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RESEARCH ARTICLE

Biochemical biomarker responses to pollution in selected sentinel organisms across the Eastern Mediterranean and the Black Sea

Tsangaris Catherine [1] **· Moschino Vanessa** [2] **· Strogyloudi Evangelia** [1] **· Coatu Valentina** [3] **· Ramšak Andreja** [4] **· Abu Alhaija Rana** [5] **· Carvalho Susana** [6] **· Felline Serena** [7] **· Kosyan Alisa** [8] **· Lazarou Yiota** [9] **· Hatzianestis Ioannis** [1] **· Oros Andra** [3] **· Tiganus Daniela** [3]

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Abstract Pollution effects were assessed by means of biochemical biomarkers (catalase, glutathione *S*-transferase and acetylcholinesterase activities, and metallothioneins content) in five species at selected coastal sites across the Eastern Mediterranean and the Black Sea. The mussel *Mytilus galloprovincialis*, a well-established sentinel species, was investigated in the Adriatic Sea, Aegean Sea, and Black Sea. The mussel *Brachidontes pharaonis* and the striped red mullet *Mullus surmuletus* were used in the Levantine Sea where *M. galloprovincialis* is not present. The white seabream *Diplodus sargus sargus* and the gastropod *Rapana venosa* were additionally sampled in the Adriatic and the Black Sea, respectively. Mussels showed catalase, glutathione *S*-transferase, and acetylcholinesterase responses to pollution in most geographical areas while the response of metallothioneins was restricted to a few sites. *R. venosa* showed marked responses of catalase and metallothioneins whereas both fish species did not generally exhibit variations in biomarker values among sites. The approach based on the reference deviation concept using the "Integrated Biological Responses version 2" index was useful for the interpretation of overall biomarker responses.

Keywords Pollution effects · Biomarkers · Acetylcholinesterase · Catalase · Glutathione *S*-transferase · Metallothioneins · Eastern Mediterranean Sea · Black Sea

Responsible editor: Cinta Porte

✉ Tsangaris Catherine
ctsangar@hcmr.gr

[1] Institute of Oceanography, Hellenic Center for Marine Research, 46.7 km, Athinon-Souniou Ave., P.O. Box 712, 19013 Anavyssos, Greece

[2] Institute of Marine Sciences—ISMAR-CNR, Arsenale, Castello 2737f, 30122 Venezia, Italy

[3] National Institute for Marine Research and Development "Grigore Antipa", Bvd. Mamaia 300, 900581 Constanta 3, Romania

[4] National Institute of Biology, Marine Biology Station, Fornače 41, 6330 Piran, Slovenia

[5] Energy, Environment and Water Research Center (EEWRC), The Cyprus Institute, 20 Konstantinou Kavafi, 2121 Aglanzia, P.O. Box 27456, 1645 Nicosia, Cyprus

[6] KAUST—King Abdullah University of Science and Technology, Red Sea Research Center, Thuwal 23955-6900, Saudi Arabia

[7] Laboratory of Zoology and Marine Biology, Department of Biological and Environmental Science and Technologies, University of Salento, Provincial road Lecce-Monteroni, 73100 Lecce, Italy

[8] Laboratory of Ecology and Morphology of Marine Invertebrates, A. N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Leninsky prospect 33, 119071 Moscow, Russia

[9] Oceanography Centre, University of Cyprus, P.O. Box 20537, 1678 Nicosia, Cyprus

Introduction

Over the last few decades, the evaluation of the effects of pollution in marine coastal and estuarine areas has been a growing concern worldwide. In the European Union, they resulted in two main directives, the EU Water Framework Directive (WFD, Directive 2000/60/EC) and the Marine Strategy Framework Directive (MSFD, 2008/56/EC). While the former had already pointed out the relevance of biological monitoring for the evaluation of water quality, biomarkers as tools for assessing pollution effects at the individual level have mainly been proposed for the MSFD (Lyons et al. 2010; Giltrap et al. 2013; Bellas et al. 2014). The recognition of the value of biomarkers in the evaluation of pollution effects in the marine environment is a step forward for assessing pollution effects. Depending on the intensity and persistence of the pollution source, the effects of pollution can be manifested at different levels of biological organization (e.g., Richardson et al. 2011; Pereira et al. 2012). The first signs will be most likely observed at lower organization levels, i.e., gene, proteins, and up to the organism level, and these changes could then lead to changes at the population and community levels (Moore et al. 2004). Biomarkers at the biochemical level can provide information on the qualitative and quantitative relationships between pollutant exposure and biological responses, and some of them can predict responses at higher levels of biological organization (Hyne and Maher 2003; Seabra Pereira et al. 2014). Such early warnings of marine pollution are extremely important, as timely detection will allow corrective measures to be undertaken, avoiding irreversible effects on the entire ecosystem.

The ability of different biomarkers to detect biological effects of pollutants in marine organisms has been shown by numerous studies, under different disturbance scenarios and across different geographical regions (e.g., Galloway et al. 2004; Lehtonen et al. 2006; Zorita et al. 2007; Gagné et al. 2008; Bellas et al. 2014). Nevertheless, studies analyzing trends over large spatial scales, particularly in the Eastern Mediterranean and Black Sea, are scarce. Although some biomarkers have been included in international environmental monitoring programs, across large geographical areas, the lack of comparability of the data is a flaw of the biomarker approach (Sanchez and Porcher 2009). Bivalves, including mussels of the genus *Mytilus* (e.g., *Mytilus galloprovincialis* in the Mediterranean Sea versus *Mytilus edulis* in the North Sea), as well as fish (e.g., *Mullus* sp., *Platichthys flesus*, *Zoarces viviparus*, and *Perca* sp.) are the most commonly used sentinel species for monitoring pollutant effects in coastal environments. This is primarily due to their wide geographical distribution, abundance, and accessibility in the field (bivalves), as well as position in the trophic chain and their key role in human nutrition (fish) (Viarengo et al. 2007; Thain et al. 2008). Considering the geographical scale encompassed by the MSFD, it is imperative that countries involved validate common indicators (e.g., suite of biomarkers) and approaches (e.g., sentinel species, methodologies) for the evaluation of the effects of pollutants in the marine ecosystem. However, the use of common sentinel species for large geographical areas may not be feasible as many of them are not cosmopolitan. On the other hand, congener species may respond differently to pollution (Moschino et al. 2011). In this regard, it is essential to analyze response patterns of a common set of biomarkers in a variety of species.

The present study assessed the effects of pollution in the Eastern Mediterranean (Adriatic Sea, Aegean Sea, Levantine Sea) and the Black Sea using a suite of biochemical biomarkers. The study utilized the well-recognized sentinel species *M. galloprovincialis* and also the alternative sentinel species *Brachidontes pharaonis*, *Rapana venosa*, *Mullus surmuletus*, and *Diplodus sargus sargus*. In each geographical area, reference and impacted sites differing in contamination levels were selected. The mussel *M. galloprovincialis* was investigated in the Adriatic, Aegean, and Black Sea. Since *M. galloprovincialis* is not present in the Levantine Sea, alternative sentinel species (the mussel *B. pharaonis* and the fish *M. surmuletus*) were investigated in this area. The fish *D. sargus sargus* and the gastropod *R. venosa* were additionally applied in the Adriatic and the Black Sea, respectively. Activities of the antioxidant enzyme catalase (CAT), the phase II biotransformation enzyme glutathione *S*-transferase (GST), and the enzyme of neurotransmission acetylcholinesterase (AChE) as well as levels of the metal binding proteins metallothioneins (MTs) were used as biochemical biomarkers. These biomarkers are among the most widely used and proposed as suitable for biomonitoring in the Mediterranean Sea (Viarengo et al. 2007). Moreover, as supporting parameters, the condition index in molluscs and the condition factor in fish were evaluated to highlight the general physiological condition and the nutritive status of the selected organisms.

The aims of the present study were (1) to investigate whether the responses to pollution of the suite of biochemical biomarkers are consistent across the study areas in a well-recognized sentinel species (*M. galloprovincialis*) and (2) to compare the biomarker responses in alternative sentinel species with those observed in *M. galloprovincialis.* In addition, an attempt was made to compare environmental stress levels at the selected sites across the study areas by integration of biomarker responses using the "Integrated Biological Response version 2" (IBRv2) index. The IBRv2 index has been proposed as an integrative tool that can be used without species limitation in large monitoring programs (Sanchez et al. 2013).

Materials and methods

Sentinel species, study sites, and animal sampling

The Mediterranean mussel *M. galloprovincialis* occurs in the low intertidal zone of exposed rocky coasts with relatively high wave energy. It is a native of the Mediterranean and Black Seas and is commonly used as sentinel in ecotoxicological investigations (Viarengo et al. 2007). *B. pharaonis* is an Indo-Pacific mussel that has colonized the Mediterranean Sea via the Suez Canal. It is abundant in midlittoral rocky habitats. Both *M. galloprovincialis* and *B. pharaonis* are sedentary filter feeders, attached by byssus threads to rocks and stones. The veined whelk *R. venosa* is a large predatory gastropod that occurs in the Black Sea down to 30 m depth in areas with sandy bottoms, as well as in rocky and muddy habitats. *R. venosa* is native of Asian waters; it was introduced into the Black Sea in the 1940s, and has also been reported in the Aegean and Adriatic Seas, North Sea, Uruguay, and Chesapeake Bay (USA). The striped red mullet *M. surmuletus* is a benthic fish species found in the Mediterranean Sea, eastern North Atlantic Ocean, and the Black Sea. Adults occur on broken and rough grounds but are also found over sand and soft bottoms at depths less than 100 m and feed on benthic organisms. The white sea bream *D. sargus sargus* is common in the Mediterranean but rare in the Black Sea. It is also present in the East Atlantic coast and South Africa. It is a demersal fish species, inhabiting littoral waters on rocky bottoms and sand close to rocks, up to 50 m depth in the Mediterranean Sea. Adults are carnivorous.

Animal sampling was carried out in seven geographical areas: along the Slovenian and Italian coasts (Venice Lagoon and Apulia coast) of the Adriatic Sea, the Greek coast (Saronikos Gulf) of the Aegean Sea, the southeast Cypriot coast of the Levantine Sea, as well as the Romanian (Constanta area) and Russian coasts (Blue Bay) of the Black Sea (Fig. 1). Two to four sampling sites were selected in each geographical area including one reference site (away from known pollution sources) as well as sites affected by anthropogenic activities (maritime traffic, industrial, agricultural, and urban activities). Specifically, four sites were sampled along the Slovenian coast (SL_S1, SL_S2, SL_S3, SL_Ref), two sites in the Venice Lagoon (IT_S1, IT_Ref1), two sites along the Apulia coast (IT_S2, IT_Ref2), four sites in Saronikos Gulf (EL_S1, EL_S2, EL_S3, EL_Ref), four sites along the Cyprus southeast coast (CY_S1, CY_Ref1, CY_S2, CY_Ref2), two sites in the Constanta area (RO_S1, RO_Ref), and two sites along the Russian coast (RUS_S1, RUS_Ref). The types of anthropogenic pressures, trophic status, temperature, and salinity during samplings as well as sentinel species sampled in each area are shown in Table 1. Hot spot sites such as ports and marinas were included in some geographical areas (the Slovenian, the Greek, and the Romanian coast).

In each geographical area, animals of similar size (Table 1) were collected between April and May 2013. Across all the areas, the length of *M. galloprovincialis* ranged between 4 and 8 cm. The length of *B. pharaonis* and *R. venosa* ranged from 2 to 4 cm and from 4 to 7 cm, respectively. The length of the fish species *D. sargus sargus* and *M. surmuletus* ranged from 25 to 29 cm and from 39 to 54 cm, respectively. Molluscs (*M. galloprovincialis*, *B. pharaonis*, and *R. venosa*) were collected by hand (including while diving). Fish were collected by spearfishing (*D. sargus sargus*) or trawling nets (*M. surmuletus*). Forty to 45 animals of each species were collected from each site (except *B. pharaonis* where 115 animals were collected per site due to the small size of individuals). The animals were transferred to the laboratory within a few hours of sampling in moist and cool conditions (molluscs) or in aerated cooled containers with seawater from the sampling site (fish). Whole soft tissues of molluscs were stored at −20 °C for condition index measurements (10 to 15 individuals per site). Fish were anesthetized on ice and sacrificed by decapitation. Fish weight and length were recorded for condition factor determination (15 individuals per site). Gills and digestive glands of molluscs and muscle and liver of fish were sampled for biomarker analyses (30 individuals per site). Tissue samples were pooled (samples of six individuals) and five pooled samples per site were frozen in liquid nitrogen. Due to the small size of *B. pharaonis*, tissues of 20 individuals were pooled in each sample (five pooled samples per site). Samples were stored at −80 °C and were transported in dry ice to the Hellenic Center for Marine Research (HCMR), Greece, where the biomarker analyses were performed.

To characterize the study sites in terms of chemical pollution, existing data on metal, polycyclic aromatic hydrocarbon

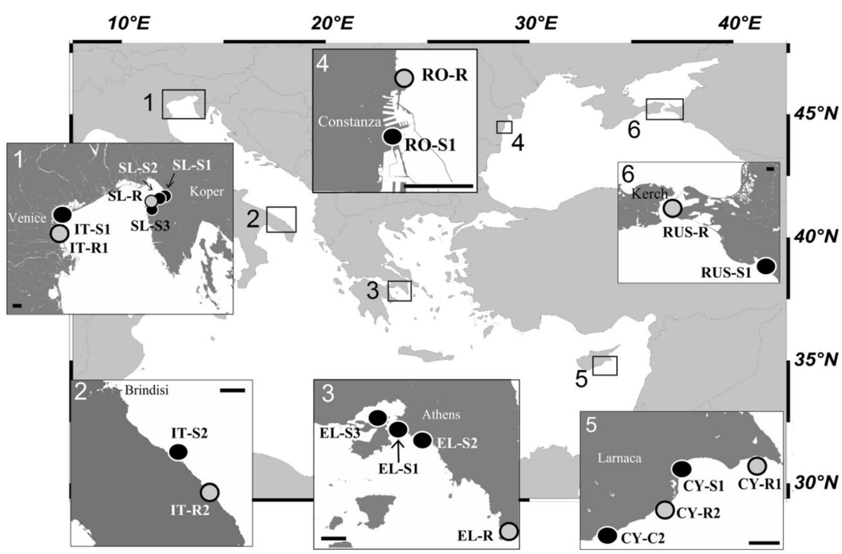

Fig. 1 Location of the sampling sites at different areas in the Eastern Mediterranean and Black Sea. *Scale bar*: 5 km. *SL* Slovenian coast, *IT* Italy (Lagoon of Venice and Apulian coast), *EL* Greece (Saronikos Gulf), *RO* Romania coast, *CY* Cyprus southeast coast, *RUS* Russian coast, *R* reference sites, *S* impacted sites

Table 1 Site description according to types of pressures and trophic status, temperature and salinity during sampling, indicator species used at each area, and size of animals sampled (length in cm)

Area	Location	Sampling site	Latitude and longitude	Type of pressure	Trophic status	*T* (°C)	*S*	Indicator species	Length (cm)
Adriatic Sea									
Slovenian coast	Bay of Koper	SL-S1	45°32′53.4″N 13°43′22.26″E	Maritime transport (port and urban traffic)	Oligotrophic to mesotrophic[1]	13	37.5	Mussel *Mytilus galloprovincialis*	5.9–6.9
	Bay of Strunjan	SL-S2	45°31′41.70″N 13°36′5.88″E	Agricultural influence	Oligotrophic to mesotrophic[1]	11	37.9		6.4–7.8
	Bay of Piran	SL_S3	45°29′41.88″N 13°34′53.16″E	Agricultural influence, fish farm	Oligotrophic to mesotrophic[1]	13	37.5		6.8–7.5
	Bouy Vida (reference)	SL_Ref	45°32′55.68″N 13°33′1.89″E	Coastal waters	Oligotrophic to mesotrophic[1]	13	37.6		6.4–8
Italian coast—Venice lagoon	Sacca sessola	IT_S1	45°24′10.60″N 12°18′43.91″E	Urban traffic (boats)	Mesotrophic to eutrophic[2]	20.0	32.8	Mussel *Mytilus galloprovincialis*	5.8
	Cà Roman (reference)	IT_Ref1	45°14′55,31″N 12°17′37.92″E	Seaward near the inlet	Oligotrophic to mesotrophic[2]	19.8	33.2		5.7
Italian coast—Apulia	Brindisi	IT_S2	40°28′37.00″N 18°14′47.00″E	Industrialized area	Oligotrophic to mesotrophic[3]	21.5	38.5	White seabream *Diplodus sargus sargus*	24.8–28.7
	Torre Guaceto (reference)	IT_Ref2	40°43′20.00″N 17°47′39.00″E	Protected area	Oligotrophic to mesotrophic[3]	21.5	38.5		25.5–29.3
Aegean Sea									
Greek coast—Saronikos Gulf	Perama bay	EL-S1	37°57′50.96″N 23°33′19.23″E	Port	Mesotrophic[4]	20	39	Mussel *Mytilus galloprovincialis*	4.3–5.3
	Marina Zeas	EL-S2	37°55′53.36″N 23°38′59.53″E	Marina	Mesotrophic[4]	21	38		4.3–5.3
	Salamina island	EL-S3	38° 0′0.05″N 23°28′35.33″E	Industrialized area	Mesotrophic[4]	20	38		4.5–5.3
	Patroklos (reference)	EL-Ref	37°39′35.97″N 23°57′20.55″E	Fish farm	Oligotrophic[4]	20	38		4.4–5.6
Levantine Sea									
Cyprus southeast coast	Larnaca	CY-S1	34°57′13.71″N 33°39′07.51″E	Gas station, urban wastes	Oligotrophic[5]	19.1	39.2	Mussel *Brachidontes pharaonis*	3–4
	Ayia Napa (reference)	CY-Ref1	34°59′11.97″N 33°58′46.53″E	Tourist resort	Oligotrophic[5]	22.3	39.2		2–3
	Vassiliko	CY-S2	34°42′58.01″N 33°19′29.65″E	Industrialized area, power plant, port	Oligotrophic[5]	21.2	39.1.	Red striped mullet *Mullus surmuletus*	38.9
	Faros-Larnaka (reference)	CY-Ref2	34°48′16.62″N 33°35′34.04″E	Rural, agricultural influence	Oligotrophic[5]	n.d.	n.d.		54.1
Black Sea									
Romanian coast—Constanta	South Constanta	RO-S1	44°08′17.76″N 28°38′56.16″E	Wastewater treatment plant, port	Eutrophic[6]	10	12.4	Mussel *Mytilus galloprovincialis*	5–6
	East Constanta (reference)	RO-Ref	44°10′0.00″N 28°41′0.00″E	Coastal waters	Mesotrophic[6]	10	10.8		5–5.5
Russian coast	Blue Bay	RUS-S1	44°33′57.30″N 37°58′56.40″E	Coastal waters, tourist resort	Eutrophic[7]	16	18	Veined whelk *Rapana venosa*	4–7
	Tuzla Spit (reference)	RUS-Ref	45°14′3.12″N 36°36′6.17″E	Protected area	Eutrophic[7]	16	17.5		4–7

[1] Mozetič et al.(2010), [2] Sfriso and Facca (2013), [3] Dell'Anno et al. (2002), [4] Pavlidou et al. (2014), [5] DFMR (2012), [6] Lazar et al. (2013), [7] BSC (2008)

T water temperature, *S* salinity, *SL* Slovenia, *EL* Greece, *IT* Italy, *RO* Romania, *CY* Cyprus, *RUS* Russia, *Ref* reference site, *n.d.* not determined

(PAHs), and polychlorinated biphenyl (PCBs) concentrations in sediments and in sentinel species (mussels and/or fish) were compiled from the literature. At areas where literature data on contaminant levels in biota from the selected study sites was scarce (Greek coast—Saronikos Gulf, Cyprus southeast coast, and Romanian coast—Constanta), additional samples of animals (mussels *M. galloprovincialis* and *B. pharaonis*) were collected for metals, PAHs, and PCBs analyses. The whole soft tissues of the mussels were stored at −20 °C until chemical analyses. Chemical analyses were performed at HCMR (*M. galloprovincialis* samples from Greek coast—Saronikos Gulf; PAHs and PCBs—pooled sample of 20 individuals per site; metals—6 pooled samples of 20 individuals per site) and at National Institute for Marine Research and Development (NIMRID), Romania (*B. pharaonis* samples from Cyprus southeast coast and *M. galloprovincialis* samples from Romanian coast—Constanta; PAHs, PCBs, and metals—pooled sample of ten individuals per each site).

Physiological status of the organisms

Condition index (CI) was determined as an indicator of the physiological status of the molluscs. CI is an ecophysiological measure of the health status of the animals that summarizes their physiological activity (e.g., growth, reproduction, secretion) under given environmental conditions (Lucas and Beninger 1985). The whole soft tissues of 10 to 15 individuals were dissected and lyophilized; shells were dried at 60 °C for 48 h and then weighed. The ratio of dry flesh weight to dry shell weight (FW/SW × 100) was used to determine CI for each sample (Davenport and Chen 1987).

Condition factor (CF) was determined as an indicator of the physiological status of the fish. CF is influenced by factors such as the nutritional and reproductive status, thus leading to weight variations (Rätz and Lloret 2003). CF was calculated as $CF = 100 \times \text{total weight}/\text{total length}^3$ (Nash et al. 2006).

Biochemical biomarker analyses

Catalase and glutathione S-transferase activity

Digestive glands (molluscs) and liver (fish) were homogenized using a Potter-Elvehjem homogenizer (Heidolph Electro GmbH, Kelheim, Germany) in 1:5 (*w*/*v*) 100 mM KH_2PO_4/K_2HPO_4, pH 7.4. Homogenates were centrifuged at 10,000×*g* for 30 min. All preparation procedures were carried out at 4 °C. CAT activity was measured through the loss of H_2O_2 that was measured colorimetrically with ferrous ions and thiocyanate on a microplate reader (Assys Digiscan reader 340) (Cohen et al. 1996). CAT activity was determined by the difference in the absorbance at 490 nm per unit of time. CAT activity results are expressed in terms of the first-order reaction rate constant (k) and protein content as follows: U/mg proteins = k/mg proteins = $[\ln (A_1/A_2)/(t_2 - t_1)]$/mg proteins where U represents units, ln is the natural log, and A_1 and A_2 are the observed mean absorbance at 490 nm at two time points, $t_1 = 1$ min and $t_2 = 4$ min. GST was measured by the method of Habig and Jacoby (1981) with 1-chloro-2,4-dinitrobenzene (CDNB) as a conjugation substrate, adapted to microplate reading by McFarland et al. (1999). Activity was expressed as nanomoles of conjugate per minute per milligram of proteins. Total protein content in the tissue extracts was measured using bovine serum albumin (BSA) as a standard (Bradford 1976).

Acetylcholinesterase activity

Gill (molluscs), muscle (*M. surmuletus*), and liver (*D. sargus sargus*) tissues were homogenized using a Potter-Elvehjem homogenizer in 1:2 (*w*/*v*) 0.1 M Tris–HCl buffer containing 0.1 % TRITON X 100, pH 7. Homogenates were centrifuged at 10,000×*g* for 20 min. All preparation procedures were carried out at 4 °C. AChE activity was assayed by the method of Ellman et al. (1961) adapted to microplate reading by Bocquené et al. (1993) on an Assys Digiscan reader 340. Enzyme activity was expressed as nanomoles of acetylthiocholine hydrolyzed per minute per milligram of proteins.

Metallothioneins content

MTs concentration was measured in digestive glands (molluscs) and liver (fish) tissues according to Viarengo et al. (1997) on a Perkin Elmer UV/VIS spectrophotometer Lamda 20. The method is based on the estimation of the sulfhydryl content of MTs proteins by spectrophotometric determination of the –SH groups using Ellman's reagent. MTs concentration was calculated utilizing reduced glutathione (GSH) as a reference standard and expressed as micrograms of MTs per gram of wet weight of tissue.

Chemical analyses in mussel tissues

Metal analyses were performed according to UNEP (1984) and IAEA-MEL (1999). The following metals were analyzed: Cd, Cu, Pb, Cr, and Zn. The accuracy and precision of the analytical methodology were verified with the standard reference material SRM 2976 which was provided by the National Institute of Standards and Technology—USA (NIST). The methodology used for PAH and PCB analysis at HCMR was described in Tsangaris et al. (2011a) PAH and PCB concentrations at NIMRD were determined according to IAEA-MEL (1995). The accuracy and precision of the analytical methodology was tested using certified reference material provided by IAEA (IAEA—432, mussel homogenate) (HCMR) and NIST (SRM—2977, mussel homogenate) (NIMRD). ΣPAHs: acenaphthene, acenaphthylene, anthracene, benzo[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo(*g*,*h*,*i*)perylene, crysene, dibenzo(*a*,*h*)anthracene, fluoranthene, fluorine, indeno(1,2,3-*c*,*d*)pyrene, naphthalene, phenanthrene, and pyrene, and ΣPCBs: 28-CB, 52-CB, 101-CB, 118-CB, 138-CB, 153-CB, and 180-CB, were determined.

Data and statistical analysis

To compare the total metal content on both sediment and biological matrices across the study areas, the metal pollution index (MPI) (Usero et al. 1996) was calculated for both sediments and organisms:

$$\mathrm{MPI} = (M_1 \times M_2 \times M_3 \ldots \times M_n)^* 1 / n,$$

where M_n is the concentration of metal n expressed in micrograms per gram of dry weight.To assess for significant changes in the response of the different biomarkers and physiological indices (condition index and condition factor), univariate analysis of variances (ANOVA) followed by Fisher's LSD multiple comparison test was applied comparing the values recorded at the reference site with those of the impacted sites in each geographical area. Prior to the analysis, data were checked for normality (Shapiro-Wilk's test) and homogeneity of variances (Levene's test). The variability of each biomarker was graphically expressed as percent alteration with respect to the reference site in each area, calculated according to the following formula:

$$\%\,\mathrm{alteration} = 100^*(\mathrm{Reference\,site}-\mathrm{Impacted\,site}) / \mathrm{Reference\,site}$$

Principal component analysis (PCA) was performed using two different data matrices: (1) percent alteration of biomarker and CI or CF values with respect to the reference site in each area for a better comparison of the variability obtained in each geographical area and sentinel species, and (2) biomarker and CI data obtained in mussel *M. galloprovincialis*, for a comparison of variability of levels and responses to pollution in this widely used sentinel species among geographical areas. STATISTICA 6.0 software (StatSoft) was used for all statistical processing.

The "Integrated Biological Response version 2" (IBRv2) index was applied to integrate biomarker data into a value representing the environmental stress level at the impacted sites in the various geographical areas (Sanchez et al. 2013). It is a modification of the "Integrated Biomarker Response" (IBR) index (Beliaeff and Burgeot 2002) based on the reference deviation concept, i.e., the deviation between a disturbed and an undisturbed state. The four biochemical biomarkers measured (CAT, AChE, and GST activities and MTs content) were introduced in the IBRv2 calculation. For the calculation of IBRv2, in each geographical area, individual biomarker data (X_i) were compared to reference data (X_0) and a log transformation was applied to reduce variance: $Y_i = \log X_i / X_0$. For each biomarker, the general mean (μ) and standard deviation (s) of Y_i for all sites were computed and Y_i was standardized as $Z_i = (Y_i - \mu)/s$. The mean of standardized biomarker response (Z_i) and mean of reference biomarker data (Z_0) were used to define a biomarker deviation index (A), $A_i = Z_i - Z_0$ for each biomarker in each site. Then, to obtain the IBRv2, the absolute value of A parameters calculated for each biomarker in each site were summed as $\mathrm{IBRv2} = \Sigma|A|$

Results

Contaminants in sediments and in sentinel species

Levels of metals, PAHs, and PCBs in sediments and in tissues of mussel and/or fish species at all sampling sites are presented in Tables 2 and 3, respectively. ERL values (effect range-low) proposed by Long et al. (1995) for chemicals in sediments are also reported in Table 2. In some cases, due to shortage of recent data in the literature, old data was used in an attempt to provide an indicative picture of the type of pollution at the sampling sites even if an in-depth chemical characterization was not possible. Overall, comparison of contaminant levels in sediments and in organisms between sites within each geographical area confirmed higher contamination at the impacted sites compared to the reference ones. Three reference sites were particular as regards contaminant levels in organisms, where even if several contaminants showed markedly lower levels than the impacted sites, this was not the case for all the contaminants analyzed, i.e., Cu, Pb, and Cr in mussels from Ca' Roman (IT_Ref1) in the Italian coast, PCBs in mussels from East Constanta (RO_Ref) in the Romanian coast, and Cd, Pb, and Cr in mussels from Ayia Napa (CY_Ref1) in the Cyprus measured southeast coast. These sites are regarded as local reference sites (Moschino et al. 2011, 2012; Coatu et al. 2013) and were thus selected as such in the present study.

The most contaminated sediments, particularly by metals, were found at the Bay of Koper (where Cu, Zn, and Hg exceeded the ERL values), Bay of Strunjan, and Bay of Piran (where Hg and Cr exceeded the ERL values) on the Slovenian coast; at Perama Bay and Marina Zeas on the Greek coast (where Cu, Pb, Cr, and Zn exceeded the ERL values); and at South Constanta on the Romanian coast (where Cd, Cu, Pb, and Cr exceeded the ERL values). Higher metal concentrations were detected in mussels from Perama Bay and Marina Zeas, Greece, and from Blue Bay, Russia. These observations are also confirmed by the MPI values calculated for both sediments and organisms (Tables 2 and 3).

Table 2 Concentrations of metals (μg/g), total polycyclic aromatic hydrocarbons (PAHs) (ng/g), and total polychlorinated biphenyls (PCBs) (ng/g) reported in sediments at the sampling locations (the Metal Pollution Index [MPI] values are also shown)

Area	Location	Cd (μg/g)	Cu (μg/g)	Pb (μg/g)	Hg (μg/g)	Cr (μg/g)	Zn (μg/g)	MPI	PAHs (ng/g)	PCBs (ng/g)
Adriatic Sea										
Slovenian coast	Bay of Koper	0.11[1]	32[1]	24[1]	0.25[1]	83[1]	100[1]	40.4	523[2]	–
	Bay of Strunjan	0.126[3]	16.6[3]	8.0[3]	0.197[4]	123[3]	56[3]	5.3	–	–
	Bay of Piran	0.091[3]	30.9[3]	18.3[3]	0.136[4]	150[3]	109[3]	7.0	414[2]	–
	Bouy Vida (Ref)	0.096[3]	15.6[3]	8.7[3]	–	66[3]	35[3]	7.9	330[2]	–
Italian coast—Venice lagoon	Sacca sessola	0.63[5]	20.7[5]	22.1[5]	0.88[5]	34[5]	90[5]	9.6	382[5]	0.61[5]
	Cà Roman (Ref)	0.43[5]	9.3[5]	9.97[5]	0.11[5]	38[5]	48[5]	4.5	384[5]	0.27[5]
Italian coast—Apulia	Brindisi	0.034[6]	3.27[6]	38.50[6]	–	8.4[6]	79.23[6]	4.9	–	–
	Torre Guaceto (Ref)	0.098[6]	19.41[6]	32.58[6]	–	29.7[6]	10.86[6]	7.2	–	–
Aegean Sea										
Greek coast—Saronikos Gulf	Perama bay	–	324[7]	374[7]	–	330[7]	973[7]	444.1	–	–
	Marina Zeas	–	104[8]	106[8]	–	231[8]	204[8]	151.0	3372[8]	–
	Salamina island	–	–	–	0.13[9]	110–320[10]	–	6.4	4800[11]	–
	Patroklos (Ref)	–	–	–	–	–	–		31.2[12]	–
Levantine Sea										
Cyprus southeast coast	Larnaca	–	–	–	–	–	–		–	–
	Ayia Napa (Ref)	–	–	–	–	–	–		–	–
	Vassiliko	3[13]	53[13]	20[13]	nd[13]	81[13]	47[13]	26.1	–	*trace*[13]
	Faros-Larnaka (Ref)	–	–	–	–	–	–		–	–
Black Sea										
Romanian coast—Constanta	South Constanta	3.77[14]	109.49[14]	55.01[14]	–	99.98[14]	–	38.8	52.46[14]	16.2[14]
	East Constanta (Ref)	0.09[14]	3.25[14]	9.72[14]	–	13.13[14]	–	2.5	0.071[14]	152.4[14]
Russian coast	Blue Bay	0.18[15]	55.24[15]	28.03[15]	0[15]	68.13[15]	–	11.7	294.49[15]	35.3[15]
	Tuzla Spit (Ref)	0.38[15]	22.23[15]	27.58[15]	0.01[15]	95.39[15]	15[15]	3.9	250.97[15]	27.1[15]
ERL[16]		1.2	34	46.7	0.15	81	150		4022	22.7

[1] ARSO (2015), [2] Bajt (2012), [3] Ščančar et al. (2007), [4] ARSO (2012), [5] Moschino et al. (2012), [6] Ianni et al. (2003), [7] Kaberi and Zeri (2004), [8] HCMR (2003), [9] Stathopoulou et al. (2001), [10] Sklivagou et al. (2008), [11] Laboratory Network for the Environmental Quality Monitoring of the Hellenic Seas (2006), [12] Tsapakis et al. (2010), [13] DFMR (2012), [14] Coatu et al. (2013), [15] BSC (2008), [16] ERL guidelines from Long et al. (1995)

Ref reference site, *nd* not detected

Biological responses

Mean values (±SE) of the biochemical biomarkers measured in different sentinel species are listed in Table 4. CAT activity showed low variability in the mussel *M. galloprovincialis* (ranging from 1.6 to 5.0 U/mg proteins), as well as in the veined whelk *R. venosa* (2.4–3.7 U/mg proteins). Higher values were observed in the fish species *M. surmuletus* and *D. sargus sargus* (10.2–11.2 U/mg proteins). The highest CAT activity was observed in the mussel *B. pharaonis*, with values ranging from 31.2 to 38.8 U/mg proteins. AChE activity in mussels *M. galloprovincialis* across areas ranged from 13 to 88 nmol/min/mg proteins. The highest AChE activity was detected in *R. venosa* (from 286.2 to 289.8 nmol/min/mg proteins) and in both fish species (from 171.1 to 295.3 nmol/min/mg proteins). *B. pharaonis* showed the lowest AChE activities (9.0–10.6 nmol/min/mg proteins). Particularly low GST activity was detected in *M. galloprovincialis* collected in the Slovenian coast (3.7–5.3 nmol/min/mg proteins), whereas mussels from the other studied areas showed higher values (34.1–83.4 nmol/min/mg proteins). The highest GST activity was exhibited by *D. sargus sargus* (265–314 nmol/min/mg proteins) and *R. venosa* (320–389 nmol/min/mg proteins). MTs content values were particularly low in *M. galloprovincialis* from the Lagoon of Venice (73–78 μg/g tissue) in comparison with those observed in mussels from the other geographical areas (130–251 μg/g tissue). The highest MTs content values were detected in *D. sargus sargus* (328 and 428 μg/g tissue in reference and impacted sites, respectively).

Comparisons between impacted and reference sites within each geographical area showed significantly lower CAT

Table 3 Levels of metals (μg/g dw), total polycyclic aromatic hydrocarbons (PAHs) (ng/g dw), and total polychlorinated biphenyls (PCBs) (ng/g dw) in biota (molluscs and/or fish) at the sampling locations (the Metal Pollution Index [MPI] values are also reported)

Area	Location	Cd	Cu	Pb	Hg	Cr	Zn	MPI	PAHs	PCBs
Mussel *Mytilus galloprovincialis*										
Adriatic Sea										
Slovenian coast	Bay of Koper	1.1[1]	–	–	0.126[1]	–	–	0.5	506[3]	–
	Bay of Strunjan	1.09[1]	6.4[1]	0.84[1]	0.155[1]	0.095[1]	108[1]	1.5	215[3]	–
	Bay of Piran	0.1[2]	7.2[1]	0.64[1]	0.017[1]	0.032[1]	95[1]	0.5	–	–
	Bouy Vida (Ref)	–	–	–	–	–	–		–	–
Italian coast—Venice lagoon	Sacca Sessola	1.04[4]	7.23[4]	2.58[4]	2.36[4]	0.72[4]	186.00[4]	4.3	136.8[4]	13.3[4]
	Cà Roman (Ref)	0.75[4]	8.83[4]	4.28[4]	1.54[4]	0.89[4]	144.75[4]	4.2	28.7[4]	7.9[4]
Aegean Sea										
Greek coast—Saronikos Gulf	Perama bay	–	16.36	–	–	1.56	98	13.6	350.7	394.5
	Marina Zeas	–	32.26	–	–	0.67	122	13.8	284.7	59.7
	Salamina island	–	6.62	–	–	0.29	120	6.1	–	–
	Patroklos (Ref)	–	7.71	–	–	0.76	145	9.5	40.9	10.9
Black Sea										
Romanian coast—Constanta	South Constanta	0.67	2.49	0.88	–	0.45	–	0.9	1430	19.46
	East Constanta (Ref)	0.73	1.36	0.33	–	0.26	–	0.5	146	84.65
Russian coast	Blue Bay	2.75[5]	39.8[5]	118.84[5]	0 [5]	42.63[5]	–	27.3	748.19 [5]	–
	Tuzla Spit (Ref)	1.77 [5]	7.7 [5]	1.15[5]	0[5]	13.15[5]	–	3.8	720.49 [5]	–
Mussel *Brachidontes pharaonis*										
Levantine Sea										
Cyprus southeast coast	Larnaca	1.15	25.34	0.03	–	2.39	–	1.2	579	16.79
	Ayia Napa (Ref)	1.73	19.29	0.11	–	4.95	–	2.1	21.6	7.31
Red mullet *Mullus barbatus*										
Adriatic Sea										
Italian coast—Apulia	Brindisi	–	2.45[6]	–	0.99[6]	–	–	1.6	3.74[6]	33.31[6]
	Torre Guaceto (Ref)	–	2.89[6]	–	0.43[6]	–	–	1.1	3.11[6]	7.43[6]
Levantine Sea										
Cyprus southeast coast	Vassiliko	0.005[7]	–	0.39[7]	0.87[7]	–	–	0.1	–	–
	Faros-Larnaka (Ref)	0.03[7]	–	0.22[7]	0.21[7]	–	–	0.1	–	–

Data without footnotes are obtained in this study

[1] Ramšak et al. (2012), [2] ARSO (2013), [3] ARSO (2012), [4] Moschino (unpublished data), [5] Database of the State Oceanographic Institute http://esimo.oceanography.ru/esp2/index/index/esp_id/10/section_id/8/menu_id/4426, [6] Corsi et al. (2002), [7] DFMR (2012)

Ref reference site

activities in *M. galloprovincialis* and *R. venosa* from the Slovenian, Greek, and Russian impacted sites (Fig. 2a). Significantly lower AChE activities at the impacted sites with respect to the reference sites were observed in mussels from the Slovenian, Greek, Italian, and Romanian coasts (Fig. 2b). On the contrary, fish from the Italian coast showed significantly higher AChE activity at the impacted compared to the reference site (Fig. 2b). Significant variations in GST between impacted and reference sites were found in mussels from the Slovenian, Greek, and Italian coasts, with higher activities at the three Greek impacted sites and lower activities at the Slovenian and Italian impacted sites (Fig. 2c). Significantly higher GST activity with respect to the reference site was also observed in fish from the impacted site in the Cyprus coast. MTs content was less variable in comparison with enzymatic activities in mussels (Fig. 2d). Significant differences in MTs content of mussels in comparison to the reference sites were observed only at SL_S2 and EL_S2 in the Slovenian and Greek coast, respectively. A markedly higher MTs content at the impacted site with respect to the reference (+231 %) was detected in *R. venosa* from the Russian coast.

CI values of molluscs and CF values of fish (mean±SE), and the statistical comparison between impacted and reference sites within each geographical area, are shown in Figs. 3 and 4, respectively. CI values in *M. galloprovincialis* showed high variability ranging from 4.5 at EL_Ref in the Greek coast to

Table 4 Biomarkers (mean±SE) in the selected sentinel species at the various sampling locations

Species	Sampling site	Biochemical biomarkers			
		CAT (U/mg proteins)	AChE (nmol/min/mg proteins)	GST (nmol/min/mg proteins)	MT (μg/g tissue)
Mytilus galloprovincialis	SL_Ref	2.2±0.2	45.1±5.6	5.3±0.4	184±13
	SL_S1	1.6±0.0	16.0±1.4	4.6±0.4	239±24
	SL_S2	1.8±0.1	13.2±1.9	3.8±0.5	251±15
	SL_S3	1.6±0.1	21.0±1.4	3.7±0.3	188±20
Mytilus galloprovincialis	EL_Ref	4.5±0.1	88.0±8.3	34.1±1.6	227±15
	EL_S1	3.2±0.3	62.1±4.3	43.7±1.7	193±11
	EL_S2	2.8±0.1	29.6±1.9	38.6±0.7	149±21
	EL_S3	3.1±0.2	79.5±5.0	47.0±4.6	200±16
Mytilus galloprovincialis	IT_Ref1	2.6±0.2	63.1±3.6	50.5±3.0	73±4
	IT_S1	2.2±0.2	50.3±6.3	36.0±4.5	78±5
Mytilus galloprovincialis	RO_Ref	4.3±0.2	45.2±4.3	66.8±4.9	130±30
	RO_S1	5.0±0.7	27.8±6.9	83.4±6.7	140±29
Brachidontes pharaonis	CY_Ref1	38.8±6.5	9.0±1.3	119.3±13.2	264±20
	CY_S1	31.2±1.1	10.6±1.6	102.3±5.4	371±59
Rapana venosa	RUS_Ref	3.7±0.2	289.8±73.0	320.3±36.9	84±8
	RUS_S1	2.4±0.3	286.2±74.1	389.1±34.6	276±24
Mullus surmuletus	CY_Ref2	10.2±0.3	199.2±15.9	83.8±8.6	280±21
	CY_S2	11.1±0.8	198.8±19.3	106.2±6.7	270±53
Diplodus sargus sargus	IT_Ref2	11.2±1.8	171.1±27.6	313.6±18.4	328±30
	IT_S2	10.6±0.8	295.3±37.5	264.7±11.5	428±51

SL Slovenian coast, *EL* Greece (Saronikos Gulf), *IT* Italy (Lagoon of Venice and Apulian coast), *RO* Romania coast, *CY* Cyprus southeast coast, *RUS* Russian coast, *Ref* reference sites, *S* impacted sites

23.7 at SL_S2 in the Slovenian coast. CI values of molluscs were significantly lower than at the reference sites at SL_S3, IT_S1, and RUS_S1, whereas at all the Greek sites and in CY_S1 they were significantly higher than at the reference sites. On the contrary, the comparison between CF values of fish did not exhibit statistically significant differences (Fig. 4).

The multivariate analysis performed on the dataset obtained from the percentage alteration of the biomarkers and CI or CF in the different geographical areas shows that factor 1 and factor 2 explain over 65 % of total variance in the data matrix (Fig. 5). Factor 1 explains 37.33 % of total variance and is characterized by negative loading of the variables CI/CF (−0.90). Factor 2 explains 27.86 % of total variance with CAT and AChE showing the higher loading values (0.81 and 0.79, respectively). The distribution of the biological alteration detected in the various sentinel organisms on the cases score plot highlights the separation of mussels *M. galloprovincialis* and *B. pharaonis* from the two fish species, *M. surmuletus* and *D. sargus sargus*, together with mussels collected along the Romanian coast in the upper right part of the plot, as well as from the gastropod *R. venosa* in the left part of the plot. In the multivariate analysis performed with the *M. galloprovincialis* biomarkers and CI dataset, factor 1 and factor 2 explain over 80 % of total variance in the data matrix (Fig. 6). Factor 1 explains 58.8 % of total variance and is characterized by positive loading of the variable CI (0.88) and negative loading of the variables CAT and GST (−0.90 and −0.88, respectively). Factor 2 explains 22.12 % of total variance and is characterized by negative loading of the variable MT (−0.88). The geographical variability in mussel biomarkers is higher than the variability between impacted and reference sites within each location, with the single exception of EL_S2 (Marina Zeas, Greece).

The values of the IBRv2 index ranged between 1.8 and 7.0 (Fig. 7). Sites showing IBRv2 values of 3 or lower (indicating lower stress levels) were the CY_ S1, CY_S2, and IT-S2. Five sites showed values between 3.7 and 5.3: SI_S1, SL_S3, EL_S1, EL_S3, and IT_S1. The highest values (≥6) were observed at SL_2, EL_ S2, and RUS_S1.

Discussion

The suite of biochemical biomarkers in the well-established sentinel species *M. galloprovincialis* revealed responses of CAT, AChE, and GST activities at the impacted sites across the different geographical areas and MTs responses only at

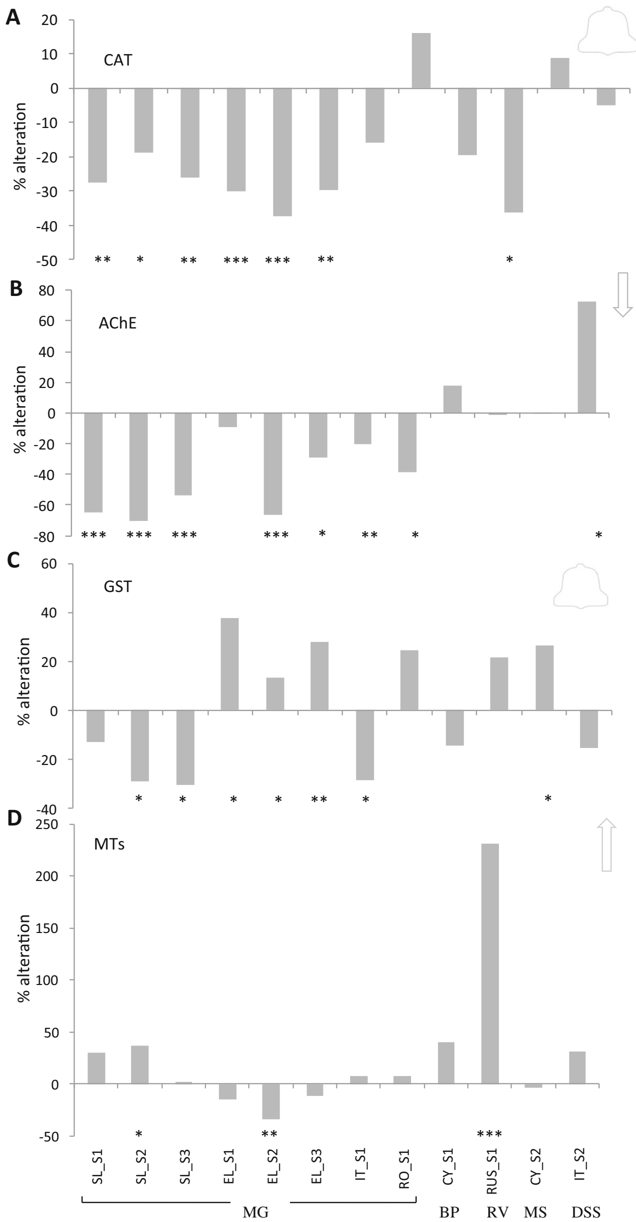

Fig. 2 Catalase activity (**a**), acetylcholinesterase activity (**b**), glutathione *S*-transferase activity (**c**), and metallothioneins content (**d**), expressed as % alteration with respect to each reference site. *SL* Slovenian coast, *EL* Greece (Saronikos Gulf), *IT* Italy (Lagoon of Venice and Apulian coast), *RO* Romania coast, *CY* Cyprus southeast coast, *RUS* Russian coast, *MG Mytilus galloprovincialis*, *BP Brachidontes pharaonis*, *RV Rapana venosa*, *MS Mullus surmuletus*, *DSS Diplodus sargus sargus.* ANOVA: *$p<0.05$; **$p<0.01$; ***$p<0.001$. *Shapes* denote type of expected response to pollution; *bell*: bell shaped, *downward pointing arrow*: inhibition, *upward pointing arrow*: induction

two sites exceeding ERL guidelines values for metals. CAT and AChE responses were consistent across areas showing lower enzymatic activities at the impacted sites, whereas GST showed either lower or higher activities at the impacted sites compared to the reference ones. CAT is an antioxidant enzyme that detoxifies hydrogen peroxide (H_2O_2), the main cellular precursor of the hydroxyl radical ($HO^\bullet$), a highly reactive and toxic form of reactive oxygen species (ROS) involved in oxidative stress, which may cause lipid, protein, and DNA damage (Kehrer 2000). CAT is widely applied as a biomarker of oxidative stress that can be induced by exposure to organic xenobiotics and metals (Livingstone 2001). The enzyme response to pollutants shows a bell-shaped trend, with an initial increase in activity due to the activation of enzyme synthesis followed by a decrease in enzymatic activity, due to the enhanced catabolic rate and/or a direct inhibitory action of toxic chemicals on the enzyme molecules (Viarengo et al. 2007). Thus, high CAT activities found in mussels and fish at polluted sites are considered an adaptive response to ROS-

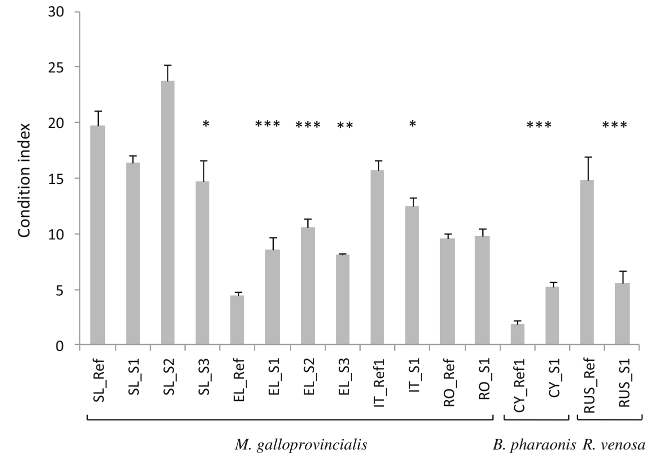

Fig. 3 Condition index (mean±SE), calculated as (dry meat weight/dry shell weight)×100, detected in molluscs (*Mytilus galloprovincialis*, *Brachidontes pharaonis*, and *Rapana venosa*) at the various sampling locations. *SL* Slovenian coast, *EL* Greece (Saronikos Gulf), *IT* Italy (Lagoon of Venice), *RO* Romania coast, *CY* Cyprus southeast coast, *RUS* Russian coast, *Ref* reference sites, *S* impacted sites. ANOVA: *$p<0.05$; **$p<0.01$; ***$p<0.001$

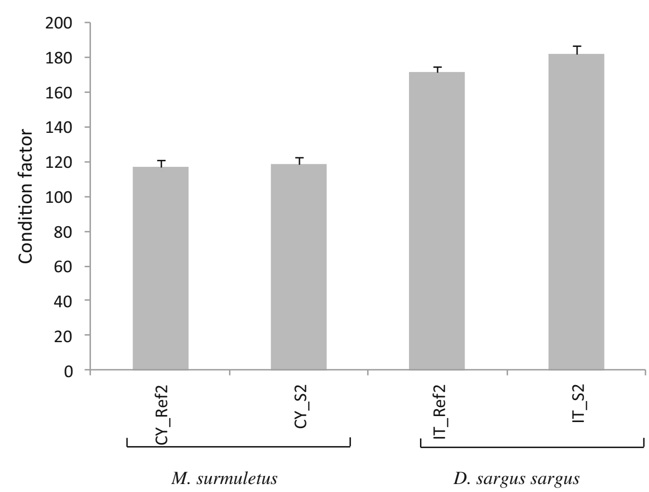

Fig. 4 Condition factor (mean±SE) calculated as (total weight/total length^3)×100, detected in fish (*Mullus surmuletus*, *Diplodus sargus sargus*) at the various sampling locations. *IT* Italy (Apulian coast), *CY* Cyprus southeast coast, *Ref* reference sites, *S* impacted sites

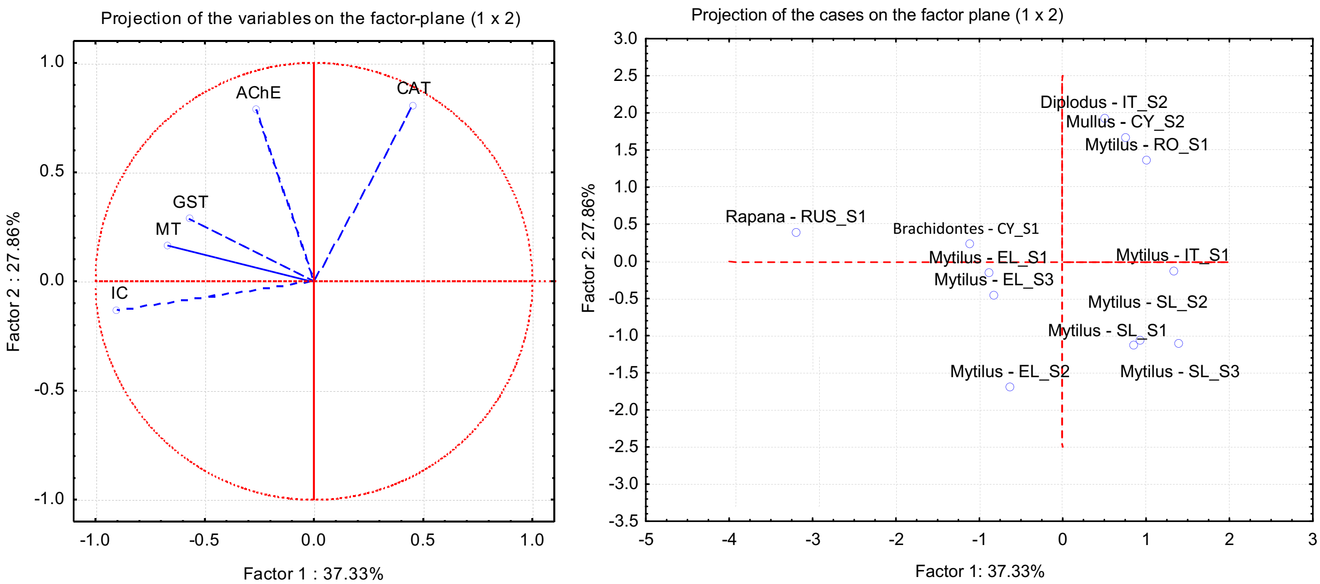

Fig. 5 PCA performed with the data obtained from the % alteration with respect to each reference site of each biomarker (CAT, AChE, GST, MTs) and condition index/factor in the various geographical areas. *SL* Slovenian coast, *EL* Greece (Saronikos Gulf), *IT* Italy (Lagoon of Venice and Apulian coast), *RO* Romanian coast, *CY* Cyprus southeast coast, *RUS* Russian coast, *S* impacted sites

inducing contaminants (Roméo et al. 2003; Cappello et al. 2013; Jebali et al. 2013) whereas low CAT activities at polluted sites are linked with increased susceptibility to oxidative stress (Regoli et al. 2004; Pampanin et al. 2005a; Tsangaris et al. 2011b; Oliva et al. 2012). Accordingly, the low CAT activities observed in this study in mussels collected from the impacted sites suggest oxidative stress experienced by these animals.

AChE is an enzyme involved in nerve impulse transmission, and its inhibition is an established biomarker of neurotoxicity (Fulton and Key 2001). Although organophosphate and carbamate pesticides are the two main classes of compounds ascribed for AChE inhibition (Fulton and Key 2001), it has also been shown that other chemicals can interact with AChE activity, such as metals, detergents, and PAHs (Lionetto et al. 2013). Thus, low AChE activities are frequently found in mussels and fish at impacted sites in various regions under different types of pollution (Lehtonen et al. 2006; Jebali et al. 2013; Bellas et al. 2014) which is in agreement with the present study.

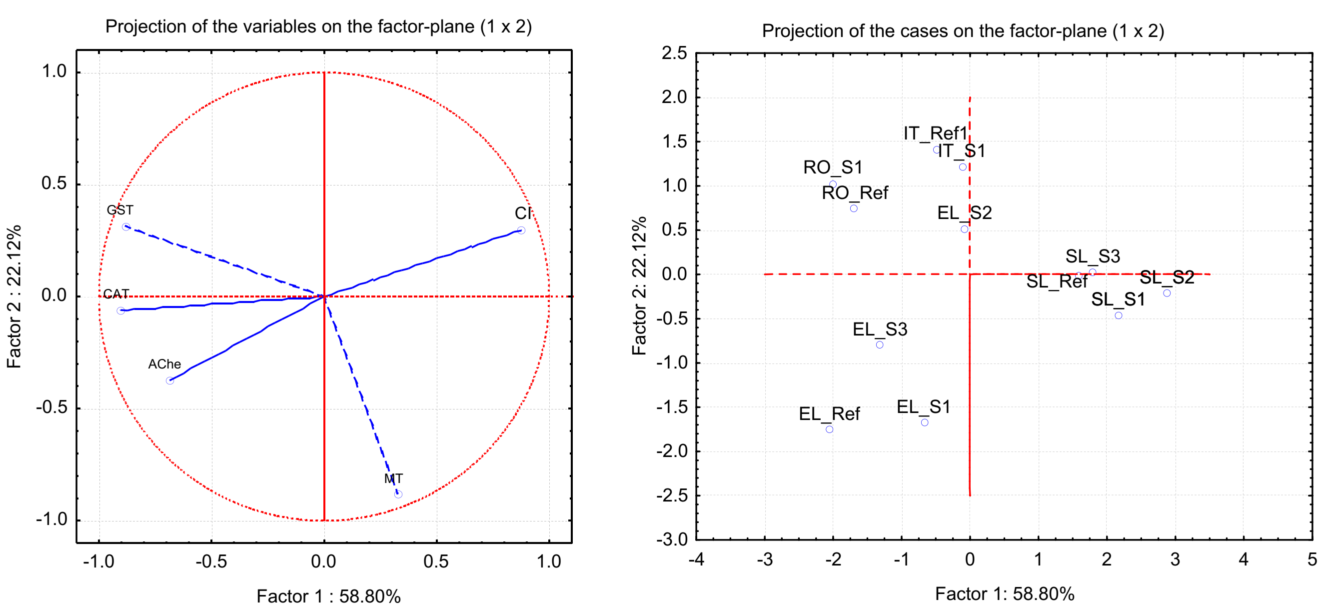

Fig. 6 PCA performed with the data of mussel *Mytilus galloprovincialis* biomarkers (CAT, AChE, GST, MTs) and condition index (CI) from the sampling sites in the various geographical areas. *SL* Slovenian coast, *EL* Greece (Saronikos Gulf), *IT* Italy (Lagoon of Venice), *RO* Romanian coast, *Ref* reference sites, *S* impacted sites

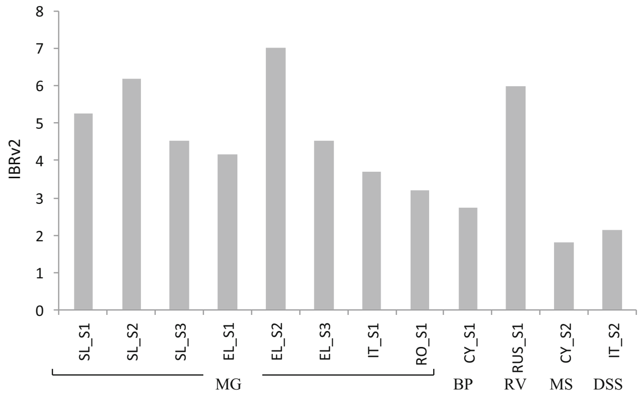

Fig. 7 IBRv2 calculated for biomarkers (CAT, AChE, GST, and MTs) measured in molluscs (*Mytilus galloprovincialis*, *Brachidontes pharaonis*, and *Rapana venosa*) and fish (*Mullus surmuletus*, *Diplodus sargus sargus*) from the various sites in Eastern Mediterranean and Black Sea coastal areas. *SL* Slovenian coast, *EL* Greece (Saronikos Gulf), *IT* Italy (Lagoon of Venice and Apulian coast), *RO* Romania coast, *CY* Cyprus southeast coast, *RUS* Russian coast, *MG M. galloprovincialis*, *BP B. Pharaonis*, *RV R. Venosa*, *MS M. surmuletus*, *DSS D. sargus sargus*

GST response to toxic chemicals follows a bell-shaped profile (Viarengo et al. 2007) and consequently increased and/or decreased GST activities are reported in specimens from impacted areas (Roméo et al. 2003; Regoli et al. 2004; Bebianno et al. 2007; Turja et al. 2014). GST is induced by organic contaminants as part of the phase II biotransformation pathway whereas GST inhibition has been reported as a more non-specific response to chemicals (Regoli et al. 2003). Thus, the GST induction observed in mussels from the Greek impacted sites may actually be due to higher concentrations of organic pollutants, as highlighted by chemical data both in the sediments and biota (Tables 2 and 3), whereas the inhibition detected in the Lagoon of Venice and Slovenian coasts might be associated with higher levels of metals, particularly Cd, Cu, and Zn.

MTs are low molecular weight, cysteine-rich proteins that play a primary role in the homeostasis of essential metals such as Cu and Zn, and in metal detoxification, as they act as chelating agents for intracellular excesses of nonessential metals, such as Ag, Cd, and Hg (Amiard et al. 2006). Their induction is therefore considered as a biomarker of metal contamination and is widely used as a tool in biomonitoring programs (Viarengo et al. 2007; Thain et al. 2008). In the present study, the lack of response of MTs in most areas can be attributed to low metal concentrations, even distribution of metals among sites or natural confounding factors (Marigómez et al. 2013; Zorita et al. 2007) and interactions with other chemicals such as PAHs (Benedetti et al. 2015) which could reduce the effect of metals on MT induction.

The fish sentinel species used in this study (*D. sargus sargus* and *M. surmulletus*) showed higher enzymatic activities compared to mussels in accordance with previous studies (Lionetto et al. 2003; Kopecka et al. 2006); however, they did not generally exhibit differences in biomarker levels between the impacted and reference sites. This lack of response, which contradicts the general expectation that fish are more sensitive than molluscs, particularly as regards AChE inhibition (Monserrat et al. 2007; Viarengo et al. 2007), is not easily explained, as it has not been possible to undertake an in-depth characterization of the chemicals present in the areas in which the fish were collected. However, our results on *D. sargus sargus* biomarkers from the Apulian coast (Italy) are in agreement with a previous study in this area by Lionetto et al. (2003) that failed to reveal differences in AChE and CAT activities of fish *Mullus barbatus* from the same sites. These authors attributed the lack of AChE response to the fact that fish were sampled offshore where the distribution of chemicals can be different compared to inshore sites, and this can also be the case in the present study. With regard to the results on *M. surmulletus* from the coast of Cyprus, the absence of biomarker responses possibly reflects no significant pollution levels in the area (DFMR 2012) highlighted by the similarly low MPI in fish from both sites (MPI=0.1, Table 3).

R. venosa whelks were used in the Black Sea since mussel populations in its northern extent have been decreasing over the last 20 years (Gudimov 2008), whereas whelks are widespread and present even in localities where no mussels are remaining. To our knowledge, this is the first time these biochemical biomarkers have been measured in *R. venosa.* This species has been proposed as a promising indicator for monitoring metal contamination as it has shown high bioaccumulation capacity of Cd and Ni (Liang et al. 2004). Although AChE and GST enzyme activities were relatively high in the whelks and comparable to those observed in fish, only CAT activity showed a significant response at the impacted site. Interestingly, *R. venosa* is the only species used in the present study showing a strong induction in MTs content in impacted compared to the reference site. This MTs induction is consistent with the metal bioaccumulation information reported from the Blue Bay and Tuzla Spit and the large differences between the two sites in MPI values (MPI=27.3 and 3.8, respectively).

The condition index in molluscs and the condition factor in fish were used as supporting parameters indicative of the trophic status and reproductive condition of the organisms. The condition index is considered as a useful tool to assess the nutritive status of bivalves and has been widely used to characterize “fitness” of cultured stocks (Lucas and Beninger 1985). Similarly, the condition factor is used to assess the condition and well-being in fish (Rätz and Lloret 2003) reflecting feeding intensity, age, and growth rates. The high condition indices found in mussels at the more contaminated sites in the Greek and Levantine coasts could be explained by the observation that polluted sites might be characterized by high nutrient loads and consequently might be highly productive environments (Meneghetti et al. 2004). Higher condition index in mussels at polluted sites can be also due to the presence of increased organic matter in the environment (Benali et al. 2015). On the other hand, both condition index and condition factor can be negatively affected by exposure to pollutants (Pampanin et al. 2005b; Kopecka et al. 2006). Thus, low condition index at the impacted sites in the Slovenian, Italian, and Russian coasts could be due to contaminant exposure.

Multivariate analysis on percent alteration of biomarker and CI/CF data with respect to reference sites highlighted similar distribution patterns on the score plot for *M. galloprovincialis* and *B. pharaonis* indicating similar biomarker response patterns. *R. venosa* showed a clear spatial separation from mussels mostly due to a strong influence of the condition index and also of the MTs response. Similar responses were observed in the two fish species, *D. sargus sargus* and *M. surmuletus*, and in the mussels from the Romanian coast.

The PCA analysis on *M. galloprovincialis* biomarker and CI data distinguished sites by geographical area, reflecting the variability in biomarker values among the different areas. It is widely acknowledged that biomarkers are influenced by natural environmental factors such as temperature, salinity, oxygen tension, trophic status as well as size, age, and reproductive condition of the sentinel organisms (Hagger et al. 2006; Holmstrup et al. 2010). Thus, in line with our results, large-scale studies show differences in biomarker ranges and baseline biomarker values among geographical areas that are mainly attributed to difference in temperature, salinity, and trophic status (Lehtonen et al. 2006; Gagné et al. 2008). This pattern complicates the establishment of baseline levels and consequently the definition of assessment criteria for several biomarkers has to be set at the level of regions (Bellas et al. 2014). Currently, background assessment criteria (BAC) and environmental assessment criteria (EAC) have been proposed for only a few biomarkers and regional areas, for example AChE in *M. galloprovincialis* for West Mediterranean Sea and Atlantic Ocean areas (Davies and Vethaak 2012). To our knowledge, baseline levels and assessment criteria for the biomarkers applied in the present study are not available for the Eastern Mediterranean Sea and Black Sea.

The use of indices to summarize biomarker responses for the evaluation of contaminant-induced stress has been increasingly employed, and this approach is useful from an environmental management perspective (Beliaeff and Burgeot 2002; Broeg and Lehtonen 2006; Hagger et al. 2009). In this study, the IBRv2 index based on the reference deviation concept was applied to integrate biomarker responses into a stress index (Sanchez et al. 2013). Preferably, calculation of the IBRv2 index should use established baseline levels of the individual biomarkers (Sanchez et al. 2013); however, an alternative approach is the use of values measured at the reference sites (Olivares-Rubio et al. 2013), and this approach was applied to assess the individual biomarker responses in this study. In this case, the suitability of the reference site is a key factor for the evaluation of biomarker responses and the IBRv2 index. Despite well-recognized uncertainties related with comparisons between impacted and reference sites, there are currently no intention/initiatives towards the standardization of reference sites at the regional level. The selection of investigated sites depends on the expert knowledge of researchers and is based on previous data. The use of common standardized reference sites, preferably several sites in a region, could minimize uncertainties, increase the robustness of the index, and allow comparisons at large spatial scales. In the present study, although IBRv2 index results did not fully correspond to the characterization of the sites with regard to contaminant levels, the highest stress levels were found at three of the sites characterized as most contaminated, i.e., SL_2 in the Bay of Strunjan, EL_S2 in Marina Zeas, and RUS_S1. Furthermore, chemical characterization of the sites in the present study was indicative and based on certain classes of chemicals, while the presence of additional contaminants, which could influence the stress response, cannot be excluded.

In conclusion, among the biomarkers applied, the present study showed responses of AChE, CAT, and GST activities at sites described as impacted in different geographical areas in the mussels *M. galloprovincialis*, suggesting usefulness for assessing pollution effects in large-scale monitoring. *B. pharaonis* mussels seemed to follow a similar biomarker response pattern as *M. galloprovincialis*. Among the alternative sentinel species used, only *R. venosa* showed marked responses of CAT activities and MTs content. In the absence of established baseline levels for the applied biomarkers in the study regions, the approach based on the reference deviation concept was useful for the interpretation of biomarker results. Results contribute to the assessment of pollution effects in the study areas and are expected to be useful in future biomonitoring programs as well as environmental risk assessments in these regions.

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