes SOD and CAT further supports the presence of an unfavourable and stressful environment at that site, which experiences high touristic pressure.

The observed variation in the OS indicators in *M. galloprovincialis* is presented in Table 2. It is worth pointing that low SOD activities were present along with high GSH levels and also high SOD activity together with low levels of GSH. These interrelations were true for both mussels and clams, and were very well demonstrated in *D. trunculus* from the Kranevo (S4) and Ahtopol Bays (S5) (Table 1). GSH is an antioxidant with low molecular weight and an important role in the antioxidant defence of cells and its major advantage, compared to the enzymatic defence, is its abundance in tissues and the ability to be mobilised as soon as OS develops (KENIYA et al. 1993). GSH can not only directly neutralise the ROS and recover other non-enzymatic antioxidants, but it is a cofactor of GPX. The observed higher GPX activity in *M. galloprovincialis* from R5 and R6 sites, along with the higher CAT and SOD activities, most probably reflect the mussels' efforts to reduce the OS (IGHODARO & AKINLOYE 2018). Thus, the GSH depletion in these specimens could be due to increased GPX activity. In clams, however, there was an inverse relationship: higher GPX activities corresponded to higher GSH levels. Prob-

ably in clams, the higher GSH levels are related to the higher activity of GR, which is involved in the restoration of GSH from the oxidised glutathione (HALLIWELL & GUTTERIDGE 2015).

The organism's enzymatic antioxidant system is activated as an adaptive response, allowing it to overcome completely or in part OS (LIVINGSTONE 2003, VLACHOGIANNI & VALAVANIDIS 2007). On the other hand, the acute or prolonged exposure to unfavourable environmental conditions can lead to the inhibition of the antioxidant enzymes, the hormesis effect (MATTSON & CALABRESE 2010). In our study, such effects were observed in *D. trunculus* sampled from the Varna Bay (S3), where the lowest values for SOD and CAT were measured, probably due to antioxidant enzyme inhibition by the anthropogenic pressures to which this site has been traditionally exposed. Varna Bay accepts pollutants from the industries, agriculture, treatment plants and ports (SHTEREVA et al. 2004) and additionally, high levels of oil and metal pollutants are often present (STANCHEVA & IVANOVA 2012), as well as chronic pollution by petrogenic and pyrolytic hydrocarbons (GORINSTEIN et al. 2003; MONCHEVA et al. 2004) and eutrophication (MONCHEVA 2003).

Results from the cluster analyses (Figs. 2, 3) for both *D. trunculus* and *M. galloprovincialis* sites, showed the presence of groups formed on the basis of similarities in the state of the marine environment at the studied sites. In support of this was also the observed separation of a northern (seemingly more stressed) group of habitats of *M. galloprovincialis* from a group of southern habitats of the species (Fig. 3). These findings supported the hypothesis that OS biomarkers in sentinel bivalve species could adequately indicate and monitor stressfulness and changes in marine environmental conditions.

In conclusion, the obtained results show that the processes indicated by the OS biomarkers (lipid peroxidation, protein oxidation, as well as antioxidant enzymes activity) are of a similar order in the studied bivalve species. Hence, the observed variations in the values of the studied biomarkers (the OS response) in the tested species reflect the difference in the environmental pressures within the habitats and OS biomarkers can be used in marine environmental assessment and monitoring. However, OS data should be combined with data from other indicators (e.g. community indices and ecosystem processes) to provide a more complete and at a higher biological resolution assessment of marine ecosystem "health" and early warning of pressures and changes in the marine environment.

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