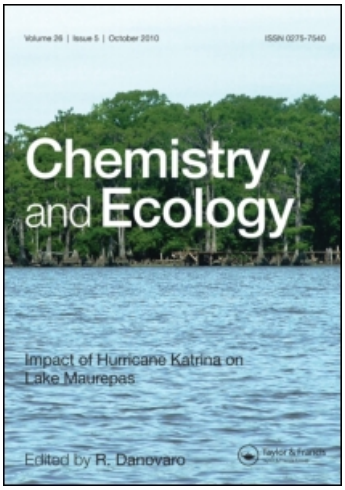

Chemistry and Ecology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713455114

Seasonal variation of biomarkers in *Mytilus galloprovincialis* sampled inside and outside Mar Piccolo of Taranto (Italy)

Roberto Caricato[a]; Maria Giulia Lionetto[a]; Trifone Schettino[a]
[a] Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali, Università del Salento, Lecce, Italy

Online publication date: 20 May 2010

To cite this Article Caricato, Roberto , Lionetto, Maria Giulia and Schettino, Trifone(2010) 'Seasonal variation of biomarkers in *Mytilus galloprovincialis* sampled inside and outside Mar Piccolo of Taranto (Italy)', Chemistry and Ecology, 26: 1, 143 — 153
To link to this Article: DOI: 10.1080/02757541003627639
URL: http://dx.doi.org/10.1080/02757541003627639

Chemistry and Ecology
Vol. 26, Supplement, June 2010, 143–153

Taylor & Francis
Taylor & Francis Group

Seasonal variation of biomarkers in *Mytilus galloprovincialis* sampled inside and outside Mar Piccolo of Taranto (Italy)

Roberto Caricato*, Maria Giulia Lionetto and Trifone Schettino

Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali, Università del Salento, Lecce, Italy

(*Received 15 May 2009; final version received 14 December 2009*)

The aim of this work was to study spatial and temporal variation in biomarkers in autochthonous *Mytilus galloprovincialis* sampled inside and outside Mar Piccolo of Taranto, a typical polluted semi-enclosed basin of the Mediterranean Sea characterised by scarce hydrodynamism. Mar Piccolo of Taranto represents a site of Italian National Interest because of the high level of pollution. A battery of biomarkers (lysosomal destabilisation, catalase, metallothioneins, acetylcholinesterase, air survival) was applied to assess pollution-induced stress effects in authoctonous mussels. The responses were analysed comparatively in two different seasons, summer and winter, in order to assess possible changes in the pollutant-induced stress syndrome throughout the year. No significant difference inside and outside Mar Piccolo was observed for metallothioneins. By contrast, the dramatically decreased acetylcholinesterase values and strongly increased catalase activity in organisms taken from Mar Piccolo in winter indicate an increased risk of exposure to anticholinesterase compounds during this season. The results suggest the importance of temporal variability in biomarker responses throughout the year for monitoring possible seasonal changes in the pollutant-induced stress syndrome of organisms living in a certain environment and, in turn, more properly detecting changes in ecotoxicological risks.

Keywords: biomarker; Mar Piccolo of Taranto; *Mytilus galloprovincialis*; Neutral Red retention assay; catalase; acetylcholinesterase; metallothioneins; stress-on-stress response; lysosomal stability

1. Introduction

Among marine organisms, bivalve molluscs, particularly mussels, are widely used as sentinel organisms in marine environmental monitoring and assessment programmes [1]. Their sessile filter-feeding lifestyle, coupled with the high bioaccumulation factor for organic and inorganic pollutants and low metabolic detoxification rates, make bivalves and *Mytilus* sp. in particular suitable for monitoring chemical pollution in the marine environment. Moreover, in the last decades, there has been increasing interest in the use of biochemical and cellular responses to pollutant exposure (i.e. biomarkers) as 'early warning' tools in marine environmental monitoring and assessment [2]. In contrast to the simple measurement of contaminants accumulating in

*Corresponding author. Email: roberto.caricato@unile.it

ISSN 0275-7540 print/ISSN 1029-0370 online

DOI: 10.1080/02757541003627639
http://www.informaworld.com

environmental matrices or in the body tissue of animals, biomarkers can offer more complete and more biologically relevant information about the potential impact of pollutants on the health of organisms living in a certain environment.

An interesting case of study of the impact of chemical contaminants on living organisms is Mar Piccolo of Taranto, a semi-enclosed basin which represents one of the sites of Italian National Interest because of its high level of pollution. Mar Piccolo of Taranto is located in the northern area of the town of Taranto. It is an inner, semi-enclosed basin (surface area of 21 km^2), with lagoon features, divided into two inlets, called the first and second inlet, which have an average depth of $\sim$5 m. The Mar Piccolo is connected with the Mar Grande through two narrow channels only. The biological balance of the Mar Piccolo ecosystem itself is very delicate because of the scarce hydrodynamism and low level of water exchange with the nearby Mar Grande, the shallow depth, thermal intensity in summer and the geological conformation. The scarce hydrodynamism and reduced water exchange with the nearby Mar Grande determine, mainly in summer, a high stratification of the water, and encourage organic matter sedimentation, which plays an important role in the transport and accumulation of pollutants in the sediments. In addition, the Mar Piccolo basin is influenced by urbanisation, harbour activities, intensive agriculture, aquaculture and commercial fishing. The main sources of environmental impact are: nine pipes discharging sewage, the Italian Navy ship-yard with its dry-docks (located in the first inlet), the fishing fleet localised in the first inlet, and small rivers and freshwater springs which drain the surrounding agricultural soil into the basin. These represent important pollutant sources of polychlorobiphenyls, organotin compounds, heavy metals, pesticides, pharmaceuticals and personal care products. Mar Piccolo of Taranto therefore represents a typical polluted semi-enclosed sea [3]. Chemical characterisation of pollutant concentrations in the basin has been investigated extensively in previous studies, although the extent of the detrimental effects of bioavailable pollutants on organisms living in Mar Piccolo is less well known.

The aim of this investigation was to study spatial and temporal variation in the biomarkers in autochthonous mussels (*Mytilus galloprovincialis*), sampled inside and outside Mar Piccolo of Taranto, in order to evaluate the stress effects of bioavailable pollutants on an autochthonous mollusc in the Taranto marine semi-enclosed basin.

Because single biomarkers cannot reflect the impairment of an organism's health, the use of a battery of responses is recommended in ecotoxicological studies of impacted areas. Therefore, a well-know battery of biomarkers, either general or specific, was applied to assess pollution-induced stress effects in autochthonous mussels: (1) lysosomal membrane stability, which was evaluated using the Neutral Red retention (NRR) assay of the haemocyte lysosomes; (2) catalase (CAT) activity; (3) tissue metallothionein (MT) concentration; (4) acetylcholinesterase (AChE) activity; and (5) the 'stress-on-stress' response. Lysosomal membrane stability and CAT activity were measured as general biomarkers, monitoring for a generic stress syndrome caused by the integrated effect of several clases of contaminant. MT concentration and AChE activity were measured as specific biomarkers, monitoring for the exposure/effects of specific chemical classes. In addition to biochemical and cellular responses, the 'stress-on-stress' response [4], expressed as a reduction in survival time in air because of marine pollution, was carried out to obtain information about the physiological state of the animals.

The responses of the biomarkers in autochthonous mussels, sampled inside and outside Mar Piccolo of Taranto, were analysed comparatively in two different seasons, summer and winter, to assess possible temporal variability in pollutant-induced stress syndrome throughout the year. Although the study of temporal variability in biomarkers represents an important aspect in the correct application and interpretation of biomarker responses in risk assessment, there are very few field studies addressing this issue in the literature.

2. Materials and methods

2.1. *Sampling stations*

The Taranto Sea is a coastal ecosystem situated in the Ionian Sea in southern Italy, inside the Gulf of Taranto. It is composed of two parts: the Mar Grande, directly connected to the Ionian Sea, and the Mar Piccolo. Sampling stations were located inside and outside Mar Piccolo of Taranto. The inside site was located in the second inlet of the Mar Piccolo (40°29′43.62″N 17°17′52.74″E), and the outside site was located in the Mar Grande (40°26′44.64″N 17°13′30.36″E). With respect to Mar Piccolo, Mar Grande is characterised by higher hydrodynamism and water exchange with the surrounding Ionian Sea.

2.2. *Animals*

Four hundred specimens of *M. galloprovincialis* Lam., both male and female, were collected from the sampling stations in summer (July 2007) and winter (January 2008). During sampling, mussels were in the same reproductive condition out of their main spawning season.

The shell lengths for the sampled mussels were: Mar Piccolo 6.00 ± 0.50 cm (summer), 5.40 ± 0.40 cm (winter); and Mar Grande 4.70 ± 0.40 cm (summer), 5.70 ± 0.50 cm (winter).

2.3. *Neutral Red retention assay*

Lysosomal NRR is determined in mussel haemolymph as an integrative measure of the general health status of the animal [5]. Haemolymph was withdrawn from the adductor muscle of 10 specimens, which were analysed individually to determine the Neutral Red retention time of the lysosomes. The NRRa method utilised was according to Lowe et al. [6]. Briefly, 40 μL of haemolymph (diluted 1:1 in saline solution containing 20 mM Hepes, 436 mM NaCl, 53 mM $MgSO_4$, 10 mM KCl, and 10 mM $CaCl_2$, pH 7.3) were dispensed on a poly-L-lysine-coated slide and incubated in a humid chamber (16 °C) for 30 min. Forty microlitres of Neutral Red solution (995 μL of saline solution and 5 μL of a 20 mg·mL^{-1} Neutral Red stock solution in dimethylsulphoxide) were added, and the slide was left in a humid chamber (16 °C) for 15 min. A cover slip was applied and the slide was observed every 15 min for the first hour and every 30 min for the next two hours. The time at which 50% of the lysosomes in the cells leached Neutral Red into the cytosol was determined.

2.4. *Catalase activity*

Catalase activity is a generic biomarker of the exposure to/effect of a pollutant mediated by oxidative stress [7].

CAT activity was measured in individual digestive glands, each sampled from 10 mussel specimens per station. CAT activity was assayed following the method of Clairborne [8] on sample homogenates obtained by homogenising the soft tissue in a 1:5 (tissue weight/buffer volume) ratio in ice-cold phosphate buffer (50 mM, pH 7). Briefly, the assay mixture consisted of phosphate buffer (KH_2PO_4 50 mM, pH 7) and hydrogen peroxide (0.036%) in a final volume of 3 mL. CAT activity was calculated as μmol H_2O_2 consumed·min^{-1}·mg^{-1} of proteins.

2.5. *Metallothionein concentration*

Metallothioneins are low molecular mass (6–7000 Da) cysteine-rich (20–30%) metal-binding proteins, whose neosynthesis represents a specific response of the organisms to heavy metal (Cu, Zn, Cd and Hg) exposure [9].

The concentration of MTs was determined in individual digestive glands, obtained from 10 mussel specimens per station, using the spectrophotometric method described by Viarengo et al. [10]. Tissue was homogenised in 3 vol. of 0.5 M sucrose, 20 mM Tris/HCl buffer, pH 8.6, with 0.006 mM leupeptine, 0.5 mM phenylmethanesulphonylfluoride and 0.01% β-mercaptoethanol. The homogenate was centrifuged at 30,000 *g* for 20 min at 0–4 °C. The resulting supernatant was treated with ethanol/chloroform; 1.05 mL of cold (−20 °C) absolute ethanol and 80 μL of chloroform were added to 1 mL aliquots of supernatant. The samples were then centrifuged at 6000 *g* for 10 min at 0–4 °C. One milligram of RNA, 40 μL 37% HCl and 3 vol. of cold ethanol were added to the collected supernatant. The sample was maintained at −20 °C for 1 h and centrifuged in a swinging rotor at 6000 *g* for 10 min. The pellet containing the MTs was washed with 87% ethanol/1% chloroform in homogenising buffer and centrifuged in 150 μL 0.25 M NaCl; 150 μL of 1 M HCl, containing EDTA 4 mM, was then added to the sample. The concentration of MTs in the extract was quantified spectrophotometrically utilising Ellman's SH reagent. The amount of MTs was calculated assuming a cysteine content in mussel MTs of 29% [10].

2.6. *Acetylcholinesterase activity*

Acetylcholinesterase catalyses the hydrolysis of acetylcholine into choline and acetic acid. Its inhibition is directly linked to the toxic action of organophosphorus and carbamate pesticides.

AChE activity was determined on individual gill tissues, obtained from 10 mussel specimens per station. Prior to AChE biochemical analysis, the tissue was ground in Tris buffer (0.1 M, pH 7.5). The homogenate obtained was centrifuged at 9000 *g* for 20 min at 0–4 °C. The supernatant was removed and used to determine AChE activity using the spectrophotometric method described by Ellman et al. [11]. Follwing this method, the increase in absorbance of the sample at 412 nm in the presence of 1 mM acetylthiocholine iodide was measured. The enzymatic reaction rate was quantified against a blank without substrate for each activity measurement. In order to subtract the spontaneous hydrolysis of the substrate, a second blank was performed without a sample. AChE activity is expressed as nmoles of product developed per minute per mg of protein.

2.7. *Stress-on-stress response*

The stress-on-stress response represents a physiological index of the state of the animals [4]. On arrival at the laboratory, mussels were placed on trays and exposed to air at 18 °C. Mortality was checked every day. Mussels were considered dead when they did not respond to squeezing of the valves, after the valves had gaped, or if they did not recover when placed in seawater.

2.8. *Protein concentration*

Protein concentration was measured using a Bio-Rad (Richmond, CA, USA) protein assay kit, using lyophilised bovine plasma gamma globulin as a standard.

2.9. *Data analysis*

Data were analysed by two-way analysis of variance (ANOVA) with Newman–Keuls *post hoc* using WinGmav 5 software™ (designed, coded and compiled by A.J. Underwood and M.G. Chapman, Institute of Marine Ecology, University of Sydney, Australia). An orthogonal experimental design was applied with two sources of variation: sampling station and sampling season. The homogeneity of variance was tested by Cochran's test prior to applying the ANOVA.

3. Results

3.1. *Lysosomal membrane stability*

Lysosomal membrane stability measured in autochthonous mussels sampled inside and outside Mar Piccolo of Taranto in summer and winter is reported in Figure 1(a). On average, the NRR

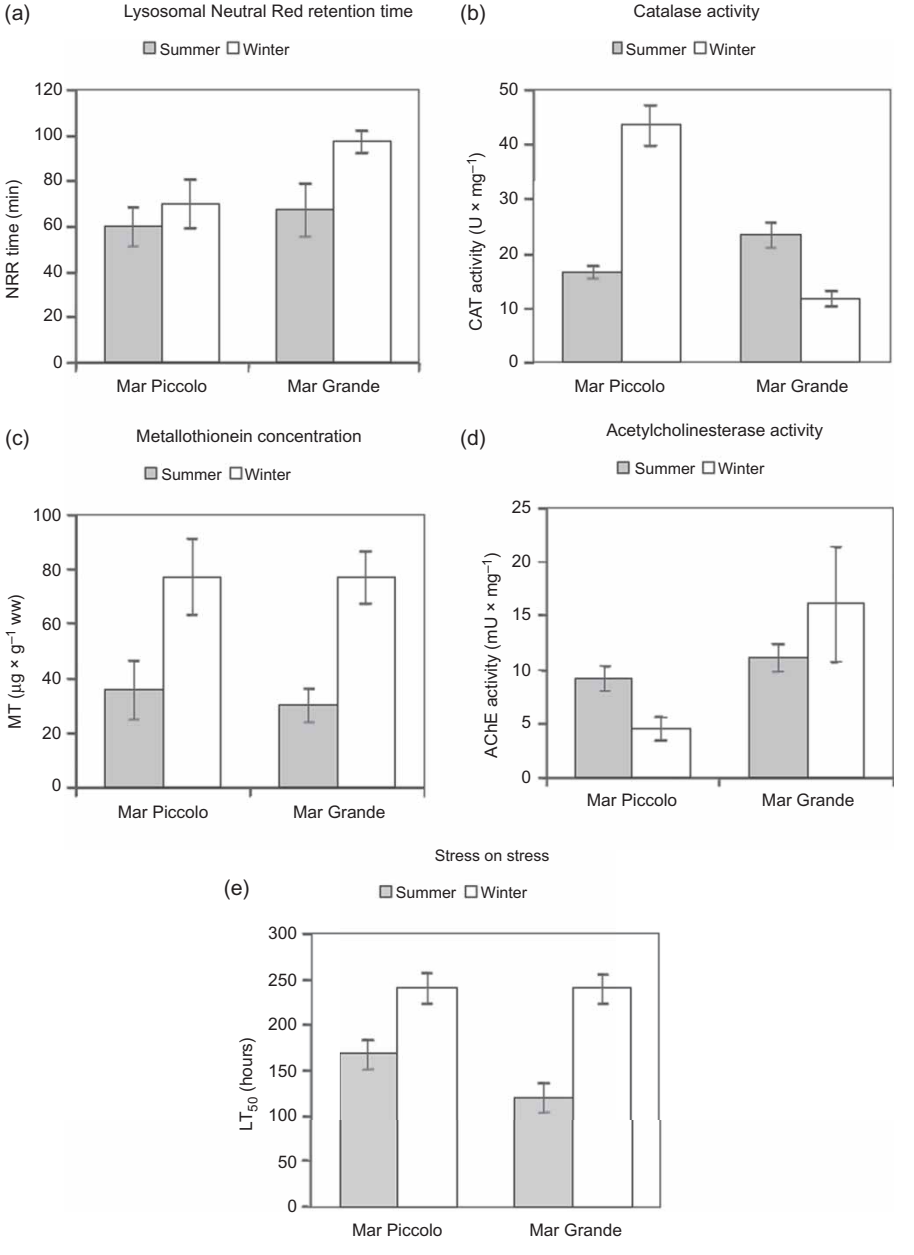

Figure 1. (a) Lysosomal Neutral Red retention time (expressed in min), (b) catalase-specific activity (expressed as enzymatic units mg^{-1} proteins), (c) metallothioneins concentration (expressed as μg MTs g^{-1} wet weight), (d) acetylcholinesterase specific activity (expressed as mU mg^{-1} proteins) determined, respectively, in haemocytes, digestive glands and gills of *Mytilus galloprovincialis* sampled in Mar Piccolo and Mar Grande of Taranto during summer and winter. (e) Median lethal time (LT_{50}; expressed in h) calculated by interpolation on survival curves. Data are expressed as mean ± SE.

times were within the range 60–100 min, with the lower mean values found in Mar Piccolo. Data were analysed by two-way ANOVA, as indicated above. As reported in Table 1, the sampling season showed a significant effect on the variability observed ($p = 0.0399$). For both sampling sites, lysosomal membrane stability appeared decreased in summer. However, the sampling site did not show a significant effect on lysosomal membrane stability, although, on average, values were lower for Mar Piccolo than for Mar Grande, especially in winter.

3.2. *Catalase activity*

As shown in Figure 1(b), CAT-specific activity measured in mussels from Mar Piccolo and Mar Grande showed different seasonal patterns. In fact, in Mar Piccolo, CAT activity appeared significantly increased in winter, whereas in Mar Grande it appeared significantly increased in summer.

As reported in Table 1, statistical analysis of the data revealed highly significant differences ($p < 0.0001$) between the sampling sites, and a highly significant effect due to the season ($p < 0.0001$). Moreover, the interaction between the two sources of variability was significant ($p < 0.0001$), because of the different effect of season on the variability at the two sites.

3.3. *Metallothionein concentration*

As shown in Figure 1(c) and Table 1, no significant differences were observed in digestive gland MT concentration between the two sampling sites. However, the sampling season had a marked effect on MT levels. In fact, in both Mar Piccolo and Mar Grande, MT levels were increased in winter with respect to summer.

3.4. *Evaluation of acetylcholinesterase activity*

Gill AChE activity levels of the sampled mussels are reported in Figure 1(d). In summer, mussels from both sampling stations displayed approximatley the same mean value; however, in winter, lower values were found in Mar Piccolo. Two-way ANOVA analysis is reported in Table 1. There were significant differences ($p = 0.0002$) between sampling stations, but no significant differences between sampling seasons. However, the interaction among the sources of variation was significant ($p = 0.0054$), suggesting a different effect of season on variability at the two sites, as observed for the CAT activity.

3.5. *Evaluation of the stress-on-stress response of mussels*

In Figure 1(e) the results obtained for the stress-on-stress response are reported as median lethal times (LT_{50}), obtained by interpolation of survival curves. Two-way ANOVA analysis is reported

Table 1. Statistical analysis of biomarker responses by two-way ANOVA.

		P				
Source of variation	Degrees of freedom	NRRt	CAT	MT	AChE	SOS
Sampling station	1.0	0.2921	<0.0001	0.6376	0.0002	0.1485
Sampling season	1.0	0.0399	<0.0001	<0.0001	0.9315	<0.0001
Interaction	1.0	0.0700	<0.0001	0.6715	0.0054	0.1485
Residual (error)	32.0					
Total	35.0					

Note: NRRt, Neutral Red retention time; CAT, catalase activity; MT, metallothionein concentration; AChE, acetylcholinesterase activity; SOS, stress-on-stress response determined as median lethal time (LT_{50}).

in Table 1. No significant differences between the two sampling sites were observed. However, there were significant differences ($p < 0.0001$) between sampling seasons, with lower values seen in summer in both Mar Piccolo and Mar Grande.

4. Discussion

As recognised in recent years by international organisations and environmental agencies, risk assessment cannot be based solely on the chemical analysis of environmental samples, because this approach does not provide any indication of the deleterious effects of contaminants on the biota [12]. Therefore, measurement of the biological effect of pollutants on living organisms (biomarkers) has become of major importance in assessing the quality of the environment [13]. Considering the complexity of biochemical and cellular responses to pollutants, the use of a multimarker approach is strongly recommended. Integrating responses from a wide spectrum of biomarkers is also of great value for obtaining better insight into the mechanisms that mediate the impairment of biological structures and functions. In this study, some of the most widely used biomarkers of pollution were determined in order to evaluate the stress effects of bioavailable pollutants in an autochthonous mollusc species, *Mytilus galloprovincialis*, in a semi-enclosed polluted basin, Mar Piccolo of Taranto. Moreover, the study was carried out at different seasons, winter and summer, to monitor possible changes in pollutant-induced stress syndrome throughout the year.

The general heath status of the animals was assessed using the NRR assay on haemolymphatic cells. On average, the NRR times found in mussels sampled from inside and outside Mar Piccolo were in the range 60–100 min, with lower values found in Mar Piccolo. The observed mean values in Mar Piccolo were ∼60 min in both sampling seasons. This value is below the normal values for NRR times reported for *M. galloprovincialis* of 90–120 min [14], suggesting the presence of a general stress condition in animals sampled in Mar Piccolo in both winter or summer. In Mar Grande, the NRR times in winter were within the normal range for this biomarker, whereas in summer, the NRR time appeared depressed, with values similar to those observed in Mar Piccolo. NRR time has been previously found [15] to be not very sensitive to seasonal variations. It has been reported to be either minimally or not at all affected by natural factors, such as temperature and salinity, but to be mainly influenced by pollutants [15,16]. Therefore, the observed reduction in NRR time could be ascribed to an increased exposure of the animals to bioavailable pollutants in Mar Grande during the summer period.

The other general biomarker investigated was CAT activity in the mussel digestive gland. The use of CAT as a biomarker has been suggested in several marine organisms [17]. Increased CAT activities have been described for several fish and invertebrate species from polluted sites [18,19], but inhibition and transitory responses are also reported according to the intensity and duration of the chemical disturbance [20,21]. Previous studies carried out on aquatic organisms indicated the sensitivity of CAT activity to environmental factors such as temperature and salinity [22–24]. Orbea et al. [24] reported an increase in the CAT activity in mussels and oysters in the summer season compared with winter. This pattern was observed in Mar Grande, where a significant increase was measured in the summer. Surprisingly, in Mar Piccolo a completely different behaviour was recorded: in winter a twofold increase in CAT activity was observed with respect to summer. It has been reported that several organic contaminants, such as pesticides and fertilisers, increase CAT activity [24–26]. Therefore, in winter, more intense rainfall may increase drainage of the surrounding agricultural soils and, in turn, increase the impact of pesticides and fertiliser on the semi-enclosed basin. In fact, it has to be considered that the Taranto Sea receives a considerable amount of sewage ($\sim$18,272 $m^3 \cdot day^{-1}$ with organic matter equal to 6.7 tonnes per day of biochemical oxygen demand [27]) from the northern part of Taranto and nearby towns,

85% of which occurs at the Second Seno. For example, the Ajedda Channel discharges waste water from eight municipalities and drains the surrounding agricultural soil into the Second Seno of the Mar Piccolo, near our sampling site.

This explanation is also supported by AChE data. AChE is specifically inhibited by organophosphate and carbamate persticides. The enzymatic activity showed a dramatic decrease (∼50%) in winter compared with summer. As reported in toxicological experiments [28], a 50% inhibition in *M. galloprovincialis* gill AChE activity corresponds to an experimental exposure of the animals to 0.1 mg·L^{-1} carbaryl (carbamate) or 0.2 mg·L^{-1} methyl parathion (organophosphate) for 24 h. These concentrations cannot be extrapolated for field conditions, because here the length of exposure or the synergic effect of a mixture of contaminants can influence the stress response of the organisms; however, our results suggest an increased exposure of the animals to AChE mainly in the winter season. Pfeifer et al. [29] reported a seasonal variation in AChE activity in blue mussels with decreased values in winter. This observation is not confirmed by our data from Mar Grande, where no significant difference was observed between the two seasons. Lionetto et al. [30] previously reported that AChE and CAT activities are inversely correlated in *M. galloprovincialis*. This was confirmed in our study, in which a dramatic decrease in AChE activity and a dramatic increase in CAT activity were observed in winter. These data corroborate the hypothesis of an increased risk of exposure to pesticides in winter in organisms living in Mar Piccolo. In addition, several field and laboratory studies show that other types of contaminant such as metals, surfactants [31] and compounds in complex mixtures [32] may inhibit the AChE activity of several bivalve molluscs. Therefore, the presence of other potential anticholinesterase compounds, related to domestic untreated discharges, cannot be excluded.

MT synthesis can be greatly enhanced by the presence of heavy metals, whereas natural factors influencing MT synthesis have to be taken into account before final conclusions can be drawn [33]. Seasonal variation in the MT content of bivalve tissues is controversial. Baudrimont et al. [34] and Bordin et al. [35] reported a decrease in MT content in summer in whole body *Corbicula fluminea* and *Macoma balthica*, respectively, whereas Viarengo et al. [10] observed an increase in MTs during summer in the digestive gland of *M. galloprovincialis*. However, Ivankocić et al. [36] found maximum MT values in winter, followed by a progressive decrease during summer/autumn in the digestive gland of *M. galloprovincialis*. In our study, two-way ANOVA analysis (Table 1) indicated a significant effect of season, but not of the sampling site, on the observed MT variability. A significant increase in MT values in the digestive gland was observed in winter in both sampling sites. In a comparative study of the levels of metals in reared mussels from either Mar Piccolo or Mar Grande, Cardellicchio et al. [37] reported seasonal changes in metal soft tissue concentrations, with maximum values in late winter/early spring, followed by a progressive decrease during the summer, partly related to the reproductive activity of mussels. Therefore, the MT results observed in our samples may reflect the natural variability in metal content in *Mytilus* tissues. The MT levels measured in mussels from either Mar Piccolo or Mar Grande were within the same range as that measured using the same method in other studies [10,38]. These results agree with heavy metal analysis in mussel soft tissue [37] and sediments [39]. In fact, soft tissue metal concentrations in mussels sampled in Mar Piccolo and Mar Grande [39] were similar to those detected in other Italian coastal zones, and within the permissible range for safe consumption by humans. In addition, metals in the sediments of Mar Piccolo are mainly present as insoluble sulphides, and not bioavailable compounds for filter feeders such as mussels [39].

A general index of physiological status measured in mussels was their ability to survive in air. Our results showed significant seasonal variation in the survival time, with higher values in winter than in summer. Moreover, no significant differences were observed between the two sites in both seasons (Table 1). Despite the fact that previous field studies have revealed that pollution exposure may decrease the ability to survive in air, other results suggest that survival in air shows a direct dependence on the concentration of pollutants only for mussels exposed for a short time

under laboratory conditions [40]. However, the exposure of mussels to pollutants for a long time may result in some level of adaptation to pollution with no significantly reduced survival times compared with reference groups [40]. This could be the case for native animals from Mar Piccolo, which are chronically exposed to pollution. This result confirms the controversial applicability of survival in air in biomonitoring based on native organisms.

5. Conclusions

The overall results of this study confirmed the utility of using multimarkers in mussels to detect biological disturbance induced by chemical contaminations and assess ecotoxicological risk. Moreover, the results highlight the importance of temporal variability in biomarker responses throughout the year for monitoring possible seasonal changes in pollutant-induced stress syndrome of organisms living in a certain environment and more properly detecting changes in the ecotoxicological risk.

This study contributes to the provision of an ecotoxicological protocol for biomonitoring Mar Piccolo of Taranto throughout the year. It revealed an increased risk of exposure to anticholinesterase in organisms living in Mar Piccolo in the winter season, probably because of the more intense rainfall, which may increase drainage from surrounding agricultural soils affected by intense agriculture.

Acknowledgements

This work was supported by Regione Puglia (POR Puglia 2000-2006 – Complemento di Programmazione, Asse III, Misura 3.12).

References

[1] A. Jernelov, *The international mussel watch: A global assessment of environmental levels of chemical contaminants*, Sci. Total Environ. 188, Supp 1 (1996), pp. 37–44.
[2] D.R. Livingstone, J.K. Chipman, D.M. Lowe, C. Minier, and R.K. Pipe, *Development of biomarkers to detect the effects of organic pollution on aquatic invertebrates: Recent molecular, genotoxic, cellular and immunological studies on the common mussel (*Mytilus edulis *L.) and other mytilids*, Int. J. Env. Poll. 13 (2000), pp. 56–91.
[3] V. Cuomo, J. Merrill, and I. Palomba, *Mariculture with seaweed and mussels for marine environmental restoration and resources production*, Oceanogr. Lit. Rev. 45 (1998), p. 702.
[4] M.P. Cajaraville, M.J. Bebianno, J. Blasco, C. Porte, C. Sarasquete, and A. Viarengo, *The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: A practical approach*, Sci. Total Environ. 247 (2000), pp. 295–311.
[5] P.W. Fernley, M.N. Moore, D.M. Lowe, P. Donkin, and S. Evans, *Impact of the* Sea Empress *oil spill on lysosomal stability in mussel blood cells*, Mar. Environ. Res. 50 (2000), pp. 451–455.
[6] D. Lowe, M. Soverchia, and M.N. Moore, *Lysosomal membrane responses in the blood and digestive cells of mussels experimentally exposed to fluoranthene*, Aquat. Toxicol. 33 (1995), pp. 105–112.
[7] F. Regoli and G. Principato, *Glutathione, glutathione-dependent and antioxidant enzymes in mussel,* Mytilus galloprovincialis, *exposed to metals in different field and laboratory conditions: Implications for a proper use of biochemical biomarkers*, Aquat. Toxicol. 31 (1995), pp. 143–164.
[8] A. Clairborne, *Catalase activity*, in *Handbook of Methods of Oxigen Radical Research*, R.A. Grenwald, ed., CRC Press, Boca Raton, FL, 1985, pp. 283–284.
[9] G. Roesijadi, *Metallothionein induction as a measure of response to metal exposure in aquatic animals*, Environ. Health Persp. 102 (1994), pp. 91–95.
[10] A. Viarengo, E. Ponzano, F. Dondero, and R. Fabbri, *A simple spectrophotometric method for metallothionein evaluation in marine organisms: An application to Mediterranean and Antarctic molluscs*, Mar. Environ. Res. 44 (1997), pp. 69–84.
[11] G.L. Ellman, K.O. Courtney, V. Anders, and R.M. Featherstone, *A new and rapid colorimetric determination of acetylcholinesterase activity*, Biochem. Pharmacol. 7 (1961), pp. 88–95.
[12] R. van der Oost, J. Beyer, and N.P.E. Vermeulen, *Fish bioaccumulation and biomarkers in environmental risk assessment: A review*, Environ. Toxicol. Phar. 13 (2003), pp. 57–159.

[13] J.S. Gray, *Biological and ecological effects of marine pollutants and their detection*, Mar. Pollut. Bull. 25 (1992), pp. 48–50.
[14] F. Regoli, G. Frenzilli, R. Bocchetti, F. Annarumma, V. Scarcelli, D. Fattorini, and M. Nigro, *Time-course variation in oxyradical metabolism, DNA integrity and lysosomal stability in mussels*, Mytilus galloprovincialis, *during a field translocation experiment*, Aquat. Toxicol. 68 (2004), pp. 167–178.
[15] A.H. Ringwood, E.C. Deanna, and J. Hoguet, *Effects of natural and anthropogenic stressors on lysosomal destabilization in oysters* Crassostrea virginica, Mar. Ecol.- Prog. Ser. 166 (1998), pp. 163–171.
[16] S. Dailianis, G.P. Domouhtsidou, E. Raftopoulou, M. Kaloyianni, and V.K. Dimitriadis, *Evaluation of neutral red retention assay, micronucleus test, acetylcholinesterase activity and a signal transduction molecule (cAMP) in tissues of* Mytilus galloprovincialis *(L.), in pollution monitoring*, Mar. Environ. Res. 56 (2003), pp. 443–470.
[17] D.R. Livingstone, *Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms*, Mar. Pollut. Bull. 42 (2001), pp. 656–666.
[18] R.T. Di Giulio, *Indices of oxidative stress as biomarkers for environmental contamination*, in *Aquatic Toxicology and Risk Assessment*, Vol. 14, M.A. Mayes and M.G. Barrons, eds., ASTM STP, American Society for Testing and Materials, Philadelphia, 1991, pp. 15–31.
[19] F. Regoli, D. Pellegrini, G.W. Winston, S. Gorbi, S. Giuliani, C. Virno-Lamberti, and S. Bompadre, *Application of biomarkers for assessing the biological impact of dredged materials in the Mediterranean: The relationship between antioxidant responses and susceptibility to oxidative stress in the red mullet (*Mullus barbatus), Mar. Pollut. Bull. 44 (2002), pp. 912–922.
[20] F. Regoli, and G. Principato, *Glutathione, glutathione-dependent and antioxidant enzymes in mussel*, Mytilus galloprovincialis, *exposed to metals in different field and laboratory conditions: Implications for a proper use of biochemical biomarkers*, Aquat. Toxicol. 31 (1995), pp. 143–164.
[21] F. Regoli, M. Nigro, and E. Orlando, *Lysosomal and antioxidant responses to metals in the Antarctic scallop* Adamussium colbecki, Aquat. Toxicol. 40 (1998), pp. 375–392.
[22] M. Dellali, M.G. Barelli, M. Romeo, and P. Aissa, *The use of acetylcholinesterase activity in* Ruditapes decussatus *and* Mytilus galloprovincialis *in the biomonitoring of Bizerta lagoon*, Comp. Biochem. Physiol. C: Pharmacol. Toxicol. 130 (2001), pp. 227–235.
[23] A. Khessiba, M. Roméo, and P. Aïssa, *Effects of some environmental parameters on catalase activity measured in mussel* (Mytilus galloprovincialis*) exposed to lindane*, Environ. Pollut. 133 (2005), pp. 275–281.
[24] A. Orbea, M. Ortiz-Zarragoitia, M. Solé, C. Porte, and M.P. Cajaraville, *Antioxidant enzymes and peroxisome proliferation in relation to contaminant body burdens of PAHs and PCBs in bivalve molluscs, crabs and fish from the Urdaibai and Plentzia estuaries (Bay of Biscay)*, Aquat. Toxicol. 58 (2002), pp. 75–98.
[25] M. Solé, C. Porte, and J. Albaiges, *The use of biomarkers for assessing the effects of organic pollution in mussels*, Sci. Total Environ. 159 (1995), pp. 147–153.
[26] A. Khessiba, P. Hoarau, M. Gnassia-Barelli, P. Aissa, and M. Romeó, *Biochemical response of the mussel* Mytilus galloprovincialis *from Bizerta (Tunisia) to chemical pollutant exposure*, Arch. Environ. Con. Tox. 40 (2001), pp. 222–229.
[27] N. Cardellicchio, C. Annichiarico, and G. Leone, *I mari di Taranto: problematiche di impatto ambientale*, Atti della Società Italiana di Ecologia 12 (1991), pp. 769–775.
[28] P. Mora, X. Miche, and J.F. Narbonne, *Cholinesterase activity as potential biomarker in two bivalves*, Environ. Toxicol. Phar. 7 (1999), pp. 253–260.
[29] S. Pfeifer, D. Schiedek, T. Joachim, and W. Dippner, *Effect of temperature and salinity on acetylcholinesterase activity, a common pollution biomarker, in* Mytilus *sp. from the south-western Baltic Sea*, J. Exp. Mar. Biol. Ecol. 320 (2005), pp. 93–103.
[30] M.G. Lionetto, R. Caricato, M.E. Giordano, M.F. Pascariello, L. Marinosci, and T. Schettino, *Integrated use of biomarkers (acetylcholinesterase and antioxidant enzymatic activities) in* Mytilus galloprovincialis *and* Mullus barbatus *in an Italian coastal marine area*, Mar. Pollut. Bull. 46 (2003), pp. 324–330.
[31] L. Guilhermino, P. Barros, M.C. Silva, and A.M. Soares, *Should the use of cholinesterases as a specific biomarker for organophosphate and carbamate pesticides be questioned?* Biomarkers 3 (1998), pp. 157–163.
[32] M. Dellali, M. Roméo, and P. Aissa, *Suivi annuel de l'activite' catalase chez des moules et des palourdes originaires de la lagune de Bizerte*, Oceanologica Acta 24 (2001), pp. 1–9.
[33] R.P. Cosson, *Bivalve metallothionein as a biomarker of aquatic ecosystem pollution by trace metals: Limits and perspectives*, Cell Mol. Biol. 46 (2000), pp. 295–309.
[34] M. Baudrimont, S. Lemaire-Gony, F. Ribeyre, J. Metivaud, and A. Boudou, *Seasonal variations of metallothionein concentrations in the Asiatic clam (*Corbicula fluminea), Comp. Biochem. Physiol. 118C (1997), pp. 361–367.
[35] G. Bordin, J. McCourt, and F. Cordeiro Raposo, *Metallothionein like metalloproteins in the Baltic clam* Macoma balthica*: Seasonal variations and induction upon metal exposure*, Mar. Biol. 129 (1997), pp. 453–463.
[36] D. Ivanković, J. Pavičić, M. Erk, V. Filipović-Marijić, and B. Raspor, *Evaluation of the* Mytilus galloprovincialis *Lam. digestive gland metallothionein as a biomarker in a long-term field study: Seasonal and spatial variability*, Mar. Pollut. Bull. 50 (2005), pp. 1303–1313.
[37] N. Cardellicchio, A. Buccolieri, S. Giandomenico, L. Lopez, F. Pizzulli, and L. Spada, *Organic pollutants (PAHs, PCBs) in sediments from the Mar Piccolo in Taranto (Ionian Sea, Southern Italy)*, Mar. Pollut. Bull. 55 (2007), pp. 451–458.

[38] S. Petrovic, B. Ozretic, M. Krajnovic-Ozretic, and D. Bobinac, *Lysosomal membrane stability and metallothioneins in digestive gland of mussels (*Mytilus galloprovincialis *Lam.) as biomarkers in a field study*, Mar. Pollut. Bull. 42(12) (2001), pp. 1373–1378.
[39] N. Cardellicchio, A. Buccolieri, A. Di Leo, and L. Spada, *Heavy metals in marine sediments from the Mar Piccolo of Taranto (Ionian Sea, Southern Italy)*, Annali di Chimica 96 (2006), pp. 727–741.
[40] R.E. Thomas, P.M. Harris, and R.D. Rice, *Survival in air of* Mytilus trossulus *following long-term exposure to spilled* Exxon Valdez *crude oil in Prince William Sound*, Comp. Biochem. Physiol. C: Comp. Pharmacol. Toxicol. 122 (1999), pp. 147–152.