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Active biomonitoring in Greek coastal waters: Application of the integrated biomarker response index in relation to contaminant levels in caged mussels

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ABSTRACT

An integrated approach using biomarkers and contaminant levels in mussels *Mytilus galloprovincialis* L. was employed to assess chemical contamination in Greek coastal waters within the framework of the MYTIMED program. Biomarkers (metallothioneins, glutathione S-transferase, catalase, acetylcholinesterase and RNA:DNA ratio) have been previously described in mussels caged at 14 sites in coastal areas influenced by different types of anthropogenic activities. This study applied a biomarker index, the Integrated Biomarker Response (IBR) to summarize biomarker responses and relate stress levels to concentrations of organochlorine compounds (PCBs, DDTs), polycyclic aromatic hydrocarbons (PAHs), and metals (Cu, Ni, Fe, Zn) measured in the mussel tissues. The IBR index indicated environmental stress at sites near cities and industries and was overall related to organic contaminants, but also elevated metal concentrations at certain sites. Slightly increased IBR values at few sites away from known pollution sources were not accompanied with increased contaminant levels suggesting stress possibly caused by natural factors. Results confirmed the usefulness of integration of biological effects measurements and chemical analysis for the assessment of chemical contamination in coastal waters.

1. Introduction

Active biomonitoring i.e. the use of sentinel organisms from a single "non-contaminated" population deployed at sites under investigation in order to measure levels and biological effects of contaminants is a useful approach for pollution assessment in coastal waters (Stien et al., 1988; Andral et al., 2004; Viarengo et al., 2007; Damiens et al., 2007; Jung et al., 2008). This approach avoids bias related to the age and the reproductive status of the organisms and allows better control of accumulation and biological effects of contaminants in a predetermined exposure period. In addition, comparisons of sites are feasible even if natural populations are scarce.

The combined approach of monitoring chemical contaminant levels, along side biological effect measurements offers enormous potential to meet the challenges outlined by the European Union Marine Strategy Framework Directive (MSFD, 2008/56/EC) for undertaking assessments of Good Environmental Status in European marine waters (Lyons et al., 2010). Biomarkers are among the emerging tools for the assessment of biological effects of contaminants in monitoring programs (Allan et al., 2006; Depledge, 2009). Biomarkers reveal environmental stress caused by chemical contaminants and also other environmental variables thus integration of biomarkers and chemical analysis is essential in order to establish links between stress and pollution (Galloway et al., 2004a; Thain et al., 2008). Combinations of multiple biomarkers reflecting effects on different endpoints and exposure to various types of contaminants are used in order to achieve a comprehensive evaluation of pollution impact (Galloway et al., 2004b; Viarengo et al., 2007). Since results of different sets of biomarkers are often difficult to interpret, integration of biomarker data into multiple-biomarker indices for the evaluation of contaminant-induced stress is increasingly employed (Belliaeff and Burgeot, 2002; Blaise et al., 2002; Chèvre et al., 2003; Broeg et al., 2005; Narbonne et al., 2005; Auffret et al., 2006; Dagnino et al., 2007; Hagger et al., 2009). These biomarker-based indices of the organisms' health status are proposed as useful tools for the assessment of ecological risk in environmental management. Biomarker indices are constructed using various univariate or multivariate methods and ranking systems. The choice of biomarkers employed depends on the basic concept of each index. A number of indices include biomarkers at different levels of biological organization ranging from general stress responses to specific effect and exposure biomarkers (Biomarker Response Index, BRI: Hagger et al., 2009; Health Status Index, HSI: Dagnino et al., 2007; Biomarker Index: Blaise et al., 2002; Chèvre et al., 2003) while others employ only biomarkers

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reflecting specific, early molecular responses to particular contaminants (Multimarker Pollution Index, MPI: Narbonne et al., 2005), toxic effects ranging from early to late responses (Bioeffect Assessment Index, BAI: Broeg et al., 2005), immunotoxic responses (Immunotoxicological Index: Auffret et al., 2006) or lysosomal responses (Lysosomal Response Index, LRI: Izagirre and Marigomez, 2009). The Integrated Biomarker Response (IBR) index is a simple graphic method developed by Belliaeff and Burgeot (2002) that uses star plots as a means of summarizing biomarker responses into a single value reflecting the level of environmental stress at each site. This index can integrate variable combinations of biomarkers of general health, toxic effects and exposure to specific contaminants (Broeg and Lehtonen, 2006). Belliaeff and Burgeot (2002) pointed out that successful application of IBR depends on a relevant choice of biomarkers in relation to the specific objectives of the study and the characteristics of the field site, while interpretation of the results has to take in account variations in abiotic and biotic factors that influence biomarker responses. A number of studies using different biomarker combinations have shown visual accordance of this index with contaminant levels (Belliaeff and Burgeot, 2002; Damiens et al., 2007; Pytharopoulou et al., 2008; Lu et al., 2010) and similar results of this index to other biomarker indices such as the BAI (Broeg and Lehtonen, 2006), the HIS and the LRI (Raftopoulou and Dimitriadis, 2010).

Mussels are commonly used as sentinel organisms in biomonitoring studies due to their wide geographical distribution and ability to accumulate contaminants in proportion to levels in the environment (Andral et al., 2004; Viarengo et al., 2007). Native populations or deployed mussels originating from a non-polluted/pristine site are commonly employed (Bocquené et al., 2004; Lehtonen et al., 2006; Gorbi et al., 2008; Gagné et al., 2008; Hunt and Slone, 2010). The use of deployed mussels has proved useful for large geographical scale monitoring since mussels can be immersed at any location and/or depth (Andral et al., 2004; Galgani et al., 2005; Galgani et al., 2011).

Within the framework of the MYTIMED program, active biomonitoring using caged mussels was applied to assess chemical contamination in the East Mediterranean Sea. Multiple biomarkers reflecting contaminant exposure and/or effects were assessed in mussels caged in Greek coastal waters (Tsangaris et al., 2010). Metallothioneins (MTs), metal-binding proteins specifically induced by exposure to certain metals such as Cu, Zn, Cd, Hg (Viarengo et al., 1999), glutathione S-transferase (GST), a phase II biotransformation enzyme that has been shown to respond to organic contaminants (PCBs, chlorinated pesticides, PAHs; Cheung et al., 2001, 2002), catalase (CAT), an antioxidant enzyme used as a biomarker of oxidative stress that can be induced by a wide range of contaminants (Livingstone, 2001), acetylcholinesterase (AchE), a biomarker of neurotoxicity indicative of exposure to carbamate and organophosphate pesticides (Fulton and Key, 2001) and also general stress (Lehtonen et al., 2006), and RNA:DNA ratio, a biochemical growth biomarker reflecting a general stress response (Wo et al., 1999) were measured. The present study aimed to apply the IBR index in combination with contaminant levels in caged mussels for the assessment of chemical contamination in Greek coastal waters. IBR values reflecting environmental stress levels, were compared to concentrations of organochlorine compounds (PCBs, DDTs), polycyclic aromatic hydrocarbons (PAHs) and metals (Cu, Ni, Fe, Zn) measured in the mussel tissues. In addition, the present study intended to further validate the suitability of the IBR index as a tool for pollution assessment in marine ecosystems.

2. Materials and methods

2.1. Sampling area and experimental design

Mussels *Mytilus galloprovincialis* were caged at several sites along the Greek coastline. The transplantation was performed by the French oceanographic vessel 'L' Europe' and the Greek oceanographic vessel 'Philia' according to standardized procedures (Andral et al., 2004). Detailed information on transplantation methodology and biomarker analyses are described in Tsangaris et al. (2010). Briefly, mussels of approximately 60 mm shell length from an aquaculture farm were placed in PVC cages, immersed at the selected sites at 8 m depth below the sea surface and recovered after three months by diving. The depth of the sites was between 20 and 30 m. Samples were conditioned immediately after collection on board. In one batch of mussels, whole soft tissues were removed from the shells and stored at −20° for chemical analyses (pooled sample of 120 individuals per site), condition index (pooled sample of 30–40 individuals per site) and RNA:DNA ratio (3 pooled samples of 3–5 individuals per site) measurements. In a second batch of mussels, gills and digestive glands were dissected and were frozen in liquid nitrogen (5 pooled samples of 6 individuals per site). AchE was measured in the gills and CAT, GST and MTs in the digestive glands.

The biomarker data used for the calculation of the IBR index are presented in Tsangaris et al. (2010). Data from 14 sites (4 sites with no data for MTs were excluded) in coastal areas influenced by different types of anthropogenic activities were used; S1 and S2 in Thermaikos Gulf, S3 in Saronikos Gulf, S4 in N. Evoikos Gulf, S5 in S. Evoikos Gulf, S6 in Korinthiakos Gulf, S7 in Maliakos Gulf, S10 in Pagasitikos Gulf, S12 in Nestos estuary, and S13, S14, S15, S16 and S18 in the Aegean Sea (Fig. 1). Types of anthropogenic impacts at the selected areas are shown in Table 1. Sites in the Aegean Sea, near islands away from known sources of pollution (S14, S15, S16 and S18) were used as reference sites.

2.2. IBR index calculation

IBR was calculated according to Belliaeff and Burgeot (2002). Data was first standardized and then the scores of all the biomarkers at a given station were represented in star plots. Calculations for a given station were as follows. For each biomarker, the mean value (X) at each station, and the general mean (m) and standard deviation (s) of X for all stations were computed. The values of X were standardized to obtain Y, where $Y=(X-m)/s$. Then Z was calculated as $Z=-Y$ or $Z=Y$ in the case of biomarker responding to pollution by inhibition or induction respectively. In the case of RNA:DNA ratio that presented a biphasic response to pollution (Tsangaris et al., 2010), Z was set as $Z=-Y$ in all stations except S4 and S5, where very high values were recorded and Z was set as $Z=Y$. Then, the minimum value (Min) of Z for all stations was obtained. The score (B) for each biomarker for a given station was computed as $B=Z+|Min|$ where $|Min|$ is the absolute value. Five biomarkers (AchE, MTs, RNA:DNA, GST and CAT) were introduced in the IBR calculation. The respective five scores for each station (B1–B5) were represented in star plots. The IBR index for each station was calculated as the area of the star plot where the scores are displayed:

$$IBR=\sum_{i=1}^{n} A_i$$

where A_i is the triangular area represented by two consecutive biomarker scores (B_i, B_{i+1}) on the star plot, and n is the number of biomarkers used in the IBR calculation.

2.3. Chemical analyses

The frozen tissues were freeze-dried and homogenized. For the analysis of metals 1.5 g of dried tissue was digested with HNO_3 under pressure using a microwave furnace CEM MDS 2100 (UNEP, 1984) and the determination of Cu, Ni, Fe and Zn was performed by flame atomic absorption spectrophotometry (Varian Spectra AA 20 Plus). The accuracy and precision of the analytical methodology were tested with the reference material NRC-Dorm2 (Dogfish muscle).

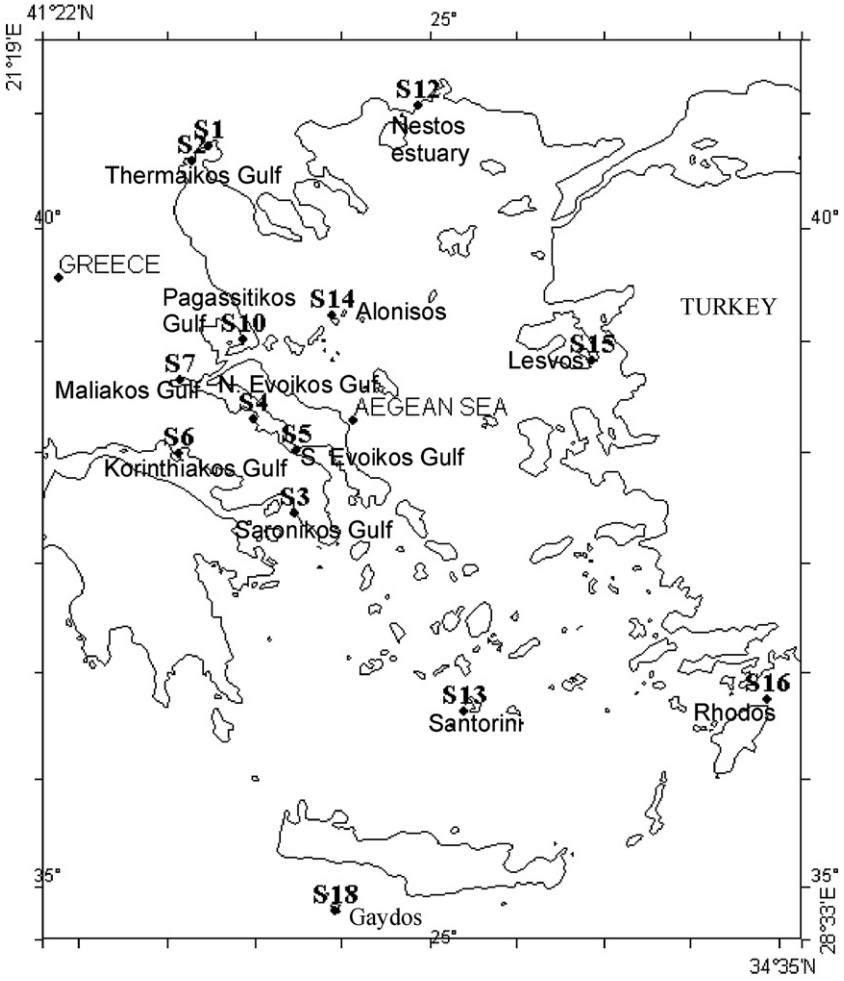

Fig. 1. Location of sampling sites at the selected areas in Greek coastal waters.

Table 1
Type of anthropogenic impacts at the selected areas in Greek coastal waters.

Site	Longitude and latitude	Area	Type of anthropogenic impact
S1	22°53.56′E 40°36.07′N	Thermaikos Gulf	Urban wastes from Thessalonica, industrial inputs
S2	22°43.72′E 40°29.37′N	Thermaikos Gulf	Urban wastes from Thessalonica, industrial inputs, agricultural practice
S3	23°42.88′E 37°52.56′N	Saronikos Gulf	Urban wastes from Athens, industrial inputs
S4	23°19.30′E 38°35.28′N	N Evoikos Gulf	Industrial inputs by ferronickel production, metalifferous slag disposal
S5	23°44.28′E 38°20.67′N	S. Evoikos Gulf	Industrial inputs via Asopos river
S6	22°35.98′E 38°19.46′N	Korinthiakos Gulf	Industrial inputs by alumina production, red mad disposal
S7	22°37.23′E 38°52.23′N	Maliakos Gulf	Agricultural practices, urban wastes
S10	23°13.02′E 39°10.47′N	Pagasitikos Gulf	Agricultural practices, urban wastes
S12	24°53.62′E 40°53.45′N	Nestos river estuary	Agricultural and aquaculture practices
S13	25°20.54′E 36°21.94′N	Santorini island, Aegean Sea	Shipwreck of cruise ship 'Sea Diamond' in April 2007
S14	24°5.01′E 39°21.38′N	Alonissos island, Aegean Sea	Natural park
S15	26°33.32′E 39°0.11′N	Lesvos island, Aegean Sea	Tourist area, absence of known pollution sources
S16	28°13.54′E 36°27.47′N	Rhodos island, Aegean Sea	Tourist area, absence of known pollution sources
S18	24°6.11′E 34°48.24′N	Gaydos island, Aegean Sea	Absence of known pollution sources

For PAH analysis 3 g of dried tissue was spiked with known quantities of deuterated PAH used as internal standards and saponified with methanolic KOH. The unsaponified material was extracted with n-hexane and the extract was cleaned-up by column chromatography. A glass column filled with 5 g of alumina deactivated with 6% water was used and the elution was performed with 20 mL of n-hexane. The eluate was condensed to 0.2 mL and put on a glass column filled with 2 g of activated silica. Two fractions were collected: The first one, contained the aliphatic hydrocarbons, with 10 mL of n-hexane and the second, contained the PAH, with 10 mL of a mixture n-hexane–ethyl acetate 9:1. The determination of PAH in this second fraction was performed by gas chromatography–mass spectrometry (Hewlett-Packard 6890 GC-MSD). The quantitation was based on the internal standards added in the beginning and the following compounds were determined: naphthalene, methyl-, dimethyl- and trimethyl-naphthalenes, acenaphthene, acenaphthylene, fluorine, dibenzothiophene, phenanthrene, methyl- and dimethyl-phenanthrenes, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b) fluoranthene, benzo(k)fluoranthene, benzo(e)pyrene, benzo(a)pyrene, perylene, indeno(1,2-cd)pyrene, benzo(ghi)perylene and dibenzo(ah) anthracene. The recoveries of the internal standard ranged as follows: Naphthalene-d8: 51–62%, acenaphthene-d10: 77–84%, phenanthrene-d10: 84–89%, pyrene-d12: 87–94%, chrysene-d12: 93–102%, perylene-d12%: 96–104%, benzo(ghi)perylene-d12: 93–103%. The accuracy and precision of the analytical methodology were tested by using a certified reference material provided from IAEA (IAEA-432, mussel homogenate).

For the organochlorine analysis 5 g of the dried tissue was Soxhlet extracted for 24 h with a mixture of dichloromethane–pentane 1:1,

the extracts were cleaned-up and fractionated on deactivated alumina columns (Satsmadjis et al., 1988) and the final determination was performed on a gas chromatograph equipped with an ECD detector (Agilent 7890). The accuracy and precision of the analytical methodology were tested by using a certified reference material provided from IAEA (IAEA-432, mussel homogenate).

2.4. Statistical analysis

Relations between the IBR index and contaminant concentrations were examined by correlation analysis (Pearson's correlation coefficient). Principal Component Analysis (PCA) was applied to discriminate sites by integration of contaminant levels according to principal axes and evaluate general patterns of contaminant bioaccumulation. PAHs, PCBs, DDTs, Cu, Ni, Zn, and Fe concentrations were the variables used in PCA. The positions of the sites in the co-ordinates of the first two principal components were then compared to the respective IBR values (Zitko, 1994). Contaminant concentrations were log $(x+1)$ transformed for statistical analysis. Statistical analysis was performed using SPSS (correlation analysis) and Primer (PCA).

3. Results and discussion

To demonstrate the contribution of each biomarker to the IBR index, the standardized data of biomarkers used in the IBR calculation (biomarker scores) are shown in Table 2. Maximum values represent sites of the highest response of each biomarker (S1 for AchE and CAT, S13 for MTs, S4 for RNA:DNA and S5 for GST), while zero values reveal sites of the lowest response (S12 for AchE, S1 for MTs, S18 for RNA:DNA, S15 for GST and S16 for CAT).

IBR index and contaminant levels in the tissues of mussels are presented in Fig. 2. The IBR index clearly discriminated stress levels among sites, showing higher values at sites near cities and industries i.e. in Thermaikos Gulf, Saronikos Gulf, N. Evoikos Gulf, S. Evoikos Gulf, Korinthiakos Gulf and Maliakos Gulf (sites S1, S2, S3, S4, S5, S6 and S7) compared to sites in the Aegean Sea (sites S13, S14, S15, S16 and S18), Pagasitikos Gulf and Nestos estuary (sites S10 and S12). The condition index of the mussels (data presented in Tsangaris et al., 2010), which is mainly influenced by the nutritional and reproductive status leading to weight variations, was not related to the IBR index ($r=0.198$, $P>0.05$). The condition index was generally in accordance with the trophic characteristics of the sites showing high values in areas where food is abundant and mussel culture is intense i.e. Thermaikos Gulf, Nestos estuary, Maliakos Gulf and N. Evoikos Gulf (SoHelME, 2005) and low values in the Aegean Sea where waters are oligotrophic (Ignatiades, 2005). Nevertheless these differences in the physiological status of mussels did not mask the overall stress response to pollution as shown by the IBR index.

Table 2
Standardized data (scores used in the calculation of IBR index) of biomarkers measured in mussels caged at 14 sites in Greek coastal waters.

Site	AchE	MT	RNA:DNA	GST	CAT
S1	2.84	0.00	1.29	1.87	3.86
S2	2.35	0.24	1.27	2.39	2.35
S3	1.30	2.80	1.10	1.90	2.53
S4	1.80	0.46	3.31	1.66	1.31
S5	1,67	0.02	2.44	3.52	2.49
S6	2.17	1.30	1.19	1.85	2.85
S7	2.43	1.71	1.28	2.30	2.21
S10	2.47	0.07	0.82	0.15	2.29
S12	0.00	0.62	1.28	0.02	1.94
S13	0.26	2.94	0.91	0.89	3.57
S14	0.48	0.35	0.84	1.81	2.08
S15	0.52	0.04	0.53	0.00	1.35
S16	0.51	0.24	1.22	1.93	0.00
S18	0.26	0.98	0.00	2.02	3.38

Concentrations of organic contaminants were generally in accordance with IBR results, particularly PCBs and DTTs that were significantly correlated to the IBR index ($r=0.769$, $P<0.01$ and $r=0.589$, $P<0.05$ respectively) (Table 3). With regard to PAHs, a positive although not statistically significant correlation to the IBR index was recorded ($r=0.513$, $P>0.05$). Highest PCBs concentrations of 12–14.7 ng/g were found at sites S1, S3 and S6. Maximum DDTs concentrations (3.4–11.1 ng/g) were recorded at sites S1, S2, S3 and S6. Peak PAHs concentrations were found at sites S1 and S3 (72.9 and 75.3 ng/g respectively).

Metal concentrations were not correlated to the IBR index (Table 3); nevertheless several peaks in Cu, Ni and Fe concentrations were evident at sites showing high IBR values. Cu concentrations were highest at sites S3 and S4 (4.8 μg/g). Fe concentrations were highest (89.5–113.4 μg/g) at sites S3, S4 and S5. Peak Ni concentrations (4.1–6.8 μg/g) were recorded not only at sites S4, S5 and S7, but also at S10 and S16 where IBR values and levels of other contaminants were low. These elevated Ni levels in areas of Pagasitikos Gulf (S10) and Rhodos Island (S16) where pollution sources are lacking can be possibly due to natural enrichment (Voutsinou-Taliadouri and Satsmadjis, 1982; Voutsinou-Taliadouri and Georgakopoulou-Grigoriadou, 1989) and did not induce an overall stress response as shown by the IBR index. Zn concentrations were homogenous with the exception of low levels at sites S10 and S18.

Overall variations in contaminant concentrations were evaluated by PCA. PCA of contaminant concentrations produced three principal components that accounted for 81.7% of the total variance. The plot of variable vectors for the first two principal components PC1 and PC2 that explained 67.3% of the total variance is shown in Fig. 3a. PC1 was mainly influenced by PCBs and DDTs on the positive part while PC2 was mainly influenced by Fe and Ni on the negative part. PC3 represented 14.4% of the total variance and was correlated with Cu. The plot of scores for different sites for the two dominant components PC1 and PC2 marked by the respective IBR values at each site is shown in Fig. 3b. Sites with low levels of contaminants are located in the upper left part of the plot (negative part of PC1, positive part of PC2) and show low IBR values. Sites with high levels of PCBs and DDTs are positioned in the positive part of PC1 and sites with high levels of Ni and Fe are located in the negative part of PC2; they are accompanied by high IBR values. These results suggest stress shown by high IBR values (5.9 to 10.3) is related to elevated levels of organic contaminants and/or metals. However at S7, where IBR value was among the highest of this study, contaminant levels were not generally elevated. Site S7 is situated in Maliakos Gulf, an area influenced by urban wastes and agricultural practices, where massive fish deaths and toxic phytoplankton blooms have been recently recorded (Pagou et al., 2010). The causes of these incidences are not clearly identified. Thus other stressors and/or contaminants not analyzed in the present study may be due for the stress response at site S7. The inner part of Maliakos Gulf is considerably affected by the discharge of Sperchios River (Chryssovergis and Panayotidis, 1995) and its ecological status based on ecological indices on phytobenthos data has been classified from bad to moderate (Spatharis et al., 2003).

IBR index at sites showing low levels of contaminants ranged between 0.4 and 4. The highest IBR values among these sites were found at S13 and S18 in the Aegean Sea (4 and 3.8, respectively), concomitantly with low condition index values reflecting stress possibly caused by natural environmental factors such as temperature and food availability as wild mussels are not present in these areas. Nevertheless regarding site S13 at Santorini island, biomarker responses could be also due to other unidentified pollutants emerging from the shipwreck of the 'Sea Diamond' cruise ship two months prior to mussel transplantation in this area.

The IBR index is regarded as a practical tool for the examination of the stress response of different populations by combination of different biomarkers and for the comparison of stress levels to contaminant

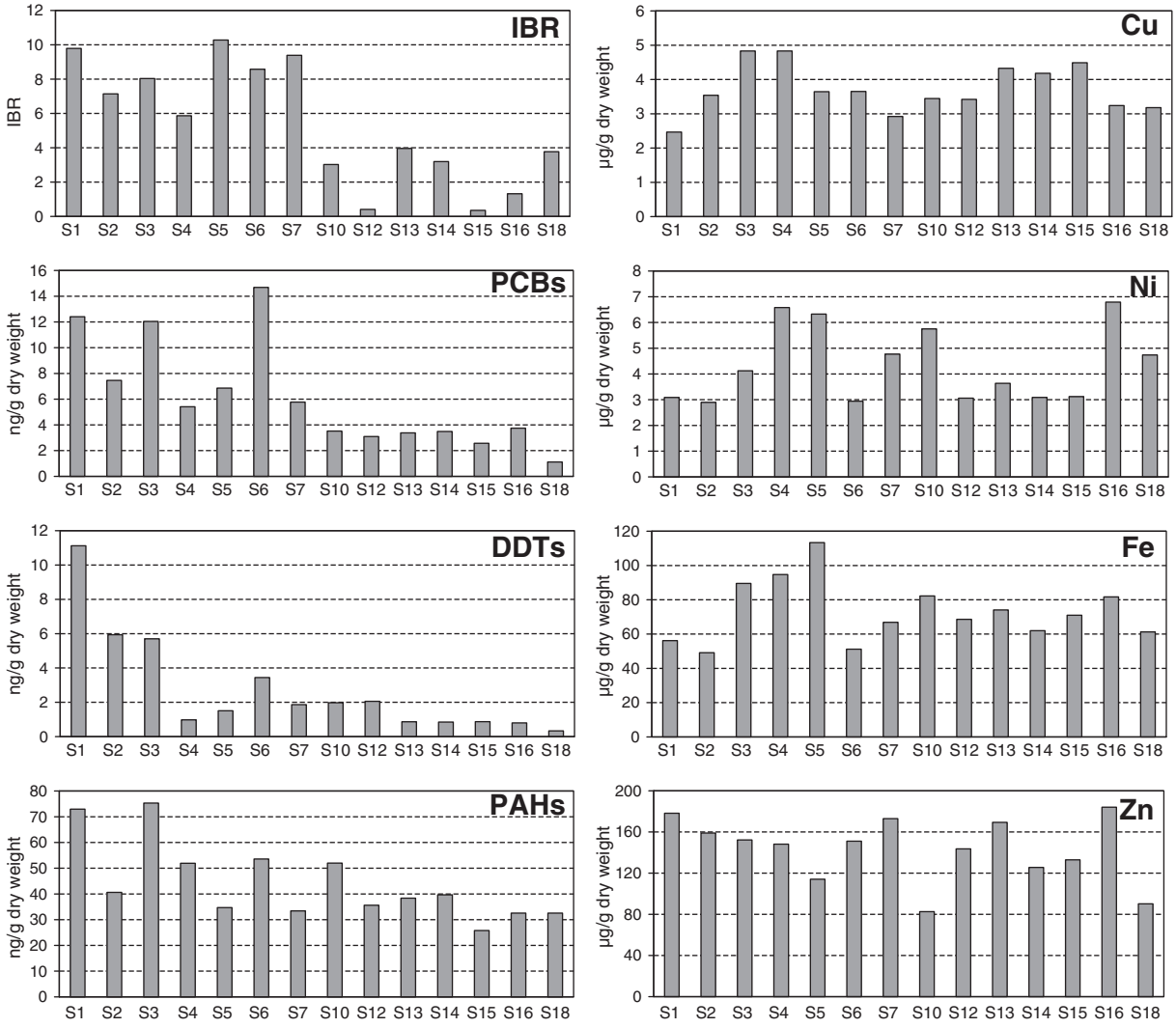

Fig. 2. IBR index, and concentrations of PCBs, DDTs, PAHs, Cu, Ni, Fe and Zn in mussels caged at 14 sites in Greek coastal waters.

levels (Bocquené et al., 2004; Broeg and Lehtonen, 2006; Damiens et al., 2007; Pytharopoulou et al., 2008; Gagné et al., 2008; Pereira et al., 2009; Oliveira et al., 2009; Raftopoulou and Dimitriadis, 2010; Wang et al., 2010; Lu et al., 2010). Several studies using different combinations of biomarkers in the IBR calculation have shown accordance of IBR with contaminant levels. In eelpout (*Zoarces viviparous*) and mussel (*Mytilus* sp.) populations of the Baltic Sea good accordance was reported between IBR and tissue levels of organochlorine compounds (Broeg and Lehtonen, 2006). In mussels (*M. galloprovincialis*) caged at several stations in the Bay of Cannes, Damiens et al. (2007) found good agreement of IBR with tissue Cu and PCB concentrations but not with PAH concentrations. In mussels (*M. galloprovincialis*) caged at an area polluted by heavy metals in the Gulf of Patras (Greece), IBR showed accordance with Cu concentrations in the digestive gland (Pytharopoulou et al., 2008). In goldfish (*Carassius auratus*) transplanted in Taihou lake (China), a visual correlation was recorded between IBR and the PCB and OCP gradient in sediments (Lu et al., 2010).

Table 3
Pearson's correlation coefficient (r) between the IBR index and contaminant concentrations in mussels caged at 14 sites in Greek coastal waters.

	PCBs	DDTs	PAHs	Cu	Ni	Fe	Zn
IBR	0.769**	0.589*	0.513	−0.240	0.037	−0.016	0.229

** $P<0.01$.
* $P<0.05$.

The relevant choice of biomarkers is a key issue for the use of integrated indices in biological effects monitoring. Selection of an appropriate battery of biomarkers can avoid false-negative responses obtained with a single biomarker and their integration into an index simplifies interpretation in biomonitoring programs (Belliaeff and Burgeot, 2002). On the other hand, inclusion of several parameters that respond to the same type of pollution or biological function will overemphasize the importance of this particular group of contaminants or function in the overall assessment of environmental stress (Broeg and Lehtonen, 2006). In the case of the IBR index, the position of the biomarkers on the star plot used for the IBR calculation should also be considered, as it affects the "relative weight" of each biomarker in the final index value (Belliaeff and Burgeot, 2002; Broeg and Lehtonen, 2006). Thus, biomarker position on the star plot has to be defined based on an objective reason such as their ability to discriminate non-impacted from impacted sites (Belliaeff and Burgeot, 2002). A limitation of the IBR index due to its mathematical basis, is that IBR values cannot be compared to values previously computed at the same site or other sites, unless all data are processed in the same calculation. Thus, the IBR is not able to give fixed values to describe environmental stress at any time or site without recalculations and furthermore, classify sites on a fixed scale. Nevertheless, the consistency of IBR results according to pollution gradients

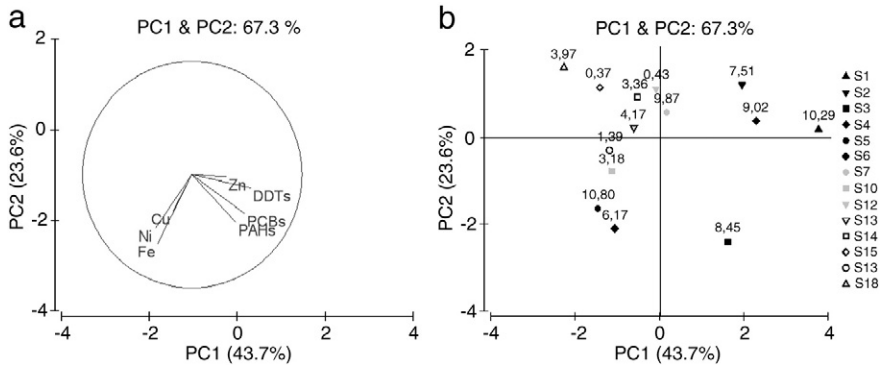

Fig. 3. Results of the PCA of the two dominant components produced by contaminant concentrations (PCBs, DDTs, PAHs, Cu, Ni, Zn, and Fe) in mussels caged at 14 sites in Greek coastal waters. (a) Plot of variable vectors. (b) Plot of scores of different sites marked by the respective IBR values at each site.

regardless of the variability in the biomarker sets used (Broeg and Lehtonen, 2006; Damiens et al., 2007; Pytharopoulou et al., 2008; Oliveira et al., 2009; Raftopoulou and Dimitriadis, 2010) supports its usefulness as a simple tool for the assessment of pollution impacts in marine ecosystems.

In the present study, biochemical biomarkers representing different biological endpoints i.e. detoxification of metals, organic contaminants and reactive oxygen species (MTs, GST and CAT), neurotoxicity (AchE) and protein synthesis (RNA:DNA ratio), were used in the IBR index. The choice of biomarkers intended to include early responses to major types of contaminants i.e. metals, PAHs, PCBs, pesticides, and general stress. The condition index may also indicate pollution stress (Pampanin et al., 2005) but it was not included in the IBR calculation since in the present study it was mostly related to the level of eutrophication. The position of biomarkers on the star plot was based on their relevance in discriminating sites receiving pollution inputs from non-impacted sites as shown by Principle Component Analysis of biomarker data (Tsangaris et al., 2010). The application of IBR using this selection of biomarkers proved useful in identifying stress in accordance to measured contaminant concentrations. Obviously, additional biomarkers in the IBR would increase the robustness of the index. For example, if ethoxyresorufin-O-deethylase (EROD) activity was measured, a better agreement of IBR with PAHs concentrations could have been detected. On the other hand, introduction of additional biomarkers representing different biological endpoints such as those indicative of endocrine disruption and genotoxicity in the IBR may have increased the robustness of the index. Furthermore, it has to be pointed out that this study was focused on associations between the IBR index and certain types of contaminants, but other contaminants that were not examined may have also influenced the stress response of mussels.

In conclusion, the IBR index proved useful tool for the assessment of contaminant-induced stress over a large spatial scale in Greek coastal waters. Tissue contaminant concentrations suggest that stress levels were overall related to exposure to organic contaminants and exposure to metals at certain sites. Results confirmed the usefulness of integration of biological effects measurements and chemical analysis for the assessment of chemical contamination in coastal waters.

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References

Allan IJ, Vrana B, Greenwood R, Mills GA, Roig B, Gonzalez C. A "toolbox" for biological and chemical monitoring requirements for the European Union's Water Framework Directive. Talanta 2006;69:302–22.

Andral B, Stanisiere JY, Sauzade D, Damier E, Thebault H, Galgani F, et al. Monitoring chemical contamination levels in the Mediterranean based on the use of mussel caging. Mar Pollut Bull 2004;49:704–12.

Auffret M, Rousseau S, Boutet I, Tanguy A, Baron J, Moraga D, et al. A multiparametric approach for monitoring immunotoxic responses in mussels from contaminated sites in Western Mediterranea. Ecotoxicol Environ Saf 2006;73:393–405.

Belliaeff B, Burgeot T. Integrated biomarker response: a useful tool for ecological risk assessment. Environ Toxicol Chem 2002;21:1316–22.

Blaise C, Gagné F, Pellerin J, Hansen P-D, Trottier S. Molluscan shellfish biomarker study of the Quebec, Canada, Saguenay Fjord with the soft-shell clam, *Mya arenaria*. Environ Toxicol 2002;3:170–86.

Bocquené G, Chantereau S, Clérendeau C, Beausir E, Ménard D, Raffin B, et al. Biological effects of the "Erika" oil spill on the common mussel (*Mytilus edulis*). Aquat Living Resour 2004;17:309–16.

Broeg K, Westernhagen Hv, Zander S, Körting W, Koehler A. The "bioeffect assessment index" (BAI). A concept for the quantification of effects of marine pollution by an integrated biomarker approach. Mar Pollut Bull 2005;50:495–503.

Broeg K, Lehtonen KK. Indices for the assessment of environmental pollution of the Baltic Sea coasts: integrated assessment of a multi-biomarker approach. Mar Pollut Bull 2006;53:508–22.

Cheung CCC, Zheng GJ, Li AMY, Richardson BJ, Lam PKS. Relationships between tissue concentrations of polycyclic aromatic hydrocarbons and antioxidative responses of marine mussels, *Perna viridis*. Aquat Toxicol 2001;52:189–203.

Cheung CCC, Zheng GJ, Lam PKS, Richardson BJ. Relationships between tissue concentrations of chlorinated hydrocarbons (polychlorinated biphenyls and chlorinated pesticides) and antioxidative responses of marine mussels, *Perna viridis*. Mar Pollut Bull 2002;45:181–91.

Chèvre N, Gagné F, Blaise C. Development of a biomarker based index for assessing the ecotoxic potential of aquatic sites. Biomarkers 2003;8:287–98.

Chryssovergis F, Panayotidis P. Communities of macrophytobenthos along an eutrophication gradient (Maliakos gulf, Aegean Sea, Greece). Oceanol Acta 1995;18:649–58.

Dagnino A, Allen JI, Moore MN, Broeg K, Canesi L, Viarengo A. Development of an expert system for the integration of biomarker responses in mussels into an animal health index. Biomarkers 2007;12(2):155–72.

Damiens G, Gnassia-Barelli M, Loquès F, Roméo M, Salbert V. Integrated biomarker response index as a useful tool for environmental assessment evaluated using transplanted mussels. Chemosphere 2007;66:574–83.

Depledge MH. Novel approaches and technologies in pollution assessment and monitoring: a UK perspective. Ocean Coast Manage 2009;52:336–41.

Fulton MH, Key PB. Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. Environ Toxicol Chem 2001;20:37–45.

Gagné F, Burgeot T, Hellou J, St-Jean S, Farcy É, Blaise C. Spatial variations in biomarkers of *Mytilus edulis* mussels at four polluted regions spanning the Northern Hemisphere. Environ Res 2008;107:201–17.

Galgani F, Chiffoleau JF, Galla PLE, Pichota Y, Andral B, Martin C. Deep-sea caging of the mussel *Mytilus galloprovincialis*: potential application in ecotoxicological studies. Chem Ecol 2005;21:133–41.

Galgani F, Martínez-Gómez C, Giovanardi F, Romanelli G, Caixach J, Cento A, et al. Assessment of polycyclic aromatic hydrocarbon concentrations in mussels (*Mytilus galloprovincialis*) from the Western basin of the Mediterranean Sea. Environ Monit Assess 2011;172:301–17.

Galloway TS, Brown RJ, Browne MA, Dissanayake A, Lowe D, Jones MB, et al. A multibiomarker approach to environmental assessment. Environ Sci Technol 2004a;38:1723–31.
Galloway TS, Brown RJ, Browne MA, Dissanayake A, Lowe D, Jones MB, et al. Ecosystem management bioindicators: the ECOMAN project — a multi-biomarker approach to ecosystem management. Mar Environ Res 2004b;58:233–7.
Gorbi S, Lamberti CV, Notti A, Benedetti M, Fattorini D, Moltedo G, et al. An ecotoxicological protocol with caged mussels, *Mytilus galloprovincialis*, for monitoring the impact of an offshore platform in the Adriatic Sea. Mar Environ Res 2008;65:34–49.
Hagger JA, Galloway TS, Langston WL, Jones MB. Application of biomarkers to assess the condition of European Marine Sites. Environ Pollut 2009;157:2003–10.
Hunt CD, Slone E. Long-term monitoring using resident and caged mussels in Boston Harbor yield similar spatial and temporal trends in chemical contamination. Mar Environ Res 2010;70:343–57.
Ignatiades L. Scaling the trophic status of the Aegean Sea, Eastern Mediterranean. J Sea Res 2005;54:51–7.
Izagirre U, Marigomez I. Lysosomal enlargement and lysosomal membrane destabilisation in mussel digestive cells measured by an integrative index. Environ Pollut 2009;157:1544–53.
Jung J-H, Kim S-J, Lee T-K, Shim WJ, Woo S, Kim D-J, et al. Biomarker responses in caged rockfish (*Sebastes schlegeli*) from Masan Bay and Haegeumgang, South Korea. Mar Pollut Bull 2008;57:599–606.
Lehtonen KK, Schiedek D, Köhler A, Lang T, Vuorinen PJ, Förlin L, et al. The BEEP project in the Baltic Sea: overview of results and outline for a regional biological effects monitoring strategy. Mar Pollut Bull 2006;53:523–37.
Livingstone DR. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar Pollut Bull 2001;42:656–66.
Lu GH, Ji Y, Zhang HZ, Wua H, Qin J, Wang C. Active biomonitoring of complex pollution in Taihu Lake with *Carassius auratus*. Chemosphere 2010;79:588–94.
Lyons BP, Thain JE, Stentiford GD, Hylland K, Davies IM, Vethaak AD. Using biological effects tools to define Good Environmental Status under the European Union Marine Strategy Framework Directive. Mar Pollut Bull 2010;60:1647–51.
Narbonne JF, Aarab N, Clérandeau C, Daubèze M, Narbonne, Champeau O, et al. Scale of classification based on biochemical markers in mussels: application to pollution monitoring in Mediterranean coasts and temporal trends. Biomarkers 2005;10(1):58–71.
Oliveira M, Maria VL, Ahmad I, Serafim A, Bebianno MJ, Pacheco M, et al. Contamination assessment of a coastal lagoon (Ria de Aveiro, Portugal) using defence and damage biochemical indicators in gill of *Liza aurata* — an integrated biomarker approach. Environ Pollut 2009;157:959–67.
Pagou K, Varkitzi I, Pavlidou A, Hatzianestis I, Psyllidou R, Kontoyannis H, et al. First occurrence of the fish killing *Raphidophyte chattonella* sp. in Maliakos gulf, Greece (Eastern Mediterranean sea). 14th International conference on Harmful Algae, Hersonisos, Crete, Greece; 2010. p. 145.. [1–5 November].
Pampanin DM, Volpato E, Marangon I, Nasci C. Physiological measurements from native and transplanted mussel (*Mytilus galloprovincialis*) in the canals of Venice. Survival in air and condition index. Comp Biochem Physiol 2005;140A:41–52.
Pereira P, de Pablo H, Rosa-Santos F, Pacheco M, Vale C. Metal accumulation and oxidative stress in *Ulva* sp. substantiated by response integration into a general stress index. Aquat Toxicol 2009;91:336–45.
Pytharopoulou S, Sazakli E, Grintzalis K, Georgiou CD, Leotsinidis M, Kalpaxis DL. Translational responses of *Mytilus galloprovincialis* to environmental pollution: integrating the responses to oxidative stress and other biomarker responses into a general stress index. Aquat Toxicol 2008;89:18–27.
Raftopoulou EK, Dimitriadis VK. Assessment of the health status of mussels *Mytilus galloprovincialis* along Thermaikos Gulf (Northern Greece): an integrative biomarker approach using ecosystem health indices. Ecotoxicol Environ Saf 2010;73:1580–7.
Satsmadjis J, Georgakopoulos–Gregoriadis E, Voutsinou–Taliadouri F. Separation of organochlorines on Alumina. J Chromatogr 1988;437:254–7.
SoHelME. In: Papathanassiou E, Zenetos A, editors. State of the Hellenic marine environment. HCMR Publ; 2005.
Spatharis S, Panayotidis P, Danielidis D, Monesanto B. Actes du Deuxieme symposium Mediterraneen sur la Vegetation Marine, Athenes; 2003. p. 217–22.. [12–13 December].
Stien X, Percic P, Gnassia-Barelli M, Roméo M, Lafaurie M. Evaluation of biomarkers in caged fishes and mussels to assess the quality of waters in a bay of the NW Mediterranean Sea. Environ Pollut 1988;99:339–45.
Thain JE, Vethaak AD, Hylland K. Contaminants in marine ecosystems: developing an integrated indicator framework using biological-effect techniques. ICES J Mar Sci 2008;65:1508–14.
Tsangaris C, Kormas K, Stroggyloudi E, Hatzianestis I, Neofitou C, Andral B, et al. Multiple biomarkers of pollution effects in caged mussels on the Greek coastline. Comp Biochem Physiol 2010;151C:369–78.
UNEP. Determination of total Cd, Zn, Pd and Cu in selected marine organisms by atomic absorption spectrophotometry. Reference Methods for Marine Pollution Studies; 1984.. [No 11, Rev 1].
Viarengo A, Burlando B, Dondero F, Marro A, Fabbri R. Metallothionein as a tool in biomonitoring programmes. Biomarkers 1999;4:455–66.
Viarengo A, Lowe D, Bolognesi C, Fabbri E, Koehler A. The use of biomarkers in biomonitoring: a 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. Comp Biochem Physiol 2007;146C:281–300.
Voutsinou-Taliadouri F, Georgakopoulou-Grigoriadou E. Heavy metal concentrations in surface stations from Pagassitikos Gulf, Greece. Toxicol Environ Chem 1989;20:53–8.
Voutsinou-Taliadouri F, Satsmadjis J. Trace metals in the Pasassitikos Gulf, Greece. Estuar Coast Shelf Sci 1982;15:221–8.
Wang C, Lu GH, Song WT, Xu S, Wang PF. Integrated biomarker response index for the assessment of environmental stress of the Yangtze River (Nanjing section). Fish Physiol Biochem 2010;36:1069–78.
Wo KT, Lam PKS, Wu RSS. A comparison of growth biomarkers for assessing sublethal effects of cadmium on a marine gastropod, *Nassarius festivus*. Mar Pollut Bull 1999;39:165–73.
Zitko V. Principal component analysis in the evaluation of environmental data. Mar Pollut Bull 1994;28:718–22.