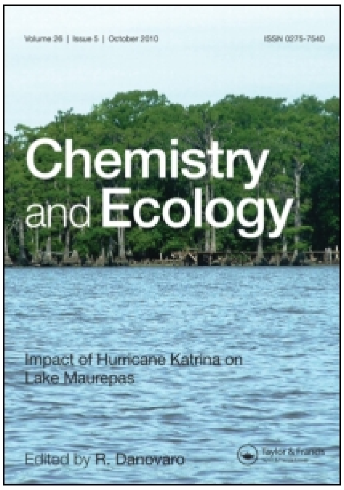

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BIOMARKER APPLICATION FOR THE STUDY OF CHEMICAL CONTAMINATION RISK ON MARINE ORGANISMS IN THE TARANTO MARINE COASTAL AREA

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This work represents a pilot study for monitoring the potential toxicological risk of commercial relevant marine resources along the South coast of Italy by using biomarkers as complementary tool to chemical analysis. The attention was focused on the industrialized area of Salento peninsula, such as Taranto, that, in spite of the presence of the big industry (oil, metal industry), sustains activities related to the sea resources, such as fishery and mussel-culture. The study was carried out in fish, such as *Mullus barbatus* and *Trachurus mediterraneus*, two important fish species for the fishery in this area, and in mussels (*Mytilus galloprovincialis*). As control area S. Maria di Leuca, area of naturalistic interest, was chosen. In fish, liver metallothionein levels (specific index of exposure to heavy metals such as Hg, Cd, Cu and Zn) and brain and muscular acetylcholinesterase (AChE) activity (specific index of exposure to organophosphate and carbamate pesticides) were measured. None of the two fish species showed significant differences in AChE activity and in pesticide trace level between the anthropogenic impact exposed site and the control group. On the other hand, metallothionein hepatic levels in *M. barbatus* were significantly increased in the organisms coming from Taranto with respect to the organisms coming from the control site, but chemical analysis, routinely performed on edible muscle for the evaluation of chemical quality of fish products, did not reveal high heavy metal concentration in the edible muscle of fish from Taranto.

Mussels exposed for one month in the Mar Piccolo of Taranto, an important mussel farming area, showed increase in the level of catalase activity, an oxidative stress index, increase in the levels of metallothioneins and inhibition of AChE activity.

The need to integrate chemical analysis with the study of biological responses to pollutants (biomarkers) in marine organisms is discussed for a better comprehension of the impact of chemical contaminants on the sea and its resources.

Keywords: Biomarkers; *Mytilus galloprovincialis*; *Mullus barbatus*; *Trachurus mediterraneus*; Taranto; Heavy metals; Pesticides

1 INTRODUCTION

Chemical contamination of marine environments is a world wide problem, but it is particularly serious along the coasts of industrialized countries, where wastes from a number of human activities reach the sea. Some of these wastes can represent a threat to marine life and possibly to man as a consumer of seafood. The quality of the fishing products gives rise to some worries especially for those species bioconcentrating chemical pollutants

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from the water or those involved in biomagnification phenomena along the trophic chain (Thibaud, 1992; Orlando, 1989). In recent years, this problem has contributed to the development of indices of biological effects (biomarker) as early warning tools of adverse environmental change. Such methods are being used in combination with analytical chemistry on a rapidly increasing basis and on a worldwide scale (Bayne *et al.*, 1988).

As reported by different authors, the evaluation of biomarkers in bioindicator organisms sampled in one or more areas suspected of chemical contamination and their comparison with organisms sampled in a control area can allow the evaluation of the potential risk of toxicological exposure of the studied community (Shugart *et al.*, 1989; Depledge, 1989; McCarthy *et al.*, 1990; Fossi *et al.*, 1992).

In the present work, biomarkers in marine organisms have been studied in parallel to chemical analysis, routinely performed for the chemical quality determination of the fishery products, in order to evaluate the potential toxicological risk from anthropogenic chemical contamination of commercial relevant marine resources in an industrialized area of the Salento Peninsula (Italy), such as Taranto. Taranto coastal area, in spite of the presence of industrial activities (oil, metal industry) and of a considerable amount of urban sewage coming from the city of Taranto and from eight nearby towns, sustains activities related to sea resources, such as fishery and mussel-culture. The study was carried out on fish (*Mullus barbatus* and *Trachurus mediterraneus*) and mussels (*Mytilus galloprovincialis*). The two fish species, which are important for fishery activities in this area, show different life style. *Mullus barbatus* is commonly found on gravel, sand and mud bottoms of the continental shelf. Due to the close association with sediments, it tends to concentrate contaminants to a higher degree than other species. For this reason it was recommended by FAO/UNEP (1993) as monitoring species.

Trachurus mediterraneus is a benthopelagic species feeding copepods and other planktonic crustaceans (Deudero and Morales-Nin, 2001). It belongs to the so-called 'pesce azzurro', collective name referring to the whole range of red meat fish which typically gleam silver and blue in the open waters of the Mediterranean; it represents a great fish resource in the Mediterranean Sea. To our knowledge, it represents the first study in which biomarker analysis is performed on *T. mediterraneus.*

Mytilus galloprovincialis is a sessile filter-feeding organism, which accumulates chemical contaminants both from the seawater and particulate food material filtered from the water. The tissue concentrations of many environmental xenobiotics can reach very high levels, thus making it a useful tool for chemical monitoring but, on the other hand, a potential risk for sea food consumers. Mussels, in particular, appear to be relatively tolerant to many metals and organic xenobiotics. This tolerance, however, does not mean that the animals are unresponsive; in fact there is considerable evidence for exposure and pathological reactions to even low concentrations of contaminants (Livingstone, 1988; Moore, 1988; Widdows and Johnson, 1988).

The biomarker responses studied are metallothioneins levels, acetylcholinesterase (AChE) and catalase activities. Metallothioneins are low molecular weight (6–7000 Da) cysteine-rich (20%–30%) metal-binding proteins, whose neosynthesis represents a specific response of the organisms to exposure to heavy metals such as Cu, Zn, Cd and Hg. It has been demonstrated that the levels of metallothioneins in the liver of fish (Hogstrand and Haux, 1991) and in the digestive gland of mussels (George and Olsson, 1994) are dose-dependently increased by exposure to heavy metals.

Acetylcholinesterase catalyses the hydrolysis of acetylcholine into choline and acetic acid. Its inhibition is directly linked with the mechanisms of toxic action of organophosphorus and carbamate insecticides. In addition to anticholinesterase insecticides, other classes of environmental contaminants, *e.g.* other pesticides (Davies and Cook, 1993; Gill *et al.*, 1990a) and heavy metals (Gill *et al.*, 1990b) have the potential to decrease AChE in exposed organisms.

Catalase is an enzyme belonging to the cellular antioxidant system that counteracts the toxicity of reactive oxygen species. Several classes of pollutants, including trace metals or organic compounds, are known to enhance the formation of reactive oxygen species. Variations in the activities of antioxidant enzymes have been demonstrated in several studies and proposed as biomarkers of pollutant mediated oxidative stress (Viarengo *et al.*, 1990; Porte *et al.*, 1991; Regoli and Principato, 1995; Hai *et al.*, 1997).

2 MATERIAL AND METHODS

Fish and mussel sampling was performed in April 2001. *Mullus barbatus* and *T. mediterraneus* were trawl-fished (about three miles offshore, trawling runs of 30 min periods) out of the Gulf of Taranto, in S. Vito place, an important fishing area near Taranto (Fig. 1). As control area S. Maria di Leuca, a low urbanized area with no industrial and commercial activities, was chosen. For both species individuals of the same size class were selected to ensure uniform sampling (mean size 14.5 ± 0.5 cm and 14 ± 1.7 cm for *M. barbatus* body length in

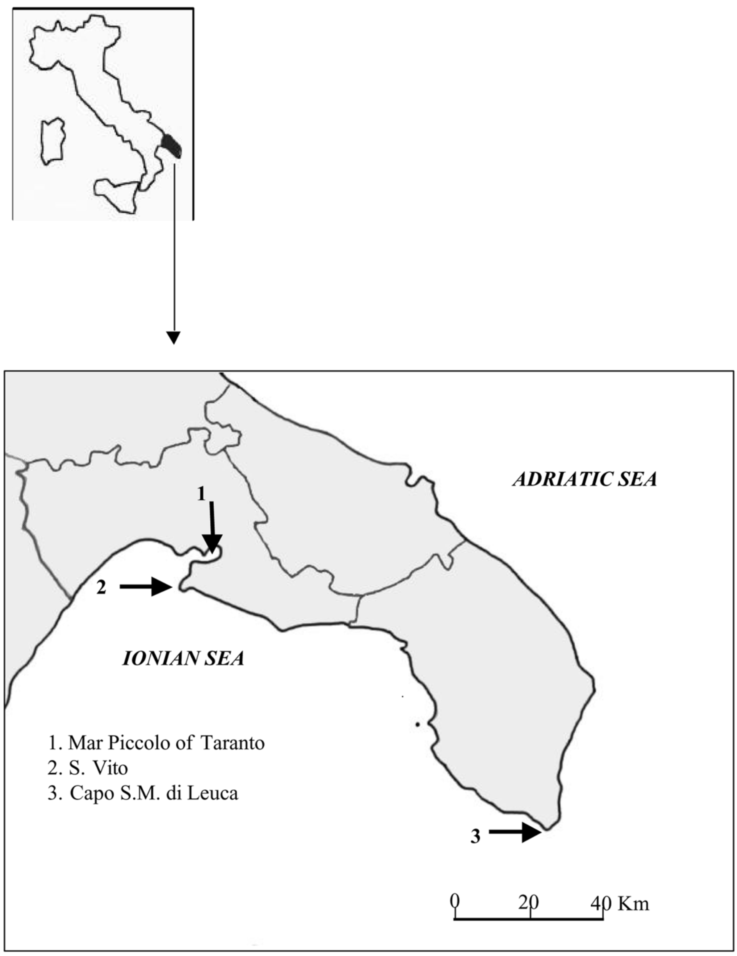

FIGURE 1 The studied area is the Ionic coast of Salento Peninsula (south part of Apulia, Italy).

Leuca and S. Vito, respectively; 19.4 ± 1.1 cm and 19.2 ± 1.9 cm for *T. mediterraneus* body length in Leuca and S. Vito, respectively).

Mussels belonging to an homogeneous stock (5.0 ± 0.5 cm shell length) were purchased from a mussel farm (Mare vivo Castro-Lecce) located in a clean site of the Salento Peninsula. They were translocated in cages (120 for each cage) implanted for 30 days in the control area and in the first 'inlet' of Mar Piccolo of Taranto (Fig. 1). Cages were immersed by scuba-diving at about 5 m depth and maintained at anchor.

Mar Piccolo of Taranto, which represents an important mussel farming area, is a coastal marine area, communicating with the Gulf of Taranto (Ionian Sea) through two channels and structured in two shelves named 'first inlet' and 'second inlet' which have a maximum depth of 13 and 10 m, respectively. It suffers especially from urban pollution since it receives a considerable amount of sewage coming from the northern area of the city of Taranto and from nearby towns.

Fish liver, brain, mussel gills and digestive glands were rapidly excised, frozen in liquid nitrogen and maintained at −80 °C till processed for analyses. Metallothionein (Mt) concentration in fish liver and in mussel digestive gland was determined by the spectrophotometric method described by Viarengo *et al.* (1997). This method involves the evaluation of the Mt concentration in a partially purified metalloprotein-containing fraction obtained by acidic ethanol/ chloroform fractionation of tissue homogenate. Briefly, tissues were homogenized in 3 volumes of 0.5 M sucrose, 20 mM Tris–HCl buffer, pH 8.6, added with 0.006 mM leupeptine, 0.5 mM phenylmethylsulphonilfluoride (PMSF) as antiproteolitic agents, and 0.01% β-mercaptoethanol as a reducing agent. For fish liver preparations the method was modified in the composition of the homogenization buffer (0.15 M sucrose, 20 mM Tris–HCl buffer, pH 8.6 and antiproteolitic agents). The homogenate was then centrifuged at 30.000*g* for 20 min at 0–4 °C. The resulting supernatant was then treated with ethanol/chloroform. Cold (−20 °C) absolute ethanol of 1.05 ml and 80 μl of chloroform were added to aliquots of 1 ml of supernatant; the samples were then centrifuged at 6000*g* for 10 min at 0–4 °C. RNA of 1 mg and 40 μl 37% HCl and 3 volumes of cold ethanol were added to the collected supernatant. The sample was maintained at −20 °C for 1 hr and centrifuged in a swinging rotor at 6000*g* for 10 min. The pellet containing the metallothioneins was then washed with 87% ethanol/1% chloroform in homogenizing buffer, centrifuged at 6000*g* for 10 min and dried under nitrogen gas stream. The pellet was resuspended in 150 μl 0.25 M NaCl. A 150 μl HCl 1N containing EDTA 4 mM were added to the sample. The concentration of metallothioneins in the extract was quantified spectrophotometrically utilizing the Ellman's SH reagent (Ellman, 1958). The amount of metallothionein was calculated assuming a cysteine content in mussel (Mackay *et al.*, 1993) and fish (Roesijadi, 1992) metallothionein of 29%.

2.1 Acetylcholinesterase Activity

The tissue (brain for fish and gills for mussels), grounded in Tris-buffer 0.1 M, pH 7.5, was homogenated and centrifuged at 9000*g* for 20 min at 4 °C. The resulting supernatant was removed and used to determine AChE activity.

Acetylcholinesterase activity was spectrophotometrically determined according to Ellman method (Ellman *et al.*, 1961) by measuring the increase in absorbance of the sample at 412 nm in the presence of 1 mM acetylthiocholine as substrate and 0.1 mM 5,5′-dithiobis-2-dinitrobenzoic acid (DTNB). 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 sample. Each AChE activity measurement was performed in duplicate. Acetylcholinesterase activity is expressed as nmoles of product developed per minute per mg of proteins.

2.2 Catalase Activity

Catalase activity was assayed by the method of Clairborne (1985) on sample homogenates obtained by homogenizing the soft tissue in 1:5 (tissue weight:buffer volume) ratio in ice-cold phosphate buffer. Briefly, the assay mixture consisted of phosphate buffer (KH_2PO_4 50 mM, pH 7) hydrogen peroxide (0.036%) in a final volume of 3 ml. Catalase activity was calculated as μmol H_2O_2 consumed $min^{-1}\,mg^{-1}$ of proteins.

2.3 Chemical Analysis

Heavy metal concentrations were analyzed according to D.M. 03/02/1989 method n.34 (cadmium, copper and zinc) and IRSA-CNR, 1985 (mercury) by using coupled plasma atomic emission spectrometry (ICP-AES). Organophosphate and organochlorine pesticides were analyzed according to FDA-PAM (1999) by multiresidual analysis by using Gas Chromatography with dual electron capture detection (GC-ECD). Heavy metal and pesticide concentrations were determined on a pool of muscle samples coming from 10 specimens of *M. barbatus* and from 10 specimens of *T. mediterraneus.* Chemical analysis were performed by Studio Effemme (Squinzano-Lecce, Italy).

2.4 Statistical Analysis

Mussel metallothionein analysis was performed on 10 pools of two digestive glands per station, while fish metallothionein analysis was performed on single liver sample of 15 specimens per station per species. Mussel AChE activity was determined on the gills of 14 specimens per station, while fish AChE activity was determined on single brain or muscle sample of 15 specimens per station per species. Mussel catalase activity was measured on 14 pooled samples per station, each composed of two digestive glands. Data are reported as mean $\pm$ S.E.M. Statistical analysis was performed by Student *t*-test. The significance of results was ascertained at $P < 0.05$.

3 RESULTS

3.1 Biomarkers in Fish

In Table I, the concentration of heavy metals in edible muscle of *T. mediterraneus* and *M. barbatus* from the two studied sites is reported. Zn and Cu tissue concentrations fall in the average levels previously found in the same species in other sites of Italian coasts (Ciusa and Ghiaccio, 1984; Ghidini *et al.*, 2000), while Cd and Hg tissue concentration

TABLE I Heavy Metal and Pesticide (Organophosphate and Organochlorine) Concentration Measured in the Muscle of *T. mediterraneus* and *M. barbatus* Coming from S.M. of Leuca and S. Vito (Taranto).

	Leuca					*S. Vito (Taranto)*				
Fish	*Cd*	*Hg*	*Zn*	*Cu*	*Pest.*	*Cd*	*Hg*	*Zn*	*Cu*	*Pest.*
Trachurus mediterraneus	0.004	0.0012	3.020	0.730	0.01	0.004	0.0012	3.780	0.780	0.01
Mullus barbatus	0.004	0.0012	2.420	0.890	0.01	0.004	0.0012	3.580	0.890	0.01

Note: Heavy metal and pesticide concentrations were determined on a pool of muscle samples coming from 10 specimens of *M. barbatus* and from 10 specimens of *T. mediterraneus.* Data are expressed as ppm wet weight. Wet weight values can be transformed to dry weight values using the conversion factor of 0.2 for fish flash (wet weight concentration = dry weight concentration $\times$ 0.2).

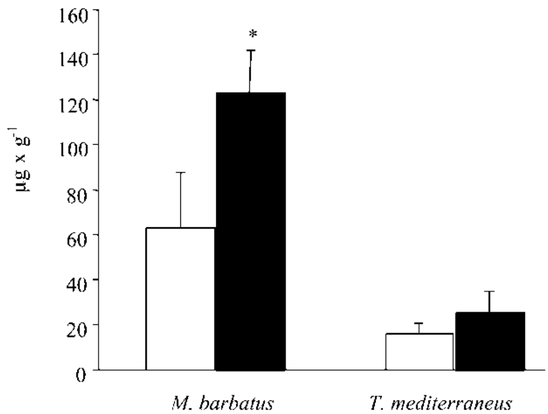

FIGURE 2 Mt levels, expressed as $\mu g\,g^{-1}$ of tissue wet weight, measured in the liver of *M. barbatus* and *T. mediterraneus* sampled in Capo S. Maria di Leuca (empty bars) and in S. Vito near Taranto (filled bars). Data are reported as mean ± S.E.M. of 15 individuals for each group. $^{*}P < 0.05$.

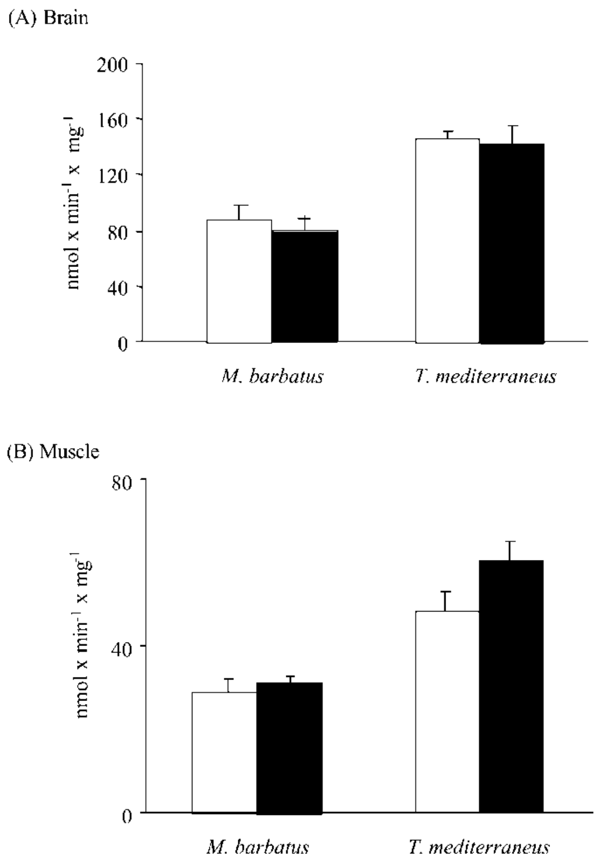

FIGURE 3 Specific AChE activity, expressed as nmoles $\min^{-1}\,mg^{-1}$ of proteins, measured in the brain (A) and in the muscle (B) of *M. barbatus* and *T. mediterraneus* sampled in capo S. Maria di Leuca (empty bars) and S. Vito near Taranto (filled bars). Data are reported as mean ± S.E.M. of 15 individuals for each group. $^{*}P < 0.05$.

were lower. Zn showed a slight increased value in the two species coming from S. Vito with respect to the control site. The chemical analysis were integrated with the measure of liver metallothioneins, biomarker of exposure to heavy metals. In *M. barbatus* specimens coming from Taranto site, liver metallothioneins are significantly increased with respect to specimens coming from the control place (Fig. 2). On the other hand, the benthopelagic species *T. mediterraneus*, characterized by lower metallothioneins levels with respect to *M. barbatus*, did not show significant differences between organisms sampled in the two sites.

Moreover, the concentration of pesticides on muscle of the sampled fish was determined by chemical analysis (Tab. I). Multiresidual analysis revealed 0.01 ppm pesticide (organophosphate and organochlorine) traces in the studied animals coming from the two sites. In parallel AChE activity, biomarkers of pesticides exposure/effect, was measured either in muscle or in brain. (Fig. 3). Comparing the activity of the two species, *T. mediterraneus* showed an higher activity either in muscle or in brain compared to *M. barbatus*, but none of the two fish species showed significant differences of the AChE activity between the site exposed to anthropogenic impact and the control site (Fig. 3).

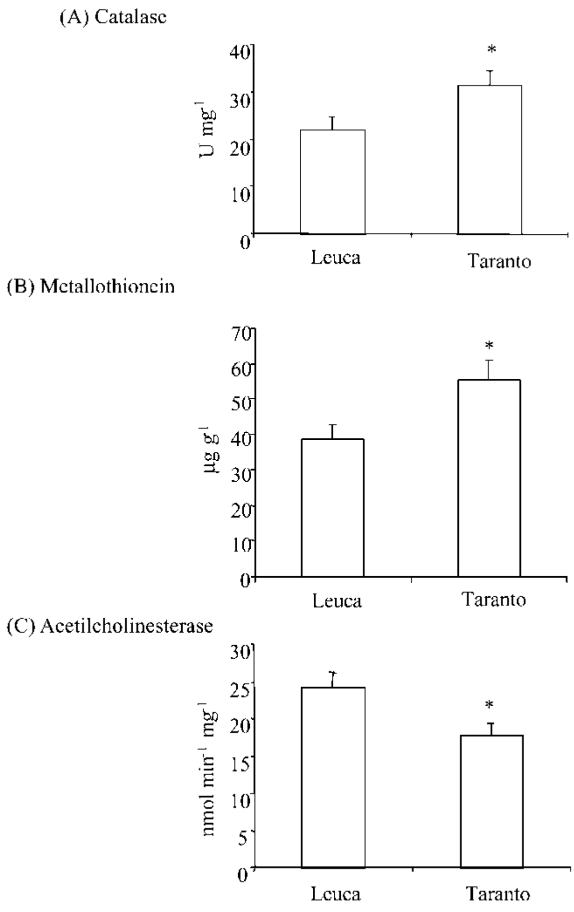

FIGURE 4 Specific catalase activity, expressed as $U\,mg^{-1}$ of proteins, measured in the digestive gland (A), metallothionein levels, expressed as $mg\,g^{-1}$ of tissue wet weight, measured in the digestive gland (B) and specific AChE activity, expressed as $nmol\,min^{-1}\,mg^{-1}$ of proteins, measured in the gills (C) of *M. galloprovincialis* exposed for 30 days in the 'first inlet' of Mar Piccolo of Taranto and in Capo S. Maria di Leuca (LE). Datas are reported as mean ± S.E.M. $^*P < 0.05$.

3.2 Biomarkers in caged mussels

As reported in Figure 4, mussels exposed for one month in the Mar Piccolo of Taranto showed a significant increase in the level of a general stress index such as catalase activity. Then, in order to identify some of the classes of contaminants responsible for the stress response observed, the analysis of specific stress indexes, such as metallothioneins and AChE, were performed. Metallothionein level in the digestive gland of mussels exposed in the Mar Piccolo of Taranto appeared significantly increased with respect to control organisms, indicating exposure to bioavailable heavy metals. Moreover gill AChE activity appeared significantly inhibited in organisms coming from the site exposed to anthropogenic impact.

4 DISCUSSION

This work represents a pilot study for monitoring the quality of fishing resources in terms of toxicological risk in an industrialized marine coastal area of the Salento peninsula, such as Taranto. Indices of biological effects (biomarkers) were measured in fish and mussels. As recognised in the last years by international organisations and environmental agencies, risk assessment cannot be solely based on chemical analysis of environmental samples, because this approach does not provide any indication of deleterious effects of contaminants on the biota. Therefore, the measurement of the biological effects of pollutants has become of major importance for the assessment of the quality of the environment (Bayne, 1989; Gray, 1992). The fish species utilized, *M. barbatus* and *T. mediterraneus*, are of commercial importance for the fishing activity along the Italian coasts and in particular in the studied area.

On these species chemical analysis, routinely performed to determine the chemical quality of fishery product, has been integrated by biomarker determination. To our knowledge, this is the first study in which the biomarker measurements are applied on *T. mediterraneus* species; therefore data obtained in this paper can represent biomarker reference values for this species. As indicated by chemical analysis, muscle concentration of mercury in *M. barbatus* and *T. mediterraneus* (0.0012 ppm) is lower with respect to values previously found in the same species in other sites of Italian coasts (Ciusa and Ghiaccio, 1984; Ghidini *et al.*, 2000) and in other areas of the Mediterranean Sea (Hornung and Kress, 1991; Giordano *et al.*, 1991; Pastor *et al.*, 1994; Focardi *et al.*, 1998) and far below the maximum value of mercury (0.5 ppm) indicated for the edible parts of fish by the European Community (G.U.C.E., 1991). Cadmium concentration found (0.004 ppm) in the two species is similar to the value reported in *M. barbatus* sampled in the Eastern Aegean Sea (Kucuksezgin *et al.*, 2001) and is lower with respect to Cd levels in fish muscle previously reported from other Mediterranean regions (Taliadouri-Voutsinou, 1982; UNEP, 1989; Giordano *et al.*, 1991; Bei *et al.*, 1992). Zn and Cu tissue concentrations fall in the average levels previously found in the same species in other sites of Italian coasts (Ciusa and Giaccio, 1984; Ghidini *et al.*, 2000). In spite of the fact that chemical analysis did not show significantly higher heavy metal concentration in the edible muscle of fish from Taranto, the biochemical determination of liver hepatic metallothioneins revealed concentrations of these proteins significantly increased in *M. barbatus* specimens coming from Taranto with respect to the organisms coming from the control site. Comparing these data with *M. barbatus* metallothionein levels found in a previous study, carried out along Salento Peninsula coasts one year before in the same season (Lionetto *et al.*, 2001), Taranto metallothioneins value is similar to levels measured in specimens sampled in an other industrialized area of Salento Peninsula (Brindisi), while control metallothionein value is similar to data found in specimens from sites not exposed to anthropogenic impact. The observed increase in metallothionein levels in *M. barbatus* specimens coming from

Taranto suggests a possible risk of exposure to bioavailable heavy metals in benthonic species; these, in fact, are closely related to sediments, which represent the accumulation compartment for environmental pollutants. On the other hand, benthopelagic species, such as *T. mediterraneus*, did not show chemical stress responses, probably due to their greater mobility and to the fact that their living style is not so closely related to the sediments. As reported in literature, induction of metallothioneins in the liver is the main form of detoxication of metals in fish (Hamilton and Mehrle, 1986) and can be sensitive to the additive exposure to several heavy metals (Cu, Zn, Cd and Hg), each in low concentration. Therefore, in the evaluation of the toxicological risk of commercial relevant fishery resources, liver metallothionein determination could represent a low cost high sensitive first screening for the early detection of exposure to heavy metals, preceding the more expensive chemical analysis, whose results are tissue and heavy metal specific; in fact, tissues such as muscle, liver, gonads or gills show different capacity for accumulating different heavy metals (Wong *et al.*, 2001; Zyadah and Chouikhi, 1999).

As regards pesticide residue, none of the two species analyzed from the two sites contained residue levels in excess of the limits stated by FAO for fish and fishery products (Nauen, 1983). Chemical analysis were paralleled by cholinesterase activity in brain and muscle: brain cholinesterase in fish consists entirely of AChE, while muscle contains not only AChE but also butyrylcholinesterase (Kirby *et al.*, 2000), that has been demonstrated to be more sensitive to certain organophosphate and carbammate (Sturm *et al.*, 1999). Brain expresses the most cholinesterase activity in the two species, with *T. mediterraneus* showing higher activity either in brain or in muscle with respect to *M. barbatus.* No significant differences in cholinesterase activity were found between organisms sampled in the two sites in both species, confirming results obtained with the residue analysis. *M. barbatus* brain acetylcholinesterase levels measured in this study are similar to the values previously found in *M. barbatus* specimens coming from other sites along the Ionian Sea coast and the South part of Salento Peninsula (Lionetto *et al.*, 2003).

In order to evaluate the quality of water of Mar Piccolo of Taranto, important mussel farming area, in term of chemical contaminant exposure risk, the biological responses to chemical stress in transplanted mussels (*M. galloprovincialis*) were utilized. Mussels exposed for one month in the Mar Piccolo of Taranto showed a significant increase in the level of catalase activity, an oxidative stress index. Moreover, the increase of metallothioneins levels suggest the risk of exposure to bioavailable heavy metals in these organisms. As regards AChE in addition to organophosphate and carbammate pesticides a number of other important contaminants have recently been shown to have anticholinesterase properties, including heavy metals (Zinkl *et al.*, 1991), hydrocarbons and detergents (Payne *et al.*, 1996). Therefore, it is probably that the anti-cholinesterase effect observed in mussels exposed to Mar Piccolo of Taranto, which receives a considerable amount of urban sewage, could be attributed to the integrated effect of several classes of contaminants.

Results obtained suggest the importance of studying biological responses to environmental chemical stress (biomarkers) in marine organisms for a better comprehension of the impact of chemical contaminants on the sea and its resources.

Acknowledgements

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