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Multiple biomarkers of pollution effects in caged mussels on the Greek coastline

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ABSTRACT

A suite of biomarkers was measured in caged mussels at areas impacted by different anthropogenic activities along the Greek coastline to assess biological effects of environmental pollution. Mussels were caged at coastal sites in the vicinity of major cities, in areas influenced by major industries, agricultural practices and in islands away from known sources of pollution. Biomarkers indicative of neurotoxicity (acetylcholinesterase, AchE), oxidative stress (catalase, CAT), phase II biotransformation of xenobiotics (glutathione S-transferase, GST), metal exposure (metallothioneins, MTs) and protein synthesis (RNA:DNA ratio) were measured to assess effects of various types of pollutants. AchE activity proved to be the most responsive biomarker with decreased values at sites influenced by agricultural, urban and industrial activities. Decreased CAT and GST activities and increased MTs levels were recorded at a number of anthropogenic-impacted sites. RNA:DNA ratio showed a biphasic response as both high and low values were found at impacted sites. Principal component analysis clearly distinguished sites receiving pollution inputs from non-polluted sites. The combination of the selected biomarkers used in caged mussels resulted useful in the assessment of the effects of environmental pollution.

1. Introduction

Biological effects of pollution are elements of major importance for the assessment of environmental quality since, by definition, pollution implies hazards to living resources. The effects of pollution can be measured at different levels of biological organization, from the molecular to the community level. Biomarkers are cellular, biochemical, molecular, or physiological changes that are measured in cells, body fluids, tissues, or organs within an organism and are indicative of xenobiotic exposure and/or effect (Lam and Gray, 2003). Biomarkers range from general to specific, reflecting general stress or exposure to specific classes of environmental contaminants. Since changes at the organism level lead to changes at the population and community levels, biomarkers can be used as early warning signals of environmental disturbance (Walker et al., 2006). Therefore biomarkers are considered useful tools and are increasingly incorporated into environmental monitoring programs (e.g. Joint Monitoring Program of the OSPAR convention; MED POL, UNEP Mediterranean Biomonitoring Program) (Lam and Gray, 2003; Viarengo et al., 2007). The EU Water Framework Directive (WFD, Directive 2000/60/EC), that specified monitoring programs required to assess the achievement of good chemical and ecological status of water bodies, pointed out the importance of biological monitoring for the determination of water quality. Biomarkers, although not incorporated in the WFD, are among the emerging biological monitoring tools considered for use in monitoring programs necessary for the implementation of the WFD (Allan et al., 2006; Mills et al., 2007).

Mussels are commonly used as sentinel organisms in biomonitoring studies (Andral et al., 2004; Viarengo et al., 2007). In addition to their wide geographical distribution and ability to accumulate contaminants, mussels can be easily caged at field sites. Wild mussels and/or transplanted mussels originating from a clean site are employed in bio-monitoring (Roméo et al., 2003; Bocquené et al., 2004; Andral et al., 2004; Pampanin et al., 2005a; Lehtonen et al., 2006). The parallel use of wild and caged mussels combines information on long-term effects and short-term effects of pollution in areas where they are naturally present. (Bolognesi et al., 2004; Pampanin et al., 2005a). Using caged mussels from a single population avoids bias related to the age and the reproductive status of the organisms that influence both contaminant bioaccumulation and biomarker responses and allows more accurate assessment of the real biological effects of contaminants over a predetermined exposure period (Andral et al., 2004; Viarengo et al., 2007).

The aim of this study was to assess biological effect/exposure of environmental pollution in Greek coastal waters over a wide spatial scale using a suite of biomarkers in caged mussels at several coastal areas impacted by different anthropogenic activities. Acetylcholinesterase (AchE) is an enzyme involved in nerve impulse transmission

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and its inhibition is an established biomarker of neurotoxicity caused by exposure to organophosphate and carbamate pesticides (Fulton and Key, 2001) while recent studies suggest it may also indicate general stress (Lehtonen et al., 2006). Catalase (CAT) is an enzyme of the antioxidant defense used as a biomarker of oxidative stress that can be induced by a wide range of contaminants including organic xenobiotics and heavy metals (Livingstone, 2001; Akcha et al., 2000; Roméo et al., 2003). Glutathione S-transferases (GST) are the most important enzymes of the phase II biotransformation of xenobiotics that have been shown to respond to organic contaminants (e.g. PCBs, chlorinated pesticides, PAHs; Cheung et al., 2001, 2002). Metallothioneins (MTs) are metal binding proteins involved in heavy metal detoxification and their induction is a biomarker of exposure to certain heavy metals, primarily Cd, Zn, Cu, and Hg (Viarengo et al., 1999). RNA:DNA ratio is a measure of protein synthesis that has been used as a biochemical biomarker of growth reflecting a general response to environmental stressors (Wo et al., 1999; Pottinger et al., 2002; Humphrey et al., 2007). While AchE, MTs, CAT and GST are commonly used biomarkers in *Mytilus* sp. (Roméo et al., 2003; Lehtonen et al., 2006) to our knowledge, this is the first application of the RNA:DNA ratio as a biomarker of the effects of pollution in this genus.

2. Materials and methods

2.1. Sampling areas and experimental design

Eighteen sampling sites at areas influenced by different types of anthropogenic activities were selected along the Greek coastline (Fig. 1). These included sites in the vicinity of major cities, Athens (S3) and Thessaloniki (S1 and S2), sites at areas influenced by major industries (S4, S5, and S6), sites at areas influenced by agricultural practices (S7, S8, S9, S10, S11, and S12) and sites in the Aegean Sea, near islands away from known sources of pollution that were used as reference sites (S14, S15, S16, S17, and S18) and on the island of Santorini (S13) where the cruise ship 'Sea Diamond' sank in April 2007 (Table 1). Pollutant concentrations previously reported in sediments at the selected areas are shown in Table 1.

Mussels were purchased from an aquaculture farm in NW Saronikos Gulf in May 2007. Mussels of approximately 60 mm shell

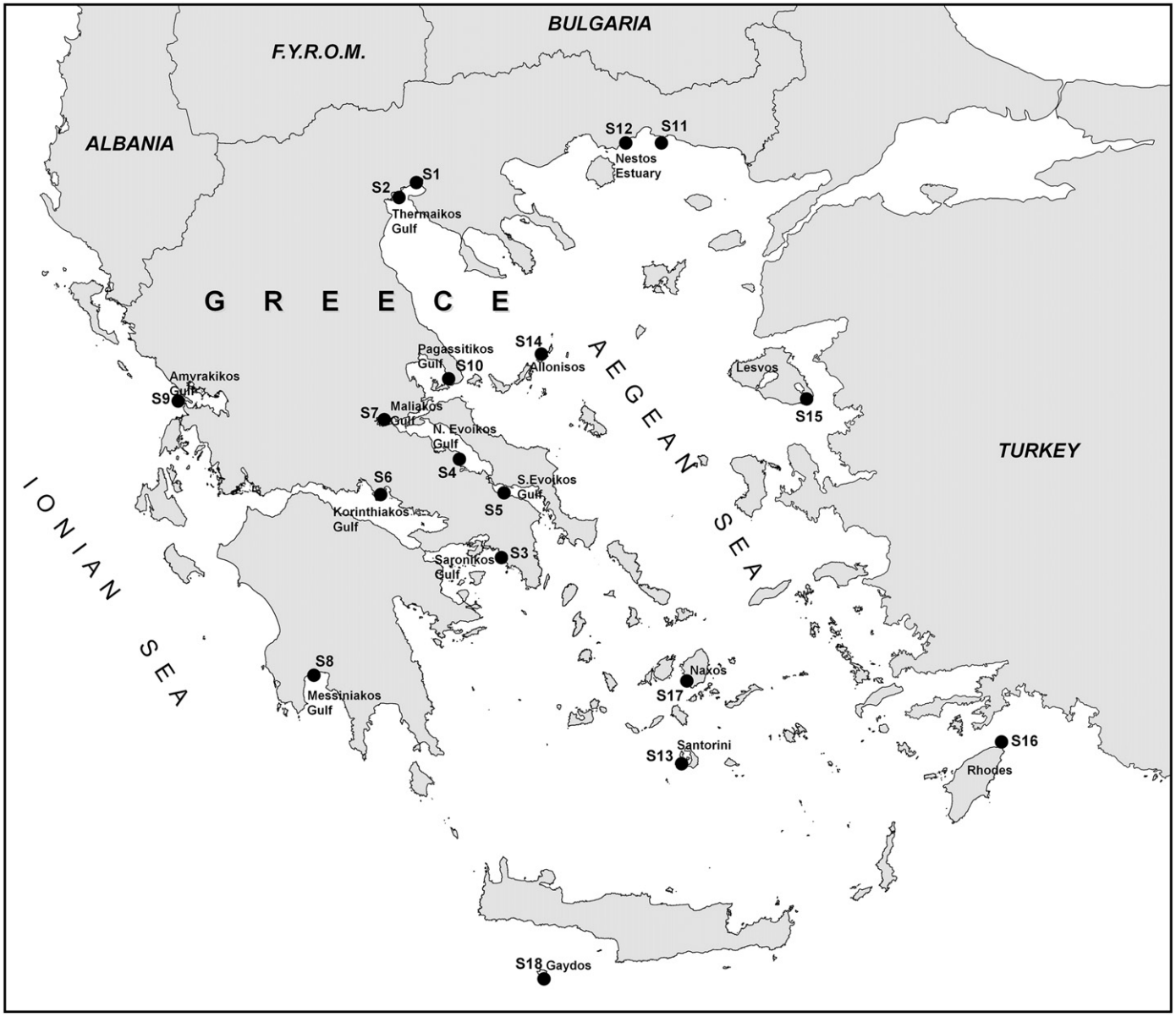

Fig. 1. Location of the 18 sampling sites in the Greek coastal region.

Table 1
Sampling sites in Greek coastal areas, types of anthropogenic impacts and levels of pollutants reported in sediments in the selected areas (AHC: aliphatic hydrocarbons, PAH: polycyclic aromatic hydrocarbons, DDTs: sum of DDT, DDD, DDE, PCBs: polychlorinated biphenyls (sum of 11 congeners)).

Station	Longitude and latitude	Area	Type of anthropogenic impact	Pollutant concentrations in sediments								
				AHC µg/g	PAH ng/g	DDTs ng/g	PCBs ng/g	Pb µg/g	Cu µg/g	Zn g/g	Cr µg/g	Ni µg/g
S1	22°53.56′E 40°36.07′N	Thermaikos Gulf	Urban wastes from Thessalonica, industrial inputs	1100[a]	1160[a]	7.1[a]	11.4[a]	146[b]	67[b]	221[b]	287[b]	
S2	22°43.72′E 40°29.37′N	Thermaikos Gulf	Urban wastes from Thessalonica, industrial inputs, agricultural practices	106[a]	218[a]	1.9[a]	0.9[a]	369[b]	17[b]	26[b]	231[b]	
S3	23°42.88′E 37°52.56′N	Saronikos Gulf	Urban wastes from Athens, industrial inputs	136[c]	2270[c]	2.1[d]	5.3[d]	22[c]	8[c]	42[c]	63[c]	34[c]
S4	23°19.30′E 38°35.28′N	N. Evoikos Gulf	Industrial inputs by ferronickel production, metalifferous slag disposal	31[e]	7760[f]	0.45[e]	8.8[e]	24[e]	14[e]	100[e]	15,000[e]	900[e]
S5	23°44.28′E 38°20.67′N	S. Evoikos Gulf	Industrial inputs via Asopos river	31[g]	7760[g]			27[h]	42[h]	106[h]	404[h]	671[h]
S6	22°35.98′E 38°19.46′N	Korinthiakos Gulf	Industrial inputs by alumina production, red mad disposal	18[b]	4330[b]	0.4[b]	19.1[b]	91[b]	32[b]	72[b]	1820[b]	850[b]
S7	22°37.23′E 38°52.23′N	Maliakos Gulf	Agricultural practices, urban wastes	12[i]	140[i]			37[j]	43[j]	105[j]		220[j]
S8	22°1.08′E 37°0.14′N	Messiniakos Gulf		18[k]	120[k]			30[k]	43[k]	70[k]	149[k]	96[k]
S9	20°42.02′E 38°58.09′N	Entrance of Amvrakikos Gulf		29[l]	480[l]	0.23[l]	0.33[l]					
S10	23°13.02′E 39°10.47′N	Pagassitikos Gulf	Agricultural and aquaculture practices	14[b]	138[b]	1.7[b]	0.9[b]	44[b]	41[b]	124[b]	267[b]	270[b]
S11	25°14.06′E 40°53.29′N	Broader Nestos river estuary		28[m]	310[m]	3.1[m]	1.9[m]					
S12	24°53.62′E 40°53.45′N	Nestos river estuary		36[m]	280[m]	16.7[m]	1.8[m]	71[b]	15[b]	102[b]	51[b]	
S13	25°20.54′E 36°21.94′N	Santorini island, Aegean Sea	Shipwreck of cruise ship 'Sea Diamond' in April 2007	8[n]	33[n]			34[o]	26[o]	69[o]	20[o]	17[o]
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	Rodhes island, Aegean Sea		10[p]	20[p]	0.14[p]	1.3[p]	6[p]	47[p]	83[p]	960[p]	500[p]
S17	25°24.01′E 36°58.15′N	Naxos island, Aegean Sea										
S18	24°6.11′ E 34°48.24′N	Gaydos island, Aegean Sea	Absence of known pollution sources									

[a] HCMR, 2008a.
[b] Laboratory Network of the Environmental Quality monitoring of the Hellenic Seas, 2006.
[c] Karageorgis and Hatzianestis, 2003.
[d] Hatzianestis and Botsou, 2005.
[e] HCMR, 2008b.
[f] Hatzianestis et al., 2005.
[g] Zenetos et al., 2004.
[h] Angelidis and Aloupi, 2000.
[i] Hatzianestis, unpublished results.
[j] Anagnostou and Kaberi, 1995.
[k] HCMR, 2007a.
[l] Lelekis et al, 2001.
[m] Cotou et al., 2002.
[n] HCMR, 2007b.
[o] HCMR, 2008c.
[p] HCMR, 2004.

length were sorted, placed in PVC cages (3 kg per cage) and maintained one week at the farm prior to immersion so that the mussels were able to fix themselves by their byssus threads. The transplantation was performed by the French oceanographic vessel 'L' Europe' and the Greek oceanographic vessel 'Philia'. During transplantation the mussel cages were kept on board in tanks provided with flowing seawater. The depth of the selected sites was between 20 to 30 m. The mussel cages were immersed with a buoy at 8 m depth below the surface and were attached by a rope to a ballast of approximately 30 kg at the bottom. The mussels were recovered after three months by diving. The transplantation period did not coincide with the spawning season of the mussels in order to avoid influences of gamete release on bioaccumulation of pollutants and biomarker responses. 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 °C for 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 pooled samples from 6 individuals (5 samples per site) were frozen and stored in liquid nitrogen for AchE, CAT, GST and MTs measurements. When transferred to the laboratory samples remained at −80 °C.

2.2. Physiological status of transplanted mussels

Condition index (C.I.) was determined as an indicator of the physiological status of the mussels. C.I. is an ecophysiological measure of the health status of the animals that summarizes their physiological activity (growth, reproduction, secretion, etc.) under given environmental conditions. The samples were pre-processed according to

standardized procedures (Andral et al., 2004). Dissected whole soft tissues from 30–40 individuals were pooled into a composed sample and then lyophilized. Shells were dried at 60 °C in the oven for 48 h and then weighed. Flesh was weighed after freeze–drying. The ratio of dry flesh weight to dry shell weight (FW/SW X 100) was used to determine C.I. for each sample. During the period of sexual dormancy, this quotient is a good indicator of mollusc growth.

2.3. AchE activity

Gill tissues were homogenized using a Potter-Elvehjem homogenizer (Heidolph Electro GmbH, Kelheim, Germany) 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 (EC 3.1.1.7) was assayed by the method of Ellman (Ellman et al., 1961) adapted to microplate reading by Bocquené et al. (1993) on an Assys Digiscan reader 340. Total protein content was measured using bovine serum albumin (BSA) as a standard (Bradford, 1976). Enzyme activity was expressed as U/mg proteins. One unit (U) of AchE activity is the amount of enzyme that causes a change in optical density of 0.001 per minute.

2.4. CAT activity

Digestive glands were homogenized using a Potter-Elvehjem homogenizer in 1:5 (w:v) 100 mM KH_2PO4/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 (EC 1.11.1.6) was measured by the method of Cohen et al. (1996) by the loss of H_2O_2 that was measured calorimetrically with ferrous ions and thiocyanate on a microplate reader. 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 ln is the natural log, 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.

2.5. GST activity

Preparation of digestive gland tissue extracts were as described for CAT. GST (EC 2.5.1.18) was measured by the method of Habig and Jakoby (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 nmol conjugate/min/mg proteins.

2.6. MTs content

MTs concentration was measured in digestive glands according to Viarengo et al. (1997) on a Perkin Elmer UV/VIS spectrometer Lamda 20. The method is based on the estimation of the sulphydryl 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 μg MTs/g wet weight tissue.

2.7. RNA:DNA

Nucleic acids were quantified spectrophotometrically in whole soft tissue. To reduce variability the whole soft tissue from 3–5 specimens was pooled for each analysis. Samples were homogenized with a mortar and pestle in liquid nitrogen and ca. 1.5 g of the homogenized material was used for nucleic acid extraction with the Qiagen RNA/DNA kit according to the manufacturer's instructions (Qiagen, USA). The concentration and purity of the extracted nucleic acids were determined with the NanoDrop ND-1000 (NanoDrop Technologies, USA). RNA:DNA was calculated as the ratio of mean RNA (ng/mg) to mean DNA (ng/mg) of 3 pooled samples per site.

2.8. Statistical analysis

Data are presented as mean ± standard error of the mean. The Kolmogorov–Smirnoff test and Levene's test were applied to test normal distribution and homogeneity of variance respectively. One-way analysis of variance (ANOVA) followed by the Fisher's LSD multiple comparison test was applied to determine differences between sites when homogeneity of variance was assumed (AchE, and MTs), while when variances were not equal (GST, and CAT) the Games–Howell test was used. Significance level was set at $P<0.05$.

Principal Component Analysis (PCA) was performed to discriminate sites by integration of biomarkers according to principal axes. Biomarkers (AchE, CAT, MTs, GST, and RNA:DNA) and C.I. were the variables used in PCA. PCA was applied to mean values of biomarkers and values from pooled samples of C.I. at each site. PCA requires that all the sites have values for all the parameters and as we did not have MTs results for S8, S9, S11 and S17 these sites were not analyzed in

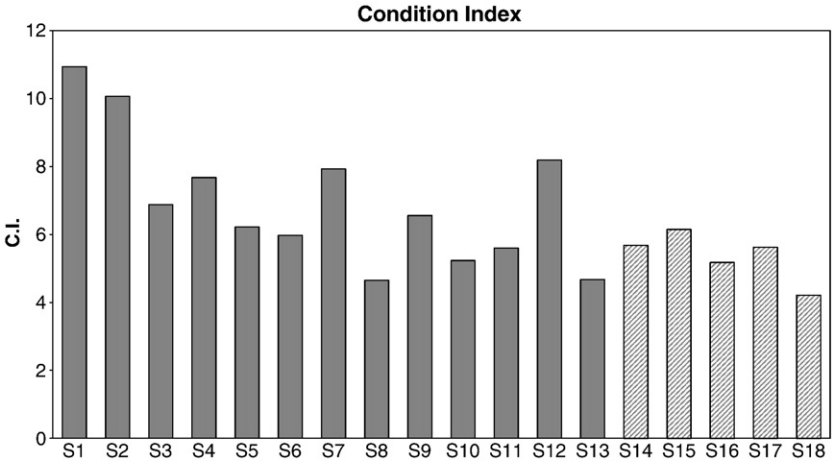

Fig. 2. Condition index (C.I.) of mussels *M. galloprovincialis* caged for three months at 18 sites in Greek coastal waters (30–40 pooled individuals per site). Dashed bars represent reference sites.

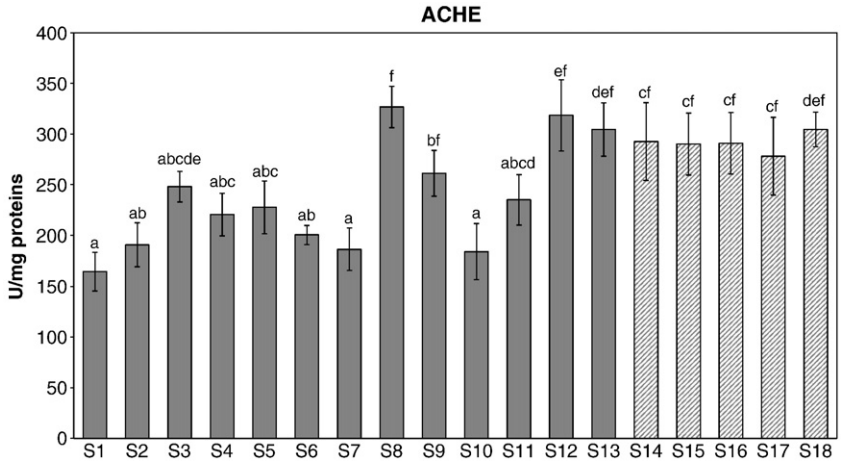

Fig. 3. Acetylcholinesterase (AchE) activities (U/mg proteins) in the gills of mussels *M. galloprovincialis* caged for three months at 18 sites in Greek coastal waters. Dashed bars represent reference sites. Mean ± standard error, $n = 5$. Bars without a common letter are significantly different (Fisher's LSD test, $P<0.05$).

PCA. Statistical analysis was performed using SPSS (univariate methods) and Statgraphics (PCA).

3. Results

3.1. Condition index

C.I. overall ranged from 4.2 to 10.9 (Fig. 2). Higher C.I. values ranging from 7.7 to 10.9 were recorded in mussels caged at S1, S2, S4, S7 and S12. Variation in the C.I. at the other sites was limited and values ranged between 4.2 at S18 and 6.9 at S3.

3.2. Acetylcholinesterase

AchE activities ranged from 165 to 235 U/mg proteins at sites influenced by anthropogenic activities (S1, S2, S3, S4, S5, S6, S7, S10, and S11) and from 278 to 305 U/mg protein at the reference sites (S14, S15, S16, S17, and S18) (Fig. 3). AchE activities recorded in mussels at S8, S9, S12 and S13 sites, were similar to those at the reference sites (261–326 U/mg protein). Significantly lower AchE activities were found in mussels caged at S1, S2, S3, S4, S5, S6, S7, 10 and S11 sites compared to the reference sites and/or S8, S12 and S13 sites.

3.3. Catalase

Overall CAT activities varied from 0.7 to 3.6 U/mg proteins (Fig. 4). CAT activities were generally lower in mussels caged at sites influenced by anthropogenic activities (0.7–1.8 U/mg protein) compared to reference sites (1.9–3.4 U/mg proteins) with the exception of S4, S8, S9, S12 and S18 sites. Significantly lower CAT activities were recorded at S1, S2, S6, S7, S11, S13 and S18 sites compared to S17 reference site and/or S16 reference site. CAT activities at S1 and S13 were also lower than those at S14 reference site and S12.

3.4. Glutathione S-transferase

Overall GST activities ranged from 35.9 to 70.4 nmol/min/mg protein (Fig. 5). GST activities did not show a clear trend at sites influenced by anthropogenic activities, although low values were noted at a few impacted sites. The lowest GST activities recorded in mussels caged at S5 site were significantly different than those at

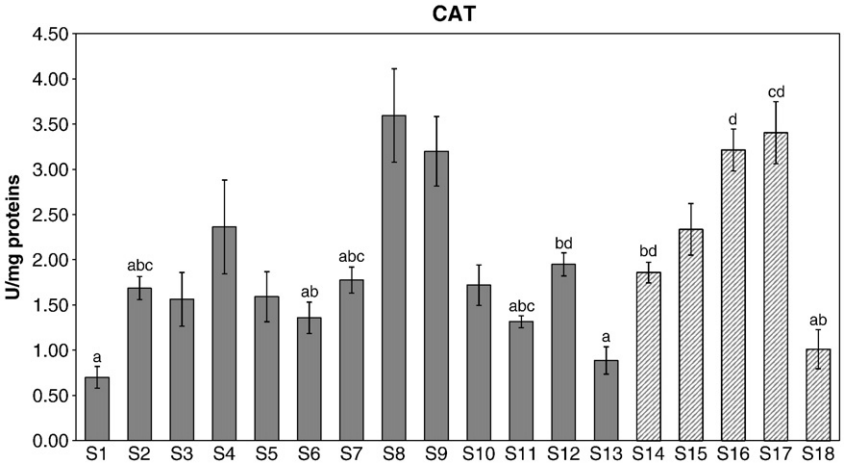

Fig. 4. Catalase (CAT) activities (U/mg proteins) in the digestive gland of mussels *M. galloprovincialis* caged for three months at 18 sites in Greek coastal waters. Dashed bars represent reference sites. Mean ± standard error, $n = 5$. Bars without a common letter are significantly different (Games–Howell test, $P<0.05$); bars without letters show no significant differences.

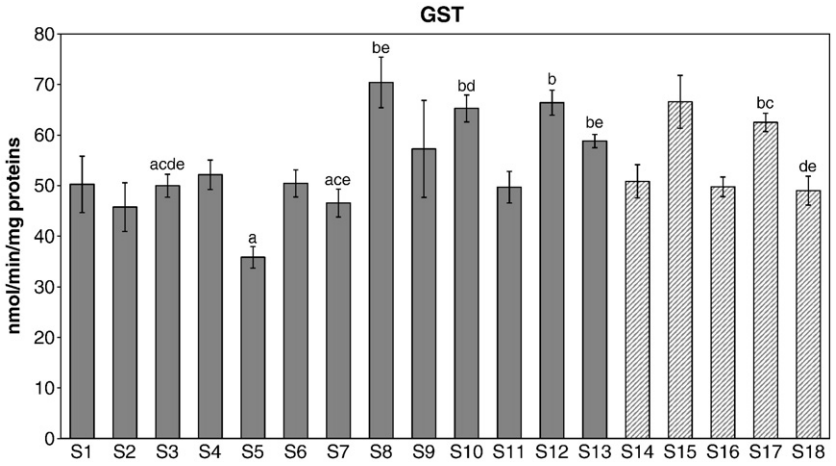

Fig. 5. Glutathione S-transferase (GST) activities (nmol conjugate/min/mg proteins) in the digestive gland of mussels *M. galloprovincialis* caged for three months at 18 sites in Greek coastal waters. Dashed bars represent reference sites. Mean ± standard error, $n = 5$. Bars without a common letter are significantly different (Games–Howell test, $P<0.05$); bars without letters show no significant differences.

S17 reference site and also S8, S10, S12 and S13 sites. Furthermore significantly lower GST activities were found at S3, S7 and S18 reference site compared to S17 reference site, S10 and/or S12 sites.

3.5. Metallothioneins

MT levels ranged between 152 and 225 μg/g tissue at four of the anthropogenically-impacted sites (S3, S6, S7, and S13), and between 94 and 138 μg/g tissue at all other sites (Fig. 6). MTs were significantly higher in mussels at S3, S6, S7 and S13 compared to all other sites with the exception of S18 reference site. MTs at S18 reference site were significantly higher with respect to the sites showing the lowest MTs levels i.e. S1, S5, S10 and S15.

3.6. RNA:DNA

RNA (ng/mg) and DNA (ng/mg) concentrations used for the calculation of RNA:DNA ratio at each site are shown in Table 2.

As regards RNA:DNA ratio, a distinct response at sites influenced by anthropogenic activities was not evident (Fig. 7). Coefficient of variation varied between 0.1 and 14.6% (average 3.3%). Overall RNA:DNA ranged from 0.01 to 2.34. Highest RNA:DNA levels were found at S4 and S5 (2.34 and 1.72 respectively) and lowest levels at S1, S2, S6, S7, S11, S12 and S16 sites (0.01–0.08). All other sites showed levels of RNA:DNA that ranged between 0.15 and 1.28.

3.7. PCA

PCA of biomarkers and C.I. produced three principal components that accounted for 76.3% of the total variance. PCs can be interpreted based on the loadings (coefficients in the linear combinations of variables making up the PCs), which explain how strongly the original variables correlate to the respective PC (Table 3). PC1 explained 31.3% of the total variance and was influenced by the C.I. on the positive part and AchE and GST on the negative part. PC2 explained 25.1% of the total variance and was correlated with MTs on the positive part and CAT and RNA:DNA on the negative part. PC3 represented 19.9% of the total variance and was positively correlated with MTs and RNA:DNA and negatively correlated with GST and C.I.

The plot of variable vectors for the two dominant components PC1 and PC2 that explained 56.4 % of the total variance is shown in Fig. 8A. The plot of scores for different sites for the two dominant components PC1 and PC2 separated four groups of sites (Fig. 8B). All reference sites S14, S15, S16, S17 and S18 were grouped together along with S10 and S12. The anthropogenically-impacted sites were separated into three groups: (a) S1 and S2, (b) S4 and S5, and (c) S3, S6, S7 and S13.

To examine how the introduction of each variable modifies PCA results, six additional PCA were applied excluding variables from the data set (not shown). PCA applied on biomarkers excluding C.I. produced a similar pattern but separation of groups was less distinct. Further exclusion of CAT and GST also led to a similar but less distinct

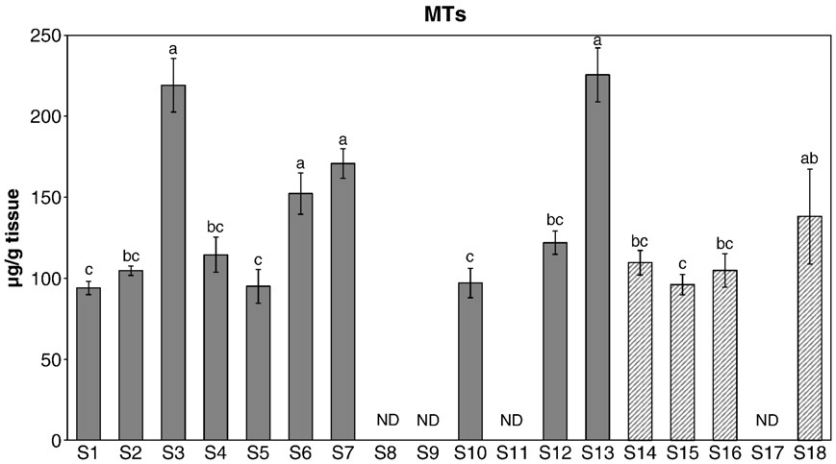

Fig. 6. Metallothionein (MTs) levels (μg/g tissue) in the digestive gland of mussels *M. galloprovincialis* caged for three months at 18 sites in Greek coastal waters. Dashed bars represent reference sites. ND: no data. Mean ± standard error, $n = 5$. Bars without a common letter are significantly different (Fisher's LSD test, $P<0.05$).

Table 2
Concentrations of RNA and DNA in mussels caged for three months at 18 sites in Greek coastal waters (mean ± standard error, $n = 3$) used for the calculation of RNA:DNA ratio.

	RNA ng/mg	DNA ng/mg
Thessaloniki	2.1 ± 0.1	158.5 ± 2.7
Axios	5.1 ± 0.1	206.2 ± 6.7
Ag. Kosmas	17.6 ± 0.2	119.0 ± 0.9
Larymna	148.5 ± 0.2	63.4 ± 0.2
Asopos	154.7 ± 0.3	89.67 ± 0.8
Korinthiakos	10.0 ± 0.5	121.0 ± 0.1
Maliakos	4.1 ± 0.1	248.0 ± 1.3
Milina	85.8 ± 0.2	248.9 ± 3.1
Kalamata	184.0 ± 0.6	152.8 ± 0.6
Preveza	308.9 ± 15.5	310.5 ± 11.1
Fanari	5.8 ± 0.1	117.6 ± 1.4
Nestos	4.2 ± 0.2	216.3 ± 2.1
Santorini	67.1 ± 0.4	238.3 ± 2.7
Alonisos	86.7 ± 1.0	261.7 ± 1.6
Lesvos	59.7 ± 0.3	107.9 ± 0.9
Rodos	6.95 ± 0.1	116.8 ± 0.6
Naxos	172.6 ± 6.9	134.6 ± 0.4
Gavdos	82.8 ± 0.2	89.1 ± 0.6

Table 3
PCA: component loadings of the variables for PC1, PC2 and PC3.

Variables	PC1	PC2	PC3
AchE	*−0.639*	−0.026	0.045
CAT	−0.241	*−0.585*	−0.260
GST	*−0.395*	0.079	*−0.599*
MTs	−0.211	*0.628*	*0.316*
RNA:DNA	0.019	*−0.505*	*0.578*
Condition index	*0.578*	0.027	*−0.372*

Significant correlation coefficients are in italics.

separation, while exclusion of AchE, MTs or RNA:DNA ratio modified the site separation and did not clearly discriminate the group of reference sites.

4. Discussion

This study applied a suite of biomarkers in mussels to assess pollution effects in Greek coastal waters. A standardized technique of caging mussels in the open sea was used in order to avoid bias related to genetic differences as well as the previous physiological and reproductive condition of the animals (Andral et al., 2004). However, variations in environmental factors such as temperature, salinity and food availability among sites would influence the physiological state of the animals. Variations in the physiological state of mussels as shown by C.I. results were generally in accordance with the trophic characteristics of the sites with high values at 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 at the reference sites in the Aegean Sea where waters are oligotrophic (Ignatiades, 2005). These findings suggest that C.I. was influenced by food availability although possible advanced spawning induced by natural or pollution stress resulting in weight loss may also have affected C.I. Nevertheless, differences in the C.I. did not mask biomarker responses to pollution since biomarker values overall varied between anthropogenic-impacted and reference sites.

AchE activity resulted the most responsive biomarker showing lower levels at nine sites influenced by anthropogenic activities compared to the reference sites. AchE inhibition has been widely used as a biomarker of neurotoxic effects by organophosphate and carbamate pesticides (Fulton and Key, 2001). Recent studies have shown that other types of pollutants such as heavy metals, surfactants and PAHs (Guilhermino et al., 1998; Akcha et al., 2000; Elumalai et al., 2002) may also inhibit AchE activity. AchE inhibition has thus been suggested as indicative of general stress (Lehtonen et al., 2006). In accordance, our results showed decreased AchE activities not only at sites in areas influenced by agricultural practices where pesticide contamination would be expected (S2, S7, S10, and S11) but also at sites in areas receiving urban and industrial wastes (S1, S3, S4, S5, and S6) where a wide variety of pollutants are found (Voutsinou-Taliadouri and Varnavas, 1993; Papatheodorou et al., 1999; Hatzianestis et al., 2000, 2003; Poulos et al., 2000; Angelidis and Aloupi, 2000; Hatzianestis and Botsou, 2003, 2006; Scoullos et al., 2007).

CAT response to toxic chemicals shows a bell-shaped trend with an initial increase in activity due to enzyme induction followed by a decrease in activity due to enhanced catabolic rate and/or direct inhibition by toxic chemicals (Viarengo et al., 2007). Such trends in CAT activities can be found in mussels at polluted sites according to the levels and duration of pollutant exposure (Regoli and Principato, 1995; Nasci et al., 2002; Roméo et al., 2003; Regoli et al., 2004; Nesto et al., 2004; Pampanin et al., 2005b). Regoli et al. (2004) showed an increase in CAT activity during the first two weeks of mussel transplantation at an industrialized harbour of NW Italy followed by a progressive decrease. Decreased CAT activities in mussels transplanted at polluted sites have been found in addition to a reduced capability of neutralizing Reactive Oxygen Species (ROS) and an increased susceptibility to oxidative stress (Pampanin et al., 2005b). Accordingly, the low CAT activities in mussels transplanted at impacted sites of this study

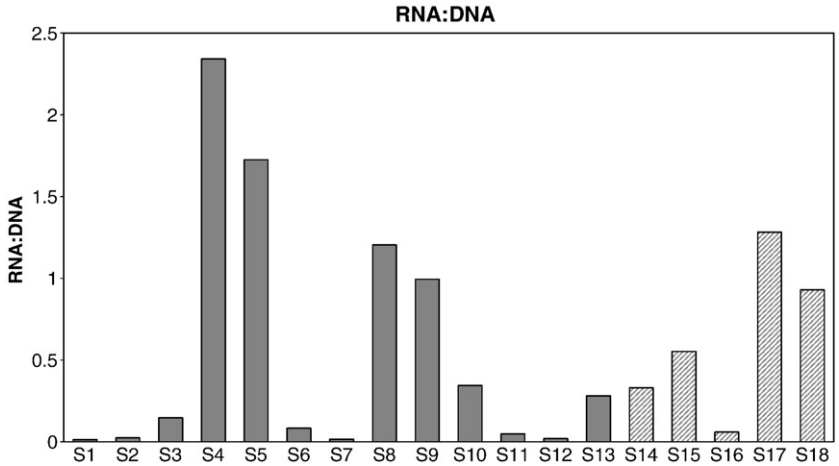

Fig. 7. RNA:DNA ratio in whole soft tissues of mussels *M. galloprovincialis* caged for three months at 18 sites in Greek coastal waters. Dashed bars represent reference sites. RNA:DNA is calculated as the ratio of mean RNA concentrations (ng/mg) ($n = 3$) to mean DNA concentrations (ng/mg) ($n = 3$) at each site.

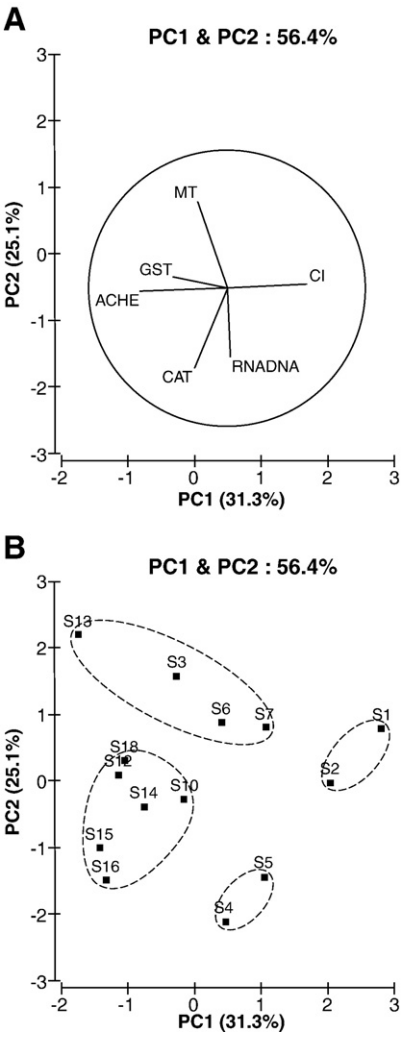

Fig. 8. Results of the PCA of the two dominant components produced by biomarkers (AchE, MTs, CAT, and GST) and condition index (C.I.). (A) Plot of variable vectors. (B) Plot of scores of different sites.

can be associated with difficulty to compensate to oxidative stress. CAT responds to a wide range of contaminants capable of ROS production such as PAHs, PCBs, heavy metals, and pesticides (Krishnakumar et al., 1997; Regoli and Principato, 1995; Akcha et al., 2000; Roméo et al., 2003; Khessiba et al., 2005) thus CAT responses were evident at sites receiving different pollutant inputs (S1, S2, S6, S7, S11 and S13).

GST response to toxic chemicals follows a similar bell-shaped trend as CAT (Viarengo et al., 2007) hence increased and decreased enzyme activities have been reported in polluted areas (Roméo et al., 2003; Regoli et al., 2004; Bocquené et al., 2004; Bebianno et al., 2007). GST is induced by organic contaminants as part of the phase II biotransformation pathway (Sheehan and Power, 1999) while GST inhibition has been indicated as more aspecific response to chemical challenge (Regoli et al., 2003). In this study, GST responses did not show a clear trend although reduced activities were found at three of the impacted sites (S3, S5 and S7).

MTs induction is a well-recognized biomarker of heavy metal exposure that is commonly applied in pollution monitoring programs (Viarengo et al., 1999, 2007). MTs levels are largely influenced by factors such as reproductive condition, duration of exposure, previous exposure and environmental parameters thus the use of transplanted mussels is suggested as the most suitable approach for the application of MTs as a biomarker of heavy metal exposure (Viarengo et al., 1999). Increased MTs values were found only at four of the impacted sites: S3, S6, S7 and S13. This is in agreement with generally low heavy metal levels in the tissues of mussels and only few differences among sites including increased Cu and/or Cd levels at S3, S6 and S7 (Andral, unpublished results).

Controversially, low CAT activities, low GST activities and high MTs levels were recorded at S18 reference site in the South Aegean Sea where the lowest C.I. values were recorded, reflecting stress possibly caused by natural factors such as temperature, salinity, food availability, etc. as wild mussels are not present in this area. Similar responses were recorded at S13 site also situated in the South Aegean Sea. Whether biomarker responses at S13 site were due to natural environmental factors or pollutants emerging from the shipwreck of the 'Sea Diamond' cruise ship two months prior mussel transplantation in this area could not be distinguished. On the other hand, at S8, S9 and S12 sites initially regarded as potentially impacted, AchE, CAT, GST and MTs values were similar to those at the reference sites suggesting the degree of impact is low. In fact contaminant levels reported in sediments at these areas are lower than at the other impacted sites (Table 1).

The RNA:DNA ratio provides an estimate of nutritional condition and recent protein synthesis (Buckley et al., 1999). Consequently, RNA:DNA ratios have been used to assess the condition and growth of bivalves (Norkko et al., 2006; Menge et al., 2007) but applications for the assessment of pollution effects are limited (Roesijadi et al., 1995; De Luca-Abbot, 2001; Lannig et al., 2006). During exposure to low pollution protein synthesis is known to increase due to induction of proteins involved in the protection of the cell against harmful conditions, such as stress proteins, MTs, antioxidant enzymes and biotransformation enzymes, which is expected to reflect in elevated transcriptional activity and thus higher RNA:DNA ratios (Lannig et al., 2006). At high pollution stress however, protein synthesis can be suppressed indicating disturbance of normal metabolic processes (Pottinger et al., 2002). Therefore increase or decrease in protein synthesis and thus RNA:DNA ratios can be expected as a result of pollutant exposure depending on the stress level. Our results indicate a biphasic response of RNA:DNA ratio to stress as both high and low values were found at impacted sites. At reference sites with the exception of S16, DNA:RNA ratio levels were in the mid range for this study as they were also at S8, S9 and S10 where most other biomarker values were similar to the reference sites. At impacted sites either very high or very low DNA:RNA ratio levels were found indicating increased or decreased protein synthesis respectively. These results indicate that changes in RNA:DNA ratio may reflect pollution stress but the use of RNA:DNA ratio as a biomarker must be used with caution and appropriate reference values must be established.

For integration of biomarker responses, PCA was applied to differentiate groups of sites as a function of principal components. The two dominant components strongly corresponded to AchE and C.I. (PC1) and MTs, CAT and RNA:DNA (PC2). PCA clearly distinguished reference from impacted sites since all reference sites (S14, S15, S16, S17 and S18) were grouped together by PC1 and PC2 reflecting similar biomarker responses. S10 and S12 were also grouped with the reference sites. The impacted sites were separated into three groups by combinations of different biomarker responses: (a) S1 and S2, (b) S4 and S5, and (c) S3, S6, S7 and S13. These results suggest that combinations of different biomarker responses reflect different types of pollution in each group of the impacted sites. This is also supported by the separation of groups of impacted sites according to geographical areas. Groups (a) and (b) that include sites in the same geographical area ((a): Thermaikos Gulf (b): Evoikos gulf,) are clearly distinct from group (c) that includes sites at different regions (Maliakos Gulf, Korinthiakos Gulf, Saronikos Gulf, and Aegean Sea) and is widespread. It is also important to point out that group (b) sites, S4 and S5, although in the same geographical area, receive industrial inputs from different industries but are both

characterized by high concentrations of heavy metals such as Cr and Ni (Voutsinou-Taliadouri and Varnavas, 1993; Angelidis and Aloupi, 2000). AchE, MTs and RNA:DNA appeared the most relevant end points applied since their introduction in the PCA was crucial for discriminating the groups of sites.

In conclusion the present study showed that a combination of biomarker responses representing different biological endpoints in caged mussels distinguished sites receiving pollution inputs by various anthropogenic activities from non-impacted sites in Greek coastal waters and are thus useful for the assessment of environmental pollution effects. Therefore, it is important to include several reference sites in field studies as certain biomarkers may show biphasic responses or can be influenced by environmental factors other than pollutant exposure. A clear picture of reference values must be established for interpretation of biomarker results.

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