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C. TSANGARIS, E. STROGYLOUDI, E. PAPATHANASSIOU

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C. TSANGARIS, E. STROGYLOUDI and E. PAPATHANASSIOU

Hellenic Center for Marine Research
Institute of Oceanography, P.O. 712, 190 13
Anavissos, Attiki, Greece

e-mail: ctsangar@ncmr.gr, estro@ncmr.gr, vpapath@ncmr.gr

Abstract

Alterations in a number of biochemical parameters in marine organisms represent specific markers of exposure to particular classes of contaminants. They are used as tools for the detection and monitoring of pollution. In this study, two biochemical markers of pollution, metallothionein (MT) content and acetylcholinesterase (AChE) activity were measured in indigenous and transplanted mussels Mytilus galloprovincialis from coastal areas within the Saronikos Gulf subject to high anthropogenic activities. Bi-annual measurements of the two biomarkers in indigenous mussel populations for two years revealed no significant differences among stations representing a pollution gradient. Limited differences in MT levels were only found between mussel populations transplanted at lesser and more impacted stations. Both biomarkers showed a variation with respect to the season of sampling, whilst during the second year of measurements a concomitant increase in metallothionein content with a decrease in acetylcholinesterase activity was noted. Our results indicate that the applied biochemical markers in indigenous mussel populations do not reflect the type of pollution in the Saronikos Gulf to a degree that can be used for pollution monitoring in the area.

Keywords: Biomarker; Metallothionein; Acetylcholinesterase; *Mytilus galloprovincialis*; Saronikos Gulf.

Introduction

Pollution responses at various levels of biological organisation are widely used in environmental monitoring since by definition pollution implies a hazard to living resources. When relating to individual organisms, responses used for the detection and evaluation of pollution, are often referred to as biomarkers. Biomarker measurements revealing exposure to and/or biological effects of chemical contaminants have been increasingly used for pollution assessment during the last decades (LIVINGSTONE *et al.* 1995, STAGG, 1998, CAJARAVILLE *et al.*, 2000, GALLOWAY *et al.*, 2002).

Very few biomarkers have been applied in Greek marine areas (COTOU *et al.*, 2001,

DOUMOUHTSIDOU & DIMITRIADIS, 2001, COTOU *et al*., 2002, TSANGARIS *et al*., 2002, DALIANIS *et al*., 2003, KALPAXIS *et al*., 2004). Most pollution monitoring programmes in Greece focus on biological indicators i.e. pollution effects at the population and community levels and/or chemical analysis in water, sediment and biota (GEORGAKOPOULOU-GREGORIADOU *et al*., 1997, NCMR, 1997, NCMR, 2001, NCMR, 2002). In the Saronikos Gulf, pollution monitoring has been performed within the framework of the MED POL programme (NCMR, 1997a) but the use of biomarker measurements in the area is scarce (COTOU *et al*., 2001, COTOU *et al.*, 2002, KALPAXIS *et al*., 2003). In this study, two biochemical markers, metallothionein (MT) levels and acetylcholinesterase (AChE) activity, were measured in Mytilus galloprovincialis indigenous or transplanted to several sites in the Saronikos Gulf subject to high anthropogenic activities. MT content is suggested as a biomarker of heavy metal exposure (VIARENGO *et al*., 1999). AChE activity is mainly considered as a biomarker of exposure to organophosphate and carbamate pesticides (BOCQUENÉ & GALGANI, 1991) and also responds to exposure to other type of contaminants including heavy metals (NAJIMI *et al*., 1997, HAMZA-CHAFFAI *et al*., 1998), undetermined compounds of complex mixtures of pollutants, detergents and surfactants (PAYNE *et al*., 1996, GUILHERMINO *et al*., 1998). The aim of this study is to investigate the response of MT content and AChE activity to contaminant levels in the Saronikos Gulf in order to evaluate their use as tools for the detection and monitoring of pollution in the area.

Materials and Methods

Field sites

Six stations in different areas of the Saronikos Gulf were selected (Fig 1). 'Piraeus 'within Piraeus harbour and 'Skaramagas', a shipyard in the eastern part of Elefsis bay, were considered as the most impacted stations. 'Megara' situated in the western part of Elefsis bay and 'Ag. Kosmas', 'Aigina' and 'Anavissos' along the coast of the eastern part of the Saronikos Gulf were regarded as less impacted stations. Industrial activities are mainly located along the eastern part of Elefsis bay and around Piraeus harbour. The wastewater treatment plant of the city of Athens is also situated in this area. Enriched levels of Cd, Cu, Zn, Pb, Cr and Fe are found in sediments in Piraeus harbour and Elefsis bay, decreasing towards the western part of Elefsis bay (VOUTSINOU–TALIADOURI *et al*., 1989) where Megara station is located. The most recent data on Hg levels in sediments from the Saronikos Gulf ranged from 0.13 to 0.27 $\mu g.g^{-1}$ in Elefsis bay, 0.04 to 0.14 $\mu g.g^{-1}$ in west Saronikos and 0.06 to 0.07 $\mu g.g^{-1}$ in east Saronikos (STATHOPOULOU *et al*., 2001). Mean values of aliphatic hydrocarbons and polycyclic aromatic hydrocarbons in sediments were found to be 531 $\mu g.g^{-1}$ and 3037 $ng.g^{-1}$ respectively in Elefsis Bay (SKLIVAGOU *et al*., 2001), 53 $\mu g.g^{-1}$ and 166 $ng.g^{-1}$ in the western Saronikos Gulf (NCMR, 1997b) and 97 $\mu g.g^{-1}$ and 1464 $ng.g^{-1}$ in Ag. Kosmas located in the eastern Saronikos Gulf (KARAGEORGIS & HATZIANESTIS, 2003). In addition significant TBT contamination has been recorded in sediments from marinas in the Saronikos Gulf with very high levels of 10 $\mu g\ Sn.g^{-1}$ in Piraeus harbour (TSELENTIS *et al*., 1999).

Sampling

Mussels *Mytilus galloprovincialis* of similar shell length (41-61mm) were sampled in the spring and autumn over a two-year period (May 2001-October 2002). Indigenous mussels were collected from 'Piraeus' and 'Skaramagas'. At 'Megara' mussels were collected from an aquaculture farm. At 'Aigina', 'Ag. Kosmas' and 'Anavissos', where there were no indigenous populations available, mussels from 'Megara' were transplanted for one month and placed in

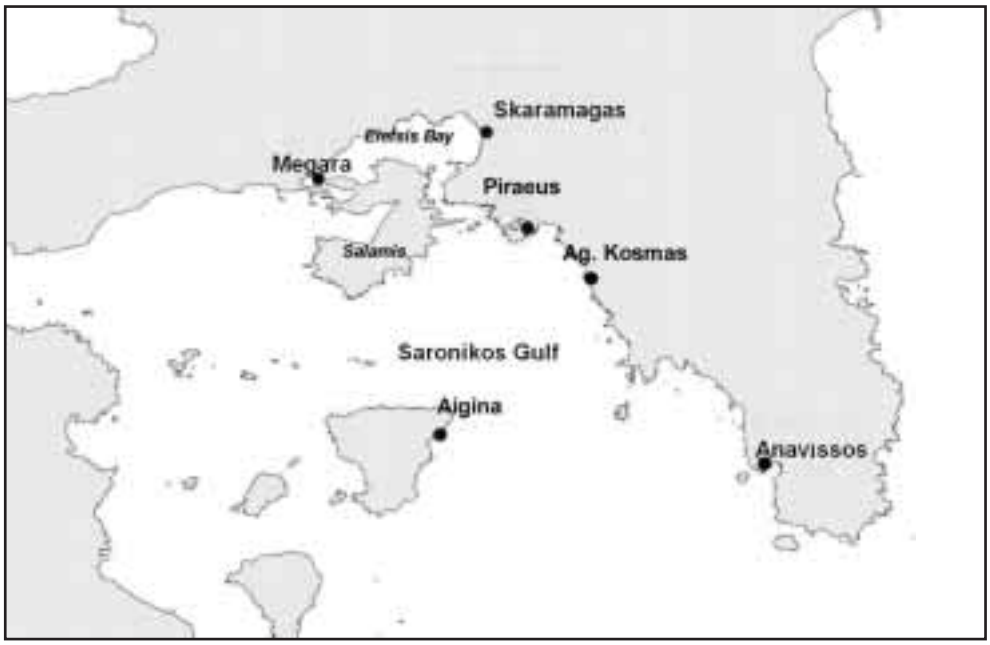

Fig. 1: Sampling stations in the Saronikos Gulf.

plastic cages at 2 to 3m below the surface. Additionally, for a comparison between indigenous and transplanted populations, mussels were transplanted as above to the 'Skaramagas' station whereas at the 'Megara' station a portion of the cultured mussels was transplanted within the immediate area. Just after collection, mussels were transferred to the laboratory where gills and digestive glands were rapidly dissected out and stored at -70°C for biochemical measurements. Whole mussel tissues were stored at -20°C for heavy metal analysis.

Acetylcholinesterase (AChE) activity

Gill tissues (1 g) from 2-5 individuals were pooled and homogenised using a Potter-Elvehjem homogeniser in 1:2 (w:v) 0.1M Tris-HCl buffer containing 0.1% TRITON X 100, pH 7. Homogenates were centrifuged at 10,000g for 20min. All preparation procedures were carried out at 4°C. AChE activity was assayed by the method of Ellman (ELLMAN *et al*., 1961) adapted to microplate reading by BOCQUENÉ and GALGANI (1992). The method is based on the increase in yellow colour produced due to the reaction of thiocholine with DTNB (dithio-bis-nitrobenzoic acid) during acetylthiocholine hydrolysis by AChE. 320µl of 0.1M Tris-HCl buffer containing 0.1% TRITON X 100, pH 7, 20µl of 0.01M DTNB and 30 µl of sample supernatant were added in each well of the microplate and the reaction initiated by addition of 10 µl of 0.1 M acetylthiocholine substrate. The enzyme kinetic was measured every 15s for 2 min at 414 nm. The assay was carried out at 25°C. Total protein content in the homogenate supernatants was measured by the Bradford method (1976) adapted for microplate reading. 280 µl of Bradford reagent and 100 µl of sample were added to each microplate well and absorbance read at 595nm. Protein concentration was calculated using a Bovine Serum Albumin (BSA) standard curve. Specific enzyme activity was expressed as U/mg protein. One unit (U) of AChE activity is the

amount of enzyme, which causes a variation of 0.001 in optical density per minute.

Metallothionein (MT) content

MT concentration was determined according to VIARENGO *et al*., (1997). The method is based on the estimation of the sulphydryl content of MT proteins by spectrophotometric determination of the -SH groups using Ellman's reagent. Pooled samples of digestive gland tissues (1g) from 2-5 individuals were prepared. MT concentration was calculated utilizing reduced glutathione (GSH) as a reference standard and expressed as mg MT/g wet weight tissue.

Heavy metal analysis

Whole mussel tissues were dissected out, freeze-dried, and digested with concentrated HNO_3 under pressure in a CEM MDS 2100 microwave device. Metal analysis was performed by Atomic Absorption Spectrophotometry (AAS). Cu, Zn and Hg concentrations were measured with a Varian Spectra AA20Plus Spectrophotometer. Hg concentrations were determined by the cold vapour technique. Cd analysis was performed with an AAS Perkin Elmer 4100 equipped with a HGA 700. Heavy metal concentrations were expressed as $\mu g.g^{-1}$ dry weight tissue. Analytical quality control was achieved using certified reference material provided by the National Research Council of Canada.

Flesh Condition Index

Flesh Condition index (FC) was calculated according to LOBEL & WRIGHT (1982) as dry whole tissue weight (mg) versus dry shell weight (gr) per individual.

Statistical analysis

Results are shown as means ± SD. Logarithmic transformation of data was performed to obtain homogeneity of variance. Significant differences between means were determined by one-way analysis of variance (ANOVA) followed by the Tukey HSD multiple comparison test. Correlation was tested by Pearson's correlation coefficient (r). Statistical analysis was carried out using SPSS software. Significance level was set at $p<0.05$.

Results

MT content and AChE activity in indigenous and transplanted mussels sampled at stations along the coast of the Saronikos Gulf are shown in Figure 2. Bi-annual measurements of the two biomarkers in indigenous mussels for two years revealed no significant differences among stations with the exception of a decreased AChE value in 'Megara' in spring 2002. In transplanted mussels, no differences in AChE activities were found among stations while MT levels showed a significant increase in Skaramagas in spring 2001 and in Anavissos in autumn 2001.

When measurements in indigenous and transplanted mussel populations were compared, significant differences were found in MT content. MT levels were significantly increased in transplanted mussels compared to indigenous populations both at Skaramagas and Megara in spring 2001 (Table 1). Furthermore, pooled data from all stations indicated higher MT levels in transplanted populations in spring 2001 (Fig 3).

Both MT content and AChE activity revealed a variation with respect to the season of sampling (Fig 2). Significantly higher MT levels in autumn than in spring were found in indigenous mussels in Megara and Skaramagas during 2002. In contrast, significantly higher MT levels were detected in spring than in the autumn in transplanted mussels in Megara and Ag. Kosmas during 2001. AChE activities in indigenous mussels were generally higher in spring than in autumn with the exception of Megara during 2002 where activities were significantly higher in autumn. In addition both MT levels and AChE activities showed a variation with respect to the year of sampling with a general increase in MT levels and

decrease in AChE activities in 2002 compared to 2001.

Heavy metal concentrations in whole tissues of indigenous and transplanted mussels from the different stations in the Saronikos Gulf are shown in Table 2. Mean values ranged from 2.36 to 31.5 μg.g^{-1} dw for Cu, from 0.24 to 1.24 μg.g^{-1} dw for Cd, from 80 to 285 μg.g^{-1} dw for Zn and from 0.07 to 0.12 μg. g^{-1} dw for Hg. In indigenous mussels, the highest Cu and Zn concentrations were found in Piraeus followed by Skaramagas and the highest Cd

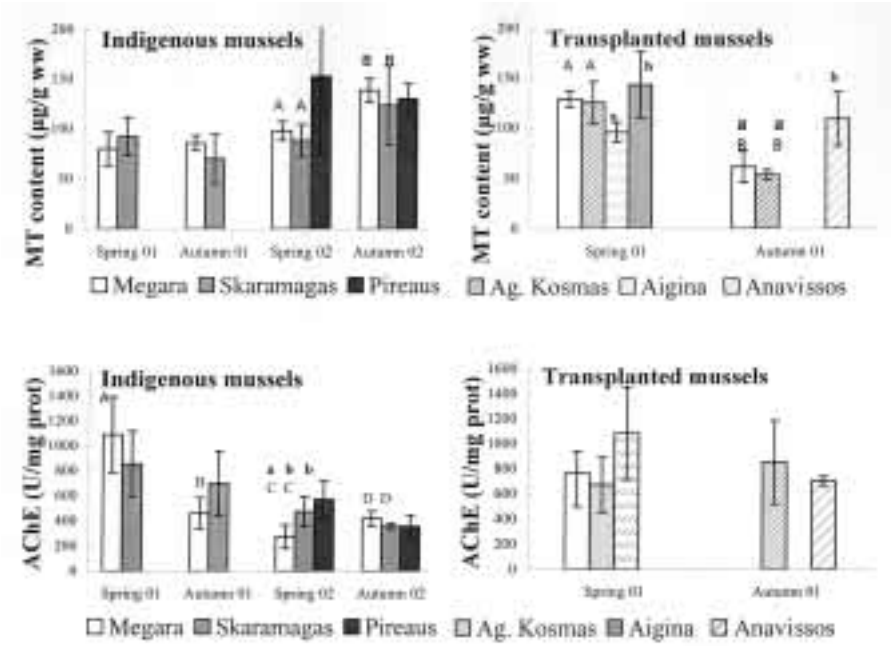

Fig. 2: MT content (μg/g ww tissue) in the digestive gland and AChE activity (U/mg protein) in the gills of indigenous and transplanted mussels *Mytilus galloprovincialis* at different stations in the Saronikos Gulf over a two-year period (2001-2002). Significant differences between stations represented by different small letters ($p<0.05$), significant differences between seasons displayed by different capital letters ($p<0.05$).

Table 1
MT content in the digestive gland and AChE specific activity in the gills of indigenous and transplanted mussels at Megara and Skaramagas during 2001 (mean±SD).

Date	Station	MT content (μg/g ww tissue)		AChE activity (U/mg protein)	
		Indigenous mussels	Transplanted mussels	Indigenous mussels	Transplanted mussels
Spring 2001	Megara	79.9±16.7^{a}	129±8^{b}	1089.9±304.2	763.5±174.2
	Skaramagas	92.1±19.5 ^{a}	143±33 ^{b}	851.4±264.9	nd
Autumn2001	Megara	85.6±7.6	61.3±15.6	458.9±125.4	nd

nd: not determined
Significant differences between populations represented by different letters (t-test, $p<0.05$).

Table 2
Heavy metal concentrations in whole tissues of mussels *Mytilus galloprovincialis* at different stations in the Saronikos Gulf during 2001 and 2002 (mean ± SD).

Station	Date	Cu (μg/g dw)		Zn (μg/g dw)		Cd (μg/g dw)		Hg (μg/g dw)
		Indigenous mussels	Transplanted mussels	Indigenous mussels	Transplanted mussels	Indigenous mussels	Transplanted mussels	Indigenous mussels
Ag.Kosmas	Spring 2001	-	4.27±0.12-	221±33	-	1.03±0.13	-	
	Autumn 2001	-	3.79±0.35-	188±12	-	0.99±0.10	-	
Aigina	Spring 2001	-	2.36±0.65-	176±17	-	0.69±0.27	-	
Anavissos	Autumn 2001	-	4.95±0.55-	197±18	-	1.21±0.08	-	
Megara	Spring 2001	5.37±2.48	4.06±0.18	199±43	120±18	0.75±0.02	0.91±0.05	-
	Autumn 2001	3.16±0.30	3.71±0.24	126±18	151±34	0.66±0.03	0.68±0.03	-
	Spring 2002	4.21±0.55	-	80±4	-	0.24±0.03	-	0.12+0.04
	Autumn 2002	6.38±0.48	-	135±8	-	0.36±0.07	-	0.08+0.05
Skaramagas	Spring 2001	11.22+1.42	14.3±2.2	197±50	190±56	1.24±0.16	1.10±0.12	-
	Autumn 2001	9.48±0.22	-	217±40	-	1.20±0.07	-	-
	Spring 2002	9.28±0.94	-	153±22	-	0.26±0.04	-	0.07+0.01
	Autumn 2002	9.98±0.17	-	244±31	-	0.41±0.08	-	0.10+0.01
Piraeus	Spring 2002	31.5±1.6	-	146±22	-	0.25±0.06	-	0.09+0.03
	Autumn 2002	17.1±0.7	-	285±59	-	0.61±0.15	-	0.11+0.04

levels were recorded in Skaramagas that were both considered to be the most impacted stations. No differences in Hg concentrations among stations were recorded. In transplanted mussels the highest Cu values were also found in 'Skaramagas' while no significant differences in Zn and Cd concentrations were detected among stations with the exception of Anavissos. In 'Anavissos', Cd, Zn and also Cu levels were elevated compared to 'Megara' and 'Ag. Kosmas', in contrast to the expected pollution gradient. In general, no differences in heavy metal concentrations were found between indigenous and transplanted mussels with the exception of increased Cd concentrations in transplanted mussels at 'Megara' in spring 2001. Heavy metal concentrations varied with respect to season of sampling, showing significantly higher values in autumn than in spring in Megara and 'Skaramagas' during 2002 with the exception of Cu in 'Skaramagas' where levels were constantly high.

The flesh condition index was used as an estimation of the physiological status of mussels (Figure 4). Indigenous mussels from 'Piraeus' and 'Skaramagas' had a significantly lower flesh condition index than mussels from the aquaculture farm at 'Megara'. Transplanted mussels in 'Ag. Kosmas', 'Anavissos' and 'Skaramagas' showed a lower flesh condition index than transplanted and indigenous cultured mussels in 'Megara'.

Correlations between biomarkers and heavy metal concentrations were not significant ($p>0.05$), while a significant correlation was found between AChE activity and MT content ($r=-0.436$, $p<0.05$).

Discussion

MT content is considered as a biomarker of exposure to heavy metal pollution and field studies using mussels have shown increases of MT content in areas contaminated with heavy metals (VIARENGO *et al*., 1999). In the present study, mean MT concentrations in indigenous

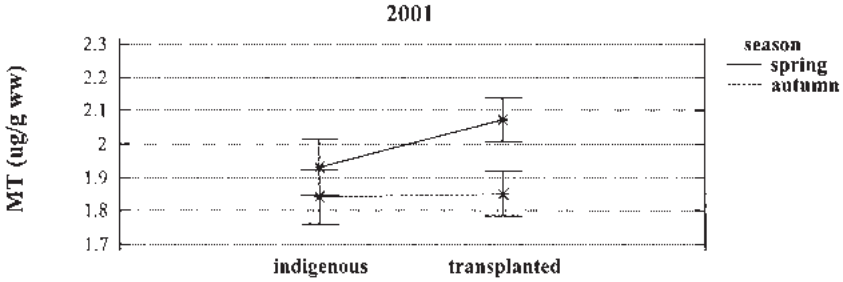

Fig. 3: Average and 95% Tukey HSD intervals for MT content in the digestive gland of indigenous and transplanted mussels from all stations during spring and autumn 2001 (log transformed values).

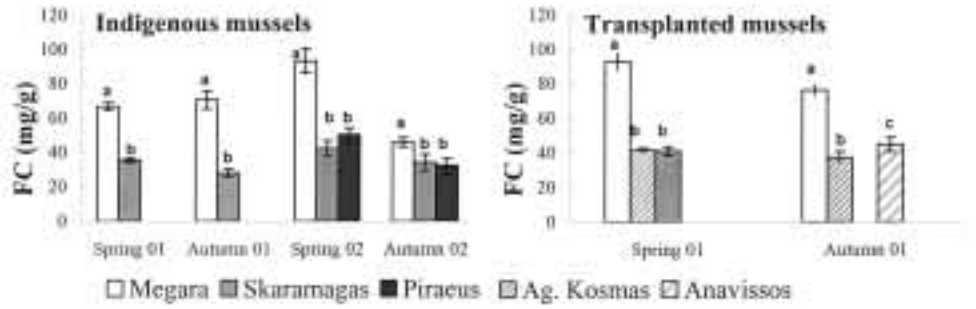

Fig. 4: Flesh Condition Index (FC) of indigenous and transplanted mussels *Mytilus galloprovincialis* at different stations in the Saronikos Gulf over a two-year period (2001-2002). Significant differences between stations are represented by different letters ($p<0.05$).

mussel populations from coastal areas of the Saronikos Gulf varied from 53 to 153mg/g and were similar to values obtained from a previous study in the framework of the MED POL programme in the same area (HCMR, 2003) and to values found in wild mussels collected from other Mediterranean and East Atlantic coastal areas (BEBIANNO & MACHADO, 1997, VIARENGO *et al*., 1997, STIEN *et al*., 1998, PETROVIC *et al*., 2001, DOUMOUHTSIDOU *et al*., 2004). Heavy metal concentrations in indigenous mussels from the Saronikos Gulf were generally low and similar to those found in non-polluted coastal areas (ROMEO *et al*., 2003, BESADA *et al*., 2002, BEIRAS *et al*., 2003) apart from few increased Cu values in 'Piraeus' although overall variation was according to the expected pollution gradient. However, significant increases in MT content parallel to the higher Cu, Cd and Zn tissue levels found in indigenous mussels from stations regarded as most impacted were not recorded. DOUMOUTSIDOU *et al*., (2004) also reported that increases in MT content in mussel populations from Northern Greece did not follow tissue heavy metal concentrations although they found MT variations among stations displaying a pollution gradient. Our results are similar to PETROVIĆ *et al.*, (2001) that failed to reveal increases of MT levels in mussels from polluted sites of the North-Eastern Adriatic and attributed their results to a generally low concentration of heavy

metals in the mussel tissues that could not induce MT biosynthesis. Furthermore GÉRET *et al.* (2002) found no increase in MT levels in the digestive gland of M. edulis exposed to Cd or Cu at tissue metal concentrations comparable to those measured in the present study. The only differences observed in MT content among mussel populations of this study are possibly related to transplantation, as MT responses differed between indigenous and transplanted mussels. BOLOGNESI *et al.*, (2004) found differences in genotoxicity biomarker responses between wild and caged mussels and suggested that caged mussels showed a higher level of DNA damage as a result of very recent exposure to genotoxic agents. Similarly the higher MT levels in transplanted compared to indigenous mussels of the present study may be a result of recent heavy metal exposure. In addition, the only cases where increased MT content was found concurrently with increased Cu, Cd and Zn tissue levels occurred in transplanted mussels ('Skaramagas' in spring 2001 and 'Anavissos' in autumn 2001). These results tend to show that the use of transplanted mussels may be a better approach for the application of MT as a biomarker of heavy metal exposure as proposed by VIARENGO *et al.* (1999), and point out the need for further research.

AChE inhibition is considered as a biomarker of organophosphate and carbamate exposure (BOCQUENÉ & GALGANI, 1991) and decreased AChE activities of mussels have been reported in relation to presence of organophosphate and carbamate pesticides (ESCARTIN & PORTE, 1997). Additionally, several field studies suggest that other environmental contaminants including heavy metals can affect AChE (AMIARD-TRIQUET *et al*., 1998, NARBONNE *et al*., 1993). AChE activities measured in the gills of mussels from coastal areas of the Saronikos Gulf ranged from 273 to 1090 U/mg protein independent of the pollution gradient. These levels were generally in the high range of those reported in mussels from Northern Greek coasts (DAILIANIS *et al*., 2003) and other Mediterranean regions (BOCQUENÉ *et al*., 1993, ESCARTIN & PORTE, 1997). The limited differences observed in AChE activity among stations were inconsistent and not in accordance with the expected pollution gradient. DAILIANIS *et al*., (2003) also reported differences in AChE activities of mussels among stations in the Thermaikos Gulf (Northern Greece) that were not consistent with respect to season of sampling and in a few cases were unexpectedly higher in stations regarded as more polluted, although in several cases they found a decrease in AChE activity at the most polluted stations. The lack of AChE response to the pollution gradient in the Saronikos Gulf may be related to the absence of highly elevated levels of contaminants capable to inhibit AChE.

Since biomarker responses are influenced by the physiological status of the bioindicator organisms, the flesh condition index was used as an indication of the physiological status of the different mussel populations of this study. Cultured and transplanted mussel populations at Megara showed a higher condition index compared to indigenous and transplanted populations at all other stations. Generally, mussel populations showed decreased body mass in areas regarded as most contaminated and in areas where they are not naturally present. These findings may be related to stress responses due to increased contaminant levels and/or variations in environmental conditions such as food availability as also reported by NESTO *et al.*, (2004) in mussels from the Venice Lagoon, Italy. In addition, differences in the reproductive status may have occurred among indigenous mussel populations while no significant differences in temperature were observed among stations within the same sampling season. Such variations in the physiological condition of mussels may have masked the net response to pollutant exposure.

Nevertheless, a significant correlation was found between AChE activity and MT content ($r=-0.436$, $p<0.05$), both parameters showed

a variation with respect to the season of sampling and during the second year of measurements a concomitant increase in MT content with a decrease in AChE activity was noted. Decreased MT content and higher AChE activities were recorded in spring although not consistently for both years at all stations as also reported by DOUMOUHTSIDOU *et al.*, (2004) and BELIAEFF & BOCQUENÉ (2004). Seasonal variation in biomarkers can be attributed to a variety of abiotic and biotic factors such as temperature, food supply and reproductive status (BAUDRIMONT *et al.*, 1997, SHEEHAN & POWER, 1999) that also affect the accumulation of contaminants (PHILLIPS, 1976). In the present study temperature differences between spring (16-17˚C) and autumn (23-24˚C) that can influence both MT and AChE (SERAFIM *et al*., 2002, BOCQUENÉ *et al*., 1993) may be among the factors related to the observed variation between seasons. On the other hand, the reason of variation in both biomarkers from one year to another is unclear and could not be attributed to differences in temperature, which was limited between years (0.5-1.0 ˚C) or variations in Cu, Cd and Zn levels in mussels. These temporal variations in biomarkers can be related to true variations in other contaminant levels or natural variability of biomarkers such as between-individual variations and seasonal changes related to physiological cycles of the organisms (BELIAEFF & BOCQUENÉ, 2004).

Although biomarker responses overall did not reflect the expected pollution gradient in the Saronikos Gulf our results are in accordance with those reported by BELIAEFF & BOCQUENÉ (2004) on biomarker responses in marine organisms from other Mediterranean areas. Results of the present study indicate that the applied biochemical markers in indigenous mussel populations do not reflect the type of pollution in the Saronikos Gulf to a degree that can be used for pollution monitoring in the area. However, further research is needed on the use of transplanted mussel populations for the application of MT in the area.

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