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ScienceDirect
Marine Pollution Bulletin 54 (2007) 1361–1371
MARINE POLLUTION BULLETIN
www.elsevier.com/locate/marpolbul

Integrated use of biomarkers (superoxide dismutase, catalase and lipid peroxidation) in mussels *Mytilus galloprovincialis* for assessing heavy metals' pollution in coastal areas from the Saronikos Gulf of Greece

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Abstract

Mussels are used as sentinel organisms and bioindicators to evaluate the toxic effects of chemical pollutants in marine organisms, especially heavy metals, representing an important tool for biomonitoring environmental pollution in coastal areas. Antioxidant defence enzymes play an important role in cellular antioxidant defence systems and protect from oxidative damage by reactive oxygen species (ROS). Indigenous mussels *Mytilus galloprovincialis* of the Saronikos Gulf of Greece were used for monitoring heavy metal pollution in three polluted sites in the area and in one unpolluted site. Seasonal variations of the activity of antioxidant defence enzymes, superoxide dismutase (SOD) and catalase (CAT), as well as lipid peroxidation (LP) were measured as biomarkers in a period of three years in relation to concentrations of trace metals in their gills and mantle and compared to mussels from an unpolluted sampling site. SOD activity increased at least 2 fold at the polluted sites when compared to the control site (the high activity was recorded in the spring time). CAT activity was increased 2–3 times at the polluted sites, with high activity in the winter and spring time, compared to the control site. LP concentration was twice higher at the polluted sites, following the same seasonal pattern. Trace metals contents in mussels collected at polluted sites were 3–4 fold higher compared to the control site and showed moderate variations along the months, with a winter maximum followed by a summer pre-spawning minimum matching the seasonal trends of temperature and salinity. Our results showed that metal pollution in the Elefsis Bay (the most polluted coastal area) causes relatively medium levels of oxidative stress in tissues of mussels due to cellular oxy-radical generation. This study, which is the first in the area, showed that seasonal variations of the activity of antioxidant defence enzymes and LP concentrations in mussels can be used as potential biomarkers of toxicity for long-term monitoring in marine coastal ecosystems.

Keywords: Heavy metals; Biomarkers; Oxidative stress; Antioxidant defence enzymes; Lipid peroxidation; Mussel *Mytilus galloprovincialis*

1. Introduction

In recent years there is increasing interest in studies of oxidative toxicity by various pollutants in aquatic organisms (Livingstone, 1998). Aerobic organisms, in the process of evolution, have adapted to the increased concentrations of oxygen which is very important to their metabolism and growth but equally damaging to biomolecules because of its oxidative potential. The pro-oxidant/antioxidant balance in biological systems and the scavenging of reactive oxygen species (ROS) are crucial for cellular homeostasis (Livingstone, 2001; Valavanidis et al., 2006). Aquatic organisms are highly susceptible to oxidative effects from environmental pollutants, especially when they can generate or enhance

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0025-326X/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.marpolbul.2007.05.018

the production of ROS, such as transition metals (Viarengo, 1989).

Aquatic organisms, especially marine bivalves, exhibit a variety of changes in enzymatic antioxidant defences after exposure to pollutants with oxidative potential (Regoli et al., 2002a,b). Prominent among these antioxidant defence system are superoxide dismutase (SOD) converting the superoxide anion into hydrogen peroxide, catalase (CAT) converting H_2O_2 into water and the glutathione S-transferase (GST), phase II detoxifying enzyme. All these enzyme antioxidant defences are used as biomarkers of oxidative damage (Regoli and Principato, 1995). Antioxidant defence enzymes are induced by various environmental pollutants under pro-oxidant conditions, initially their concentrations increase to counteract oxidative stress but prolonged exposure causes their depletion, leading to oxidative damage to basic biological molecules, such as lipid peroxidation, protein and DNA damage (Bebianno et al., 2005).

Metals, such as Cd, Ni, Cr, Pb and Hg are toxic in aquatic organisms mainly because of the oxidative potential whereas other metals, such as Fe, Zn, Cu, Se and Mn are essential for their metabolism but become toxic when their concentrations are excessive (Chang et al., 1996). Numerous studies investigated the bioaccumulation and oxidative damage caused by heavy metals in the gills and digestive glands of mussels and in fish (Canesi et al., 1998; Arabi and Alaeddini, 2005).

Bivalve mollucs, such as mussels and oysters are filter-feeding sedentary species prone to the accumulation of pollutants. They have been used extensively in recent years as sentinel organisms and sensitive bioindicators for pollutants associated with ROS generation and oxidative damage (Frenzilli et al., 2004). Seasonal and spatial variability of antioxidant defence enzymes, metallothionein concentrations, protein damage and other parameters in mussels have been used in numerous studies for marine monitoring pollution studies (Orbea et al., 2002; Manduzio et al., 2003). An international ''Mussel Watch'' was established many decades ago to monitor various pollutants in the world ocean, especially metals, PAHs, radioactive elements and other pollutants of concern (Goldberg and Bertine, 2000). Mussels, such as *Mytilus galloprovincialis* are used in the Mediterranean countries to evaluate contaminated coastal environments (Lionetto et al., 2001). The present ''Mussel Watch'' programme in Greece use mainly the mussel *M. galloprovinciallis* as a bioindicator of heavy metals (mainly as metallothionein content) contamination in various harbors and estuarine ecosystems which are influenced by anthropogenic activities (Kalpaxis et al., 2004; Zangrandi et al., 2005; Catsiki and Florou, 2006).

The Saronikos Gulf is a marine enclosed area near Athens and Piraeus directly influenced by the sewage outfall of the two metropolitan cities, industrial discharges (shipyards) and intensive shipping activities of the port of Piraeus and surrounding harbors. The start of the operation (1995) of the wastewater treatment plant of the city of Athens in Psyttalia was a turning point, contributing to the decreasing levels of pollutants discharged.

Marine pollution monitoring of the Saronikos Gulf started in 1986 within the framework of the MED-POL (MAP/UNEP National Monitoring Program) in 16 monitoring stations (NCMR, 1997; HCMR, 2003; Papathanasiou and Zenetos, 2005). Heavy metal levels of the Saronikos Gulf were measured by researchers of the University of Athens and the results were published (Scoullos and Pavlidou, 1997; Scoullos et al., 2007). Biomarkers such as metallothionein (MT) levels and acetylocholinesterase activity (AchE) were measured in *M. galloprovincialis* in relation to heavy metals, but results are very limited (Cotou et al., 2001; Tsangaris et al., 2004).

This is the first study of systematic biomonitoring for three years, using as bioindicators indigenous mussels *M. galloprovincialis* and as biomarkers the seasonal variations of SOD and CAT activities and LP concentrations in the gills and mantle in three polluted sites, in relation to concentrations of certain heavy metals.

2. Materials and methods

2.1. Site description

In the first year of our project we investigated various sites in the Saronikos Gulf, taking samples and measuring trace metal concentrations in mussels. We aimed for indigenous mussels and seasonal availability for their collection. Indigenous mussels *M. galloprovincialis* were sampled in the period 2004–2006 in three sites of the Saronikos Gulf. Sites S1 and S2 are from the Elefsis Bay area, a highly polluted area, while site S3 is located in the Aegina island (in the middle of the Gulf), which is considered an area with low metal pollution. The fourth site,''control'' (S4) was from an unpolluted reference site (commercial marine farm, located in the area of Stylida, in the Maliakos Gulf) characterized by its low concentrations of metals in surface water as well in mussels.

The Saronikos Gulf is directly influenced by the sewage outfall of the metropolitan cities Athens and Piraeus, industrial discharges and shipping activities. Until 1995 the untreated urban sewage of 4 million inhabitants was discharged in the shallow waters of the gulf on top of intensive navigation and significant industrial (steel, refineries, tanneries) and shipyard activities (Elefsis Bay). The start of the operation (1995) of the wastewater treatment plant in Psyttalia (small island near Salamis) was a turning point. Other measures in the port of Piraeus and cessation of some of the industrial and shipyard activities in the area decreased the pollution level of the gulf's waters (Sklivagou et al., 2001; Mavrakis et al., 2004).

2.2. Sample collection and preparation of mussels

Indigenous mussels *M. galloprovincialis* were sampled in the period 2004–2006. They were characterized by similar

maximum length and size: 45 ± 10 mm shell length, 8–10 g soft wet tissue. Mussels were collected in the spring (March–June), summer (July–September), autumn (October–November) and winter (December–February) months. Temperature of seawater of sites 1, 2, 3 and 4 was: winter 9–11 °C (surface layer), spring 15–20 °C, summer 20–25 °C and autumn 12–14 ° C. Salinity in sites 1–4 was within the optimal range 32–35 ‰ in winter, 35–37 ‰ in the spring, 36–38 ‰ in the summer and 33–34 ‰ in the autumn. Immediately after collection, mussels were transferred fresh inside an ice cool box to the laboratory where gills and mantle were rapidly dissected and treated accordingly as described in the following paragraphs. Whole mussel tissues were stored at −20 °C for short-term storage for heavy metal analysis. Every year, 150 mussels were collected from each sampling site, of which 120 were used (4 × 30 for every season). Site 3 (Aegina) in winter was a very difficult location to collect mussels due to dangerous conditions. Thirty specimens of mussel tissues (gills and mantle) were rapidly dissected, 15 for biochemical analyses and 15 for chemical determination of protein concentration and heavy metals. Dissections were completed within 2–4 h of collection. The dissected tissues were blotted dry and weighed and then were homogenized (special electric homogenizer) in 1:5 w/v homogenization buffer (0.05 phosphate buffer, pH 7.4). The homogenate was centrifuged twice at 10,000*g* at 4 °C for 20 min. The supernatants were diluted 1:5 v/v with phosphate buffer, collected in aliquots and kept at −20 °C for short-term measurements. We made sure that most of the enzymatic measurements were carried out within 5–6 h on the day of collection.

2.3. Enzymatic biomarkers

Superoxide dismutase (SOD) activity was determined by the degree of inhibition on the reduction of cytochrome *c* by superoxide anion generated by the xanthine oxidase/hypoxanthine system (Flöhe and Ötting, 1984). Measurements were recorded at a wavelength of 550 nm. The reaction is taking place in 0.05 M Na_2PO_4/$NaHPO_4$ buffer, pH 7.4, with 48 μM xanthine/0.2 units of xanthine oxidase and with 96 μM EDTA. One unit of SOD activity is defined as the amount of sample that inhibits by 50% the reduction of cytochrome *c*. The SOD activity in gills and mantle was expressed as U/mg wet tissue.

Catalase activity (CAT) was measured following the decrease of absorbance at 240 nm due to H_2O_2 consumption (Aebi, 1984; Greenwald, 1985). The reaction takes place in phosphate buffer was 50 mM at pH 7.0 at 25 °C (reagent A) containing 50–100 units of CAT per ml (immediately prepared before use). Reagent A was H_2O_2 (0.036% w/w). The CAT activity is the difference in the absorbance at that wavelength per unit of time. The results of CAT activity are expressed in terms of the first order rate constant (k)/protein content, as follows: enzyme units = k/protein = [ln (A_1/A_2)/t]/protein where ln is the natural log, A_1 and A_2 are the observed mean absorbance at the two selected time points, and t is the time differential between two points (expressed in min), protein content is in mg (mean value μmol H_2O_2 min^{-1} mg protein) (Aebi, 1984; Cohen et al., 1996). CAT activity was expressed as U/mg of protein.

2.4. Lipid peroxidation concentration and total protein quantification

For the evaluation of LP concentration we measured malondialdehyde (MDA) by the TBARs method at 535 nm (Buege and Aust, 1978). 2 ml of the reaction mixture [thiobarbituric acid (0.375%), trichloroacetic acid (15%) and hydrochloric acid (0.25 N)] were mixed in 1:1:1 and were added to 1 ml of the heat denatured supernatant. TBARs levels was estimated at 535 nm using MDA as standard. The concentration of LP compounds was expressed as nmoles of MDA per g of wet mass.

Total protein concentrations was measured on the supernatants (10,000*g* during 20 min at 4 °C) of the homogenized gills and mantle (Stoscheck, 1990). The protein concentration is the difference in absorption at 280 and 260 nm. Total protein concentrations was expressed as mg g^{-1} wet weight tissue.

2.5. Trace metal concentrations in surface seawater (Saronikos Gulf)

Metal concentration in the Saronikos Gulf were measured routinely for a long period (starting in 1987) in 14 sites spread throughout the area of the gulf and recently (2004) in two locations near Psyttalia. We pooled data for selected years of dissolved trace metal concentrations in surface seawater, such as Cd, Cu, Mn, Ni, Zn and Pb, that are indicative of the trend in metal concentrations in the various sections of the gulf and do not represent a detailed analysis which is found in a recent paper (Scoullos et al., 2007). The eastern part (Elefsis Bay, stations 1–3) is considered as the most polluted area, whereas the western (4–6 and 11), the eastern (stations 7, 8_A, 8_B, 9 and 10), the southern (stations 12 and 13) parts are considered less polluted (Fig. 1). Measurements in stations (14 and 15), near the outlets of the sewage treatment plant in Psyttalia started in 2004 and there are very limited results.

Analysis of trace metals was performed by Atomic Absorption Spectrometry (AAS, Varian SpectrAA 640 Graphite Furnace, with Zeeman background correction and Varian SpectrAA 200 Flame AAS).

2.6. Trace metal analysis in mussels

Fresh gills and mantles were excised from 15 mussel specimens collected from each sampling station and were subdivided into four samples. Each sample was homogenized, weighed and digested with concentrated nitric acid (65%) in Teflon vessels for 2 h at 140 °C and then processed for analysis by atomic absorption spectroscopy (AAS).

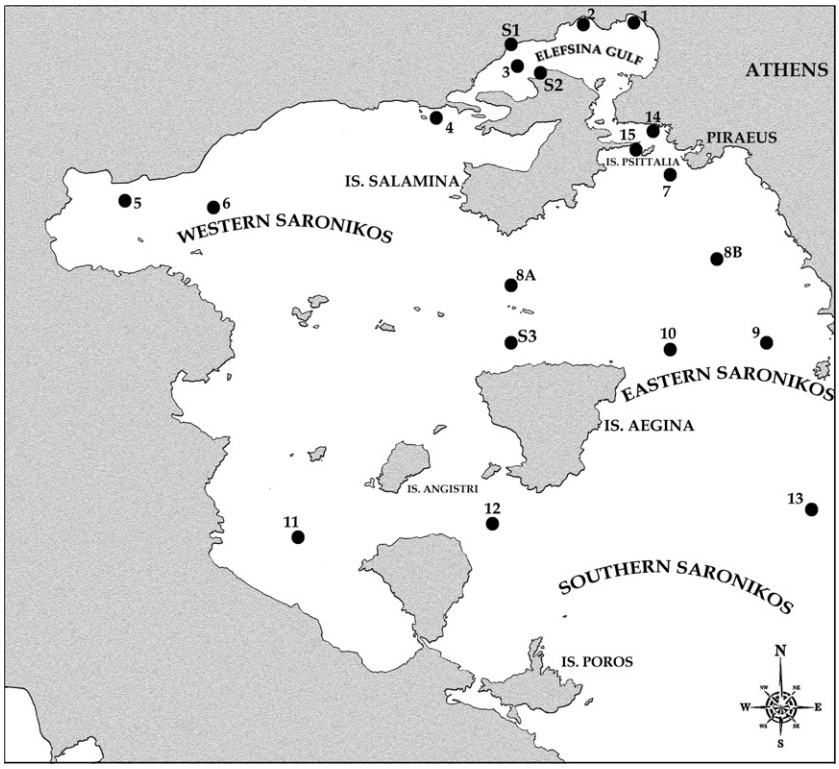

Fig. 1. The area map of Saronikos Gulf with sampling sites for trace metal in surface seawater and sampling sites S1, S2 and S3 for mussels (*S4 is not shown in this map).

Varian SpectrAA 640 GF AAS with Zeeman background correction was used for Cd, Cr, Ni, Pb and Cu. For measurements of Fe a Varian SpectrAA 200 Flame AAS was used.

2.7. Statistical analysis

Data are expressed as mean ± standard deviation (SD) of four independent experiments, each performed in triplicate. Statistical comparisons were made using one-way analysis of variance, ANOVA (SPPS software for Windows), assuming homogeneity of variance. The level of significance was set at 5%. Values of $^{*}p < 0.05$ were considered significant, $^{**}p < 0.01$ and $^{***}p < 0.001$.

3. Results and discussion

Total protein concentration in mussel's gills and mantle was expressed as mg g^{-1} wet mass. Measurements were: mantle (M): 1.80–2.18 (min.–max.) mg g^{-1} wet mass and gills (G): 1.82–2.09 mg g^{-1} wet mass.

3.1. Metal concentrations in seawater of the Saronikos Gulf

Trace metals in surface seawater of Saronikos Gulf were found mainly in the dissolved form than in the particulate phase. Percentages for dissolved metals were: Cd (94–96%), Ni (91–93%), Zn (84–86%), Pb (71–75%), Mn (67–70%) and Cu (66–70%).The cutting point of these measurements was 1995 when the Psyttalia sewage water treatment plant started operation and heavy metal concentrations started to decline (Scoullos et al., 2007).

Dissolved trace metals (Table 1) in various parts of Saronikos Gulf showed that Zn, Cu, Mn, Ni, Pb and Cd were at high concentrations in the Elefsis Bay, compared to other parts of the Saronikos Gulf. From 1996 concentrations of metals dropped substantially by 2–5 times in the most polluted areas, with the exception of Zn. Levels of metals in 2004–2005 were the lowest detected ever since the beginning of the MED-POL programme twenty year ago (Scoullos et al., 2007). The initial progress is put to test by the increasing population generated from the two metropolitan areas Athens and Piraeus and the increased use of water by industry and commercial enterprises and the intensive shipping activities, which inevitably produced more waste water reaching the Saronikos Gulf (Firfilionis et al., 2004).

3.2. Trace metals in soft tissues of mussels

Trace metal concentrations in mussels collected in 2004–2006 from 3 different locations (S1, S2) in the northern (Elefsis Bay) and central section (S3) of the Saronikos Gulf. The fourth site (S4) was used as a reference area (marine farm) considered as ''clean'' and was located in Stylida (Maliakos Gulf). The results are presented in Table 2. All experimental measurements were carried out in triplicate.

Table 1
Indicative mean values of the concentrations of dissolved trace metals (μg/L) in five areas (sampling sites 1–13) of the Saronikos Gulf for selective years: 1987, 1994, 1996, 2000 and 2004–2005

Area (sampling site[a])	1987	1994	1996	1998	2000	2004[b]
Elefsis bay (1–3)	Cd: 0.15	0.08	0.035	0.035	0.04	0.04
	Cu: 2.0	1.3	1.2	0.9	0.45	0.4
	Mn: 2.1	2.0	1.8	0.7	0.4	0.6
	Ni: 1.6	1.6	1.2	0.55	0.6	0.8
	Pb: 0.95	0.97	0.8	0.4	0.37	0.25
	Zn: –[b]	4.5	4.0	2.5	3.4	3.5
Eastern Saronikos (7, 8_A, 8_B, 9 and 10)	d: 0.13	0.05	0.055	0.025	0.027	0.025
	Cu: 1.0	1.2	0.95	0.30	0.2	0.2
	Mn: 2.0	0.6	0.4	0.35	0.25	0.4
	Ni: 1.1	0.9	0.4	0.35	0.3	0.4
	Pb: 0.9	0.6	0.35	0.32	0.3	0.35
	Zn: –[b]	–[b]	4.0	2.5	3.4	3.5
Western Saronikos (4, 5, 6 and 11)	Cd: 0.12	0.05	0.055	0.03	0.033	0.028
	Cu: 0.8	1.0	1.0	0.5	0.2	0.2
	Mn: 2.1	1.25	0.85	0.45	0.25	0.4
	Ni: 1.1	0.8	0.5	0.45	0.4	0.5
	Pb: 0.85	0.65	0.3	0.7	0.25	0.3
	Zn: –[b]	–[b]	3.3	1.3	2.5	1.8
Southern Saronikos (12 and 13)	Cd: 0.15	0.09	0.07	0.035	0.04	0.03
	Cu: 1.0	1.4	0.8	0.6	0.1	0.15
	Mn: 2.5	1.5	0.8	0.6	0.25	0.3
	Ni: 0.9	0.85	0.45	0.55	0.3	0.4
	Pb: 0.45	1.1	0.4	0.5	0.1	0.43
	Zn: –[b]	–[b]	3.0	1.25	1.4	1.0

[a] The majority of measurements was from the Laboratory of Environmental Chemistry (Chemistry Department, University of Athens). Sampling for metals in sites 14 and 15 (outlet of Psyttalia sewage treatment station) started in 2004 but are very limited.
[b] Zn measurements were not performed in these locations in 1987 and 1994.

The results from Table 2 showed that most metal concentrations in soft tissues of mussels from the two sites near the Elefsis Bay (sites S1 and S2) were 2–3 fold higher in gills and mantle, compared to the unpolluted reference site (S4) in Stylida (Malaikos Gulf). Site S3 (near the island of Aegina) was much less pollutted than S1 and S2 and for most metals the levels were similar to reference site S4. Metals such as Cd, Ni and Cu showed higher concentrations in the spring and summer, whereas Cr, Pb and Fe showed higher concentrations in the summer, probably as a result of intensive navigation and industrial activities in the summer months. Gills had higher concentrations for all metals compared to mantle. Fe was found in very high levels in the mantle in the summer and autumn months, but is known for its low toxicity to mussels, compared to the highly toxic metals Cu, Cd, Zn and Pb (Yap et al., 2004).

Similar results were obtained by Catsiki and Florou (2006) in the Saronikos and Thermaikos Gulf (Thessaloniki) with *Mytilus galloprovinciallis*. In general, it has been observed that mussels tend to bioaccumulate toxic heavy metals to a lesser degree at warmer periods. It has been suggested that the main influence is their reproductive cycle, increasing in the period before spawning (related to changes in flesh weight during gamete spawning) after November and during winter months. Similar trends were recorded for Cu, Cd and Zn with moderate variations along the months with a slight winter maximum followed by a summer pre-spawning minimum, matching seasonal trends of temperature/salinity (Soto et al., 2000; Adami et al., 2002).

Metal concentrations for the whole edible part of the mussels are below 1 μg g^{-1} wet mass, which is the permissible level for metals in sea food (European Union Decision 93/351) (Marcotrigiano and Storelli, 2003).

3.3. Seasonal measurements of SOD activity

Measurements of seasonal SOD activity in gills and mantle of mussels (*M. galloprovincialis*) from four sampling locations are presented in Fig. 2a and b.

The association between antioxidant defence enzymes in mussels and exposure to pollutants with oxidative potential (metals) is confirmed from the measurements of SOD and CAT activities, although the oxy-radical metabolism and cellular damage is a complex cause–effect relationship.

Measurements from sites S1 and S2 showed significant increased SOD activity in the spring period compared to the reference site S4. The seasonal SOD activity was higher in the gills than in the mantle. This is expected because gills are the prominent organ bioaccumulating metals. The SOD activity in sites 1 and 2 was in the range of 1020–2750 U SOD g^{-1} in gills and 900–1920 in the mantle compared with values in the range 550–1030 (gills) and 560–1070 (mantle) in sites 3 and 4. The enhanced SOD activity,

Table 2
Summary data for seasonal variations of heavy metal concentrations (μg/g, wet mass) in mantle (M) and gills (G) of mussels (*M. galloprovincialis*) in three sites of Saronikos Gulf in 2004–2006 and in Site 4 (marine farm, Stylida, Maliakos Gulf) (mean values ± SD)

Metals (Seasonal measurements)	Site 1 (Loutropyrgos)	Site 2 (Salamis)	Site 3 (Aegina)	Site 4 (Marine farm, Stylida, Maliakos Gulf)
Cadmium (Cd)				
Summer[a]	0.09 ± 0.03 (M)	0.04 ± 0.01 (M)	0.04 ± 0.02 (M)	0.02 ± 0.0 (M)
	0.41 ± 0.08 (G)	0.39 ± 0.08 (G)	0.30 ± 0.1 (G)	0.15 ± 0.05 (G)
Autumn[a]	0.04 ± 0.01 (M)	0.03 ± 0.01 (M)	0.01 ± .0.005 (M)	0.01 ± 0.006 (M)
	0.32 ± 0.05 (G)	0.42 ± 0.10 (G)	0.14 ± 0.01 (G)	0.11 ± 0.02 (G)
Winter[a]	0.03 ± 0.1 (M)	0.07 ± 0.02 (M)	–[b]	0.05 ± 0.0 (M)
	0.25 ± 0.04 (G)	0.43 ± 0.15 (G)	–[b]	0.21 ± 0.05 (G)
Spring[a]	0.055 ± 0.06 (M)	0.03 ± 0.01 (M)	0.04 ± 0.01 (M)	0.05 ± 0.01 (M)
	0.36 ± 0.12 (G)	0.34 ± 0.04 (G)	0.15 ± 0.03 (G)	0.21 ± 0.00 (G)
Chromium (Cr)				
Summer	2.35 ± 0.8 (M)	1.4 ± 0.5 (M)	0.34 ± 0.1 (M)	0.17 ± 0.04 (M)
	4.21 ± 1.5 (G)	3.7 ± 1.2 (G)	0.33 ± 0.15 (G)	0.29 ± 0.02 (G)
Autumn	0.39 ± 0.05 (M)	0.37 ± 0.1 (M)	0.2 ± 0.04 (M)	0.2 ± 0.06 (M)
	0.57 ± 0.08 (G)	0.47 ± 0.2 (G)	0.3 ± 0.03 (G)	0.35 ± 0.1 (G)
Winter	0.13 ± 0.04 (M)	0.2 ± 0.07 (M)	–[b]	0.12 ± 0.05 (M)
	0.37 ± 0.12 (G)	0.4 ± 0.2 (G)	–[b]	0.4 ± 0.12 (G)
Spring	0.14 ± 0.06 (M)	1.36 ± 0.5 (M)	0.12 ± 0.05 (M)	0.14 ± 0.07 (M)
	0.21 ± 0.07 (G)	2.64 ± 0.7 (G)	0.42 ± 0.2 (G)	0.55 ± 0.2 (G)
Nickel (Ni)				
Summer	0.23 ± 0.09 (M)	0.27 ± 0.1 (M)	0.06 ± 0.01 (M)	0.07 ± 0.02 (M)
	0.53 ± 0.05 (G)	0.29 ± 0.08 (G)	0.28 ± 0.05 (G)	0.35 ± 0.08 (G)
Autumn	0.15 ± 0.04 (M)	0.34 ± 0.1 (M)	0.16 ± 0.07 (M)	0.08 ± 0.02 (M)
	0.43 ± 0.14 (G)	0.5 ± 0.15 (G)	0.34 ± 0.1 (G)	0.30 ± 0.07 (G)
Winter	0.07 ± 0.02 (M)	0.3 ± 0.1 (M)	–[b]	0.07 ± 0.03 (M)
	0.23 ± 0.05 (G)	0.6 ± 0.2 (G)	–[b]	0.30 ± 0.08 (G)
Spring	0.28 ± 0.15 (M)	0.74 ± 0.2 (M)	0.15 ± 0.08 (M)	0.19 ± 0.06 (M)
	0.59 ± 0.24 (G)	1.87 ± 0.6 (G)	0.30 ± 0.1 (G)	0.45 ± 0.1 (G)
Lead (Pb)				
Summer	10.52 ± 2.5 (M)	3.08 ± 0.8 (M)	0.4 ± 0.1 (M)	0.22 ± 0.1 (M)
	21.10 ± 8.7 (G)	7.7 ± 2.3 (G)	0.75 ± 0.2 (G)	0.68 ± 0.3 (G)
Autumn	5.4 ± 1.6 (M)	0.58 ± 0.15 (M)	0.73 ± 0.2 (M)	0.25 ± 0.06 (M)
	8.7 ± 2.8 (G)	0.89 ± 0.3 (G)	0.36 ± 0.08 (G)	0.60 ± 0.2 (G)
Winter	0.28 ± 0.1 (M)	0.38 ± 0.1 (M)	–[b]	0.22 ± 0.04 (M)
	0.84 ± 0.24 (M)	0.79 ± 0.35 (G)	–[b]	0.43 ± 0.1 (G)
Spring	0.34 ± 0.12 (M)	0.26 ± 0.1 (M)	0.3 ± 0.09 (M)	0.34 ± 0.1 (M)
	0.93 ± 0.3.4 (G)	0.95 ± 0.3 (G)	0.48 ± 0.2 (G)	0.46 ± 0.2 (G)
Copper (Cu)				
Summer	1.4 ± 0.3 (M)	1.10 ± 0.5 (M)	0.8 ± 0.4 (M)	0.54 ± 0.2 (M)
	1.5 ± 0.4 (G)	1.43 ± 0.6 (G)	1.10 ± 0.5 (G)	1.29 ± 0.3 (M)
Autumn	1.3 ± 0.5 (M)	0.91 ± 0.4 (M)	0.72 ± 0.2 (M)	0.65 ± 0.2 (M)
	2.17 ± 0.8 (G)	2.25 ± 0.9 (G)	1.48 ± 0.4 (G)	1.4 ± 0.3 (G)
Winter	0.93 ± 0.3 (M)	0.91 ± 0.3 (M)	–[b]	0.4 ± 0.1 (M)
	2.25 ± 0.7 (G)	2.25 ± 1.10 (G)	–[b]	0.9 ± 0.3 (G)
Spring	0.82 ± 0.3 (M)	0.84 ± 0.3 (M)	0.85 ± 0.2 (M)	1.15 ± 0.6 (M)
	2.22 ± 0.5 (G)	2.09 ± 1.2 (G)	0.95 ± 0.3 (G)	1.35 ± 0.5 (G)
Iron (Fe)				
Summer	98.4 ± 30.5 (M)	63 ± 23 (M)	15 ± 6.5 (M)	16.5 ± 6 (M)
	151 ± 58 (G)	130 ± 37 (G)	29 ± 7 (G)	30.2 ± 10 (G)
Autumn	90 ± 26 (M)	45.0 ± 20 (M)	23.8 ± 5.6 (M)	15.5 ± 5 (M)
	130 ± 35 (G)	48.0 ± 26 (G)	27.7 ± 4.8 (G)	18.4 ± 6.5 (G)
Winter	22.2 ± 7 (M)	20.5 ± 5.5 (M)	–[b]	12.3 ± 4 (M)
	32.9 ± 9 (G)	30.6 ± 6.8 (G)	–[b]	15.8 ± 6 (G)
Spring	19.0 ± 7.5 (M)	30.1 ± 7.5 (M)	12.5 ± 4.5 (M)	14.9 ± 4.7 (M)
	21.8 ± 9.8 (G)	43.8 ± 8.5 (G)	18.6 ± 7.6 (G)	19.1 ± 5.5 (G)

Number of measurements 6–8 for each sampling site (determinations in triplicate).
[a] Summer (July–September), Autumn (October–November), Winter (December–February), Spring (March–June).
[b] Winter measurements for mussels from Site 3 (Aegina) are absent because of the difficulties to collect mussels in this area.

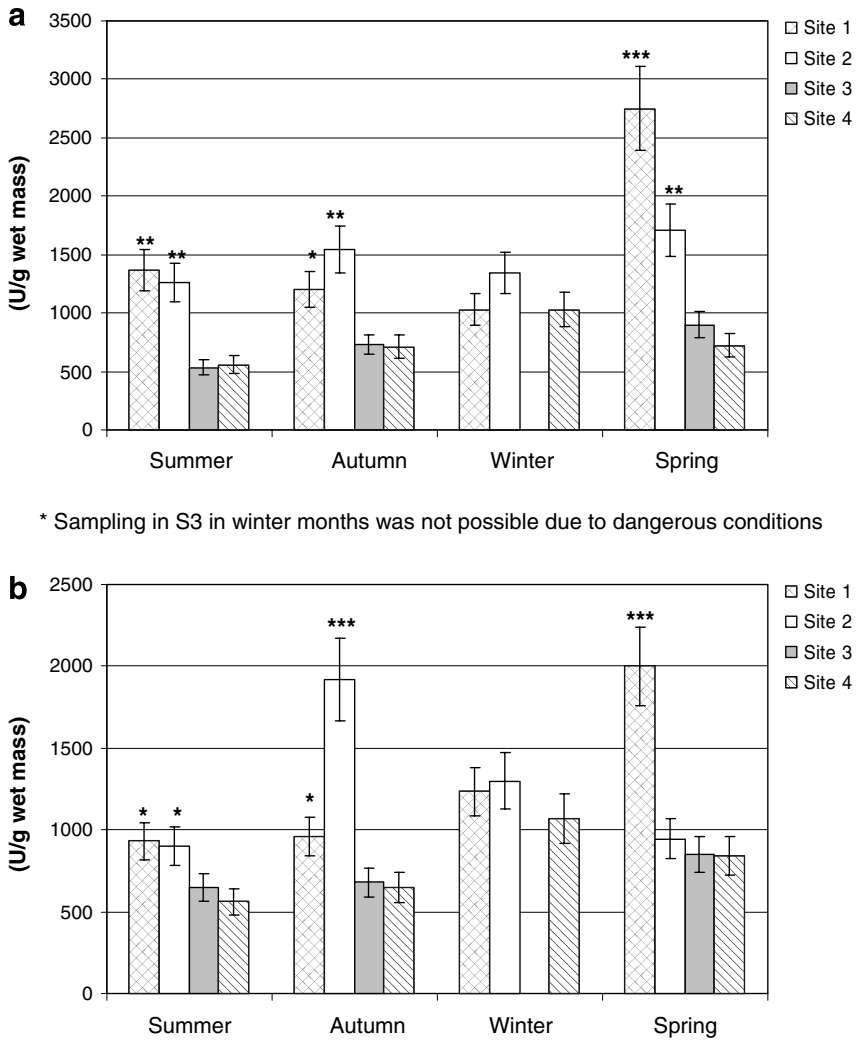

Fig. 2. Seasonal variations of superoxide dismutase (SOD) activity in gills (2a) and mantle (2b) of mussels from sampling sites S1, S2, S3 and S4. Mean values ± SD ($n = 6$–8, determinations in triplicate). Values of $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

which are 2–3 fold higher compared to the reference site S4, was considered as a result of oxidative stress from xenobiotics. There are no decreases of SOD activity, indicating that levels of pollutants with oxidative potential are relatively modest. There is a good correlation of these increases with comparable increases in concentrations of metals in mussel's gills and mantle. Seasonal variations of SOD and CAT activity are consistent with other findings, showing decreased activities from autumn to late winter-early spring, and increases again in the summer months (Regoli, 1998). Elevated activity of SOD and CAT was reported in *M. galloprovincialis* in the Adriatic Sea (Borković et al., 2005). Seasonal changes in SOD and CAT activities were measured in the digestive gland of the brown mussel *Perna perna* (Filho et al., 2001). The effect of trace metals on antioxidant defence enzymes are more evident when different populations (polluted and non polluted areas) are compared. Seasonal variations of trace metals seems that they do not influence directly to the same extent the antioxidant defence enzyme due to other biological factors (spawning, gametogenesis, growth) which regulate fluctuations of both defences (Viarengo et al., 1991).

3.4. Seasonal measurements of CAT activity

Seasonal measurements of CAT activity in mussels in Sites 1–4 was presented in Fig. 3a and b.

The biological importance of CAT is more evident from various studies due to the fact that H_2O_2 is the main cellular precursor of the hydroxyl radical ($HO^{\bullet}$) which is a highly reactive and toxic form of ROS. The removal of H_2O_2 is an important strategy of marine organisms against oxidative stress (Regoli et al., 2002a,b). Increased activities of CAT have been reported in several fish and invertebrate species (Di Giulio et al., 1993; Stephensen et al., 2000), whereas inhibition of CAT has been suggested as a transitory response to acute pollution (Regoli and Principato, 1995).

Our results of CAT activity in the gills and mantle of mussels, from sites S1 and S2 showed a 2 fold increase,

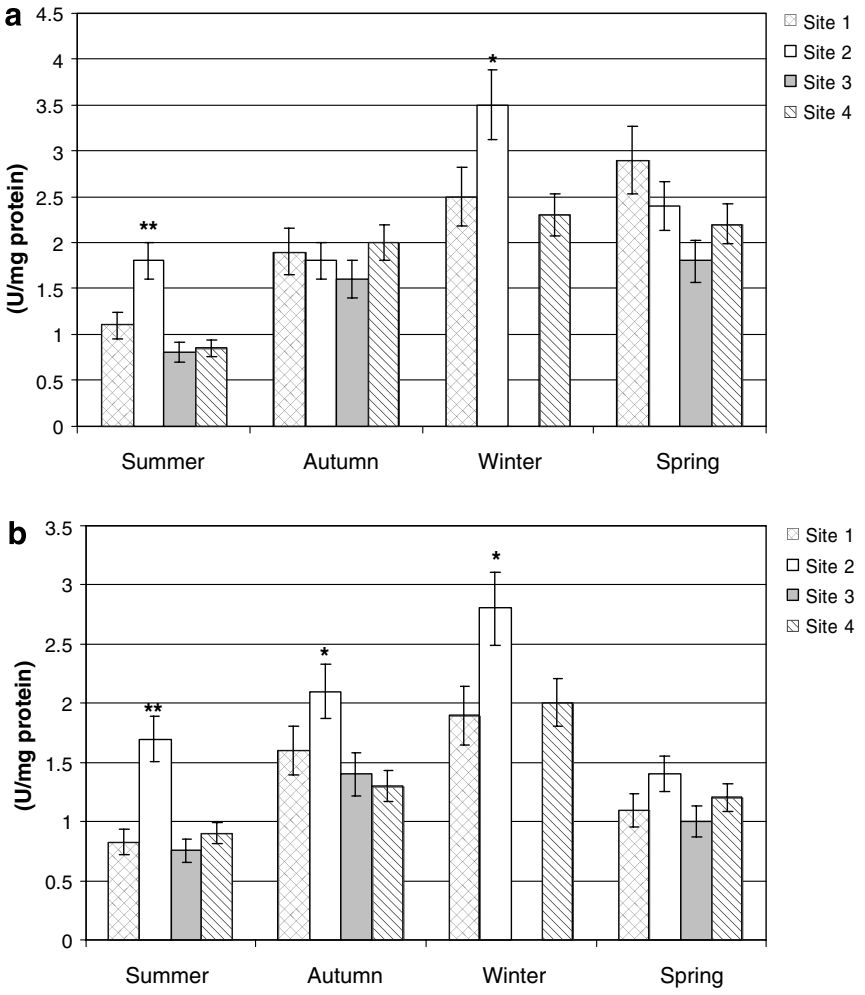

Fig. 3. Seasonal variations of catalase (CAT) activity in gills (3a) and mantle (3b) of mussels from sampling sites S1, S2, S3 and S4. Mean values ± SD ($n = 6$–8, determinations in triplicate). Values of $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

compared to mussels from sites S3 and S4. The CAT activity in the polluted sites (S1 and S2) was higher in the winter–spring period (2.89–3.5 in the gills and 1.90–2.8 in the mantle) than in the summer–autumn period. There are no decreases in CAT activity in the gills and mantle of the mussels, indicating that pollution levels in S1 and S2 are relative moderate.

CAT is considered by many scientists as an important and sensitive biomarker of oxidative stress, better than SOD, revealing biological effects on the redox status of the marine organisms (Regoli et al., 2002a,b). Our data for increases in CAT activity and to a lesser extent in SOD, demonstrate a "disturbance" from pollutants in the Elefsis Bay with seasonal variations reflecting the intensity of pollution in the area. But, these induced antioxidant defence enzyme increases were not enough to reduce lipid peroxidation levels in the polluted sites.

3.5. LP concentration

The summary of results for seasonal LP concentration in mantle and gills of mussels are presented in Fig. 4a and b.

Our results for LP showed a consistent trend for higher levels in polluted sites S1 and S2, whereas smaller increases were detected in site S3. By comparison the mussels from the site S4 had much lower levels of MDA, as expected. Gill tissues of mussels showed a 2 fold increase in concentration of LP compared to mussels' mantle. LP concentrations varied from 300 to 800 nmol g^{-1} w.w.t (mantle) and 500–1000 nmol g^{-1} w.w.t (gills), compared to 200–360 (mantle) and 500–570 (gills) from the reference site S4.

The fact that CAT activity and LP concentration are both elevated at the polluted sites suggest that there is excess of H_2O_2 which diffuses into the cells causing oxidative damage. Although CAT removes most of the H_2O_2 by increasing its activity levels, it cannot compete with the excess presence of Fe in the gills and mantle which generate $HO^{\cdot}$ radicals via the Fenton reaction, thereby causing increased concentration of LP. Therefore we conclude that CAT activity in mussels is not sufficient to eliminate H_2O_2 before the formation of hydroxyl radicals as it has been suggested by others (Bebianno et al., 2005).

Increased concentration of LP was observed in mussels exposed in polluted areas when compared to less polluted

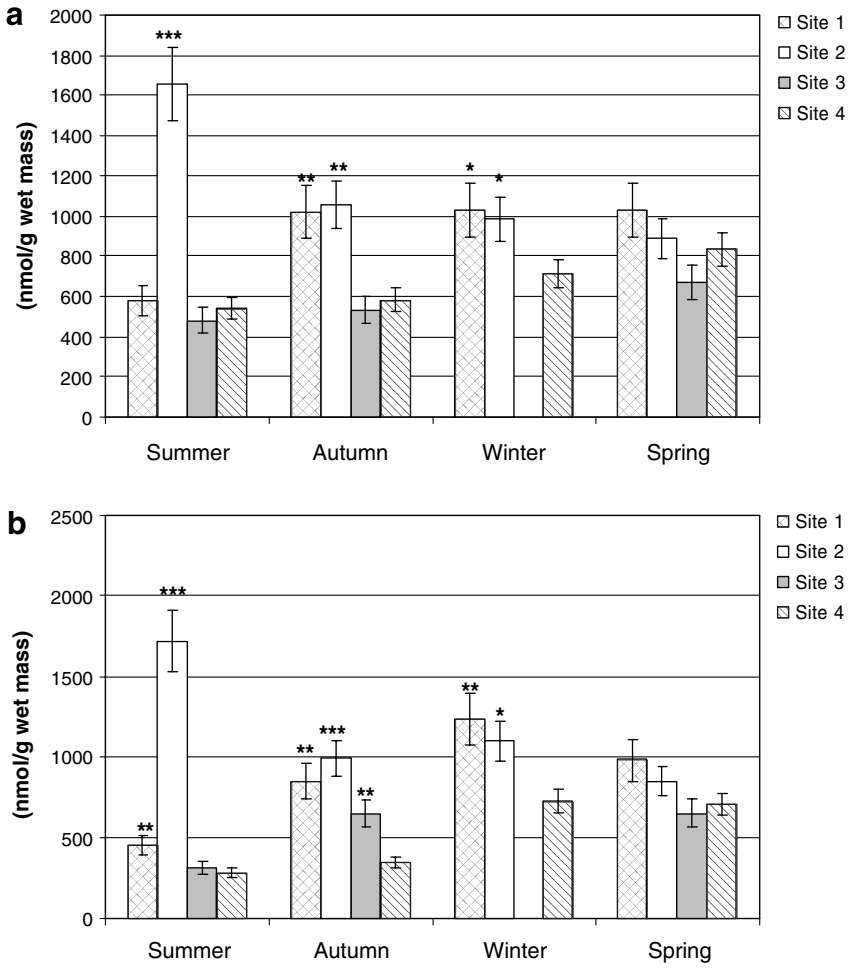

Fig. 4. Seasonal variations of lipid peroxidation (LP) concentrations measured in gills (4a) and mantle (4b) of mussels from sampling sites S1, S2, S3 and S4. Mean values ± SD ($n = 6$-8, determinations in triplicate). Values of $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

sites (Lau and Wong, 2003; Pampanin et al., 2005). Pampanin et al. (2005) observed an inverse correlation between LP concentration and CAT activity in transplanted mussels (*M. galloprovincialis*) in Venice Lagoon after five weeks exposure. Concentration of LP was significantly higher ($p < 0.05$) for polluted areas of Venice Lagoon compared to the reference site (marine farm). CAT activity was reduced due to increased levels of pollution indicating the importance of antioxidant action of CAT for cell membrane damage.

4. Conclusions

It is recognized in recent years that risk assessment of environmental pollution cannot be based solely on chemical analysis because does not provide a clear indication of toxic effects of pollutant on the aquatic biota (Livingstone, 2001). Although higher aquatic organisms are necessary for an unambiguous conclusion of the ecosystem health, mussels can be used as sentinel organisms for the evaluation of an early warning response to long-term ecological damage.

The present work, as far as we know, is the first systematic biomonitoring study in which the integrated use of general enzymatic stress indices were used to evaluate the seasonal impact of heavy metals on aquatic organisms of the Saronikos Gulf. We are fully aware of the limitations of our study, as long as the number of antioxidant enzyme defence activities used and the choice of soft tissues for analysis, but we had to restrict the scope of our investigation, so that we can have some useful quantitative results and compare them with other studies.

Mussels *M. galloprovincialis* have a number of properties that make them useful sentinels for environmental monitoring and can provide a measure of environmental pollution with observable cellular and physiological responses (Lau and Wong, 2003). It is known that mussel's biochemistry and physiology is influenced by seasonality. Depending on the availability of nutrients, reproductive status and growth rate, the activity of antioxidant defence enzymes and other biomarkers fluctuate significantly throughout the year (Borković et al., 2005).

The results of our study showed that the start of operation of the waste water treatment plant in Psyttalia (1995)

was very beneficial to the Saronikos Gulf. But the initial progress, which is facing recently some technical and administrative problems, is put to test by the increasing levels of pollutants in the area. Thus, it is important for vigilance and continuation of measurements of heavy metals and other physicochemical characteristics of the seawater and sediments. Our study, despite its limitations, showed that levels of pollution are not very high and that measurements of oxidative stress in sentinel organisms can be a useful tool of monitoring in an integrated programme of environmental measurements. Despite the higher levels of pollution in the Elefsis Bay (sites S1 and S2), the Aegina island region (site S3) is relatively unpolluted, when compared to the control site (S4).

Acknowledgement

This work was funded partially by the University of Athens Research Fund. We would like to thank Dr. Catsiki of the Hellenic Marine Research Centre for her help and advice concerning the collection of mussels. Also, we would like to thank Assoc. Prof. Vasilios Roussis of the Pharmacy Department of the University of Athens for his advice concerning experimental methods and the treatment of mussels.

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