A Multimarkers Approach to Assess the Environmental Pollution on Biological Resources Subject of Commercial Fishing: the Case of Mullus barbatus (Linnaeus, 1758) in the Southern Ty...

Chapter · November 2011

9 authors, including:
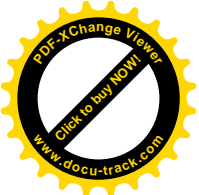
A Multimarker Approach to Assess the Environmental Pollution on Biological Resources Subject of Commercial Fishing: the Case of *Mullus barbatus* (Linnaeus, 1758) in the Southern Tyrrhenian Sea

V. Micale[1], L. Pirrera[1], T. Bottari[1], B. Busalacchi[1], A. Perdichizzi[1], F. Perdichizzi[1], A. Profeta[1], P. Rinelli[1], I. Corsi[1]

1, Institute for Coastal Marine Environment, CNR, Messina, Italy

2, Department of Environmental Sciences, University of Siena, Siena, Italy

valeria.micale@iamc.cnr.it

Abstract

A set of biomarkers were used to evaluate the environmental quality of a marine area along the Sicilian Tyrrhenian coast, subjected to a high anthropic impact. Biological responses to environmental stress were investigated in a commercially valuable fish, the red mullet *Mullus barbatus* (L. 1758), which has also been recommended as one of the most important sentinel organisms for the Mediterranean sea (UNEP/RAMOGE, 1999). Liver cytochrome P4501A levels, measured as ethoxyresorufin-O-deethylase activity, glutathione S-transferase and UDP-glucuronosy transferase activities were detected as specific biomarkers of exposure to PCBs and PAHs. In order to investigate the impact of other anthropogenic chemicals widely present in marine coastal areas due to agricultural run-off, acetylcholinesterase activity was also measured. Vitellogenin gene induction was assayed in adult male specimens as a biomarker of exposure to estrogenic compounds. Gonad developmental stage and gonad alterations, such as intersexuality and/or fibrosis, were also investigated histologically to assess potential disturbances in reproduction.

1 Introduction

The NW Mediterranean Sea is the recipient of extensive urban and industrial wastewater discharges from bordering countries, through continental runoff, sewage sludge disposal, and atmospheric deposition. It is estimated that up to now more than 100.000 chemical compounds and xenobiotics have been produced by chemical industry, many of which are persistent: because of their ability to bind to organic matter, they may accumulate up the food chain, especially in species at the apex of the chain itself [1, 2], constituting a potential risk factor for the entire marine biotic component, but also for humans, as final consumer. The primary toxicity of a contaminant is exercised primarily at biochemical and molecular levels and only later the effects may be observed, by a cascade mechanism, through the hyerarchical ladder of
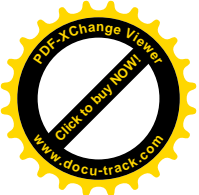
the organization (organelle, cell, tissue, individual) until reaching the population [3]. Regarding biochemical responses of fish to aquatic pollutants, the mixed function oxidase system P450 is known to play a major role in the oxidative metabolism/biotransformation of toxic compounds such as chlorinated and aromatic hydrocarbons [4, 5, 6]. The induction of EROD activity has been successfully used in assessing environmental pollution as specific markers of exposure to PCBs and PAHs in a variety of fish species [5, 7].

The inhibition of AChE activity is a specific biological effect of exposure to agricultural pesticides including organophosphates (OPs) and carbamates (CBs) [8] but more recently, its field application in biomonitoring programmes on fish species has been reported to be less specific, thus it is now emerges as a more general marker of exposure to neurotoxic contaminants including heavy metals and organochlorines [9, 10].

Some xenobiotics compounds, called Endocrine Disruptors Compounds (EDCs), can interfere at various levels and through different mechanisms, with the normal hormonal regulation of organisms. Numerous studies on EDCs highlight the reproductive disorders induced by them, such as decrease in the number and quality of eggs [11, 12, 11], changes in the number and viability of sperms [13, 14], alterations in sexual differentiation and development [15], intersexuality [16, 17, 14], stimulation of estrogen receptors in immature males, stimulation of hepatic vitellogenesis in males and juveniles [18, 19, 17, 20]. In ecotoxicological studies the use of a wide battery of biological responses is recommended since single biomarkers cannot reflect impairment of an organism's health and/or adaption to impaired environmental conditions [21, 22].

The selected bioindicator fish species, *Mullus barbatus*, is widely used in environmental monitoring programs in the Mediterranean Sea [23, 24, 25, 26, 14, 27, 28, 29]. The red mullet is a benthic species, wide distributed and easy to find, considered of high commercial value and with well-known biological features [30, 31], which because of its territorial characteristics (habitat, feeding mode and degree of mobility),is suitable for biomonitoring investigations [32, 33]. Due to their close association with sediments these fish tend to concentrate contaminants to a higher degree than other species.

In this paper, a set of biomarkers were used to evaluate the environmental quality of a coastal marine area with high anthropic impact. The effects of exposure to chemical contaminants were investigated in a sentinel species, the red mullet *M. barbatus* (L., 1758) and responses to environmental stress were detected.

2 Materials and methods

2.1 Study area

The area selected to carry out this study lies in the Tyrrhenian sea, along the northern coast of Sicily (Figure 1). The selection of the sampling sites was as follows: site 1, a low impacted area, close to cape Tindari, characterized by being a not taken zone; site 2 (7 Km far from site 1), a moderately impacted area within the gulf of Patti, recipient of both urban and agricultural drains; and site 3 (30.65 Km far from site 1), just in front of Milazzo, affected by the presence of an oil refinery, as well as by a most important commercial harbour.
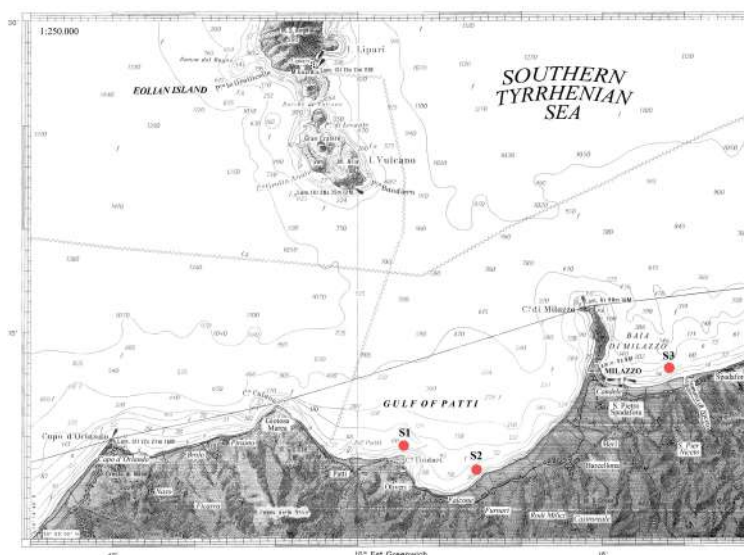
Figure 1: Study area with sampling sites.

| Target gene | Primer sequence | | Amplicon size | Annealing |
	Foward	Reverse	(nucleotides)	temperature (°C)
Vtg	CAGGGAGAAGATGACCCAGAT	AGAGATGCCCTCACCCTTTG	126	59
β-actin	CAGGGAGAAGATGACCCAGAT	GATACCGCAGGACTCCATACC	~430	57

Table 1: Primer pair sequences, amplicon size and annealing temperatures used for vitellogenin expression analysis.

2.2 Sampling

Red mullets, *Mullus barbatus* (mean LT ± SD) were collected by trawl surveys in June 2007 and January 2008 at 50 m depth. After sexing macroscopically, total length (TL, cm) and body weight (BW, g) of males were recorded. Liver and brain were frozen in liquid nitrogen and stored at -80 °C, until laboratory analysis. Gonads were fixed for 24 h in Bouin's solution and stored in 70° ethanol for subsequent histological analysis.

2.3 Biochemical and catalytic assays

Cytosolic and microsomial fractions were prepared according to Corsi et al. [34]. Liver microsomal 7-ethoxyresorufin-O- deethylase (EROD) activities were measured in duplicate according to the spectofluorimetric assays of Burke and Mayer [35] using a Perkin-Elmer LS50B luminescence spectrofluorimeter. UDP-glucuronosyltransferase activity was performed according to Aitio[36] modified by Collier et al. [37]. The glutathione S-transferase activity was measured by the
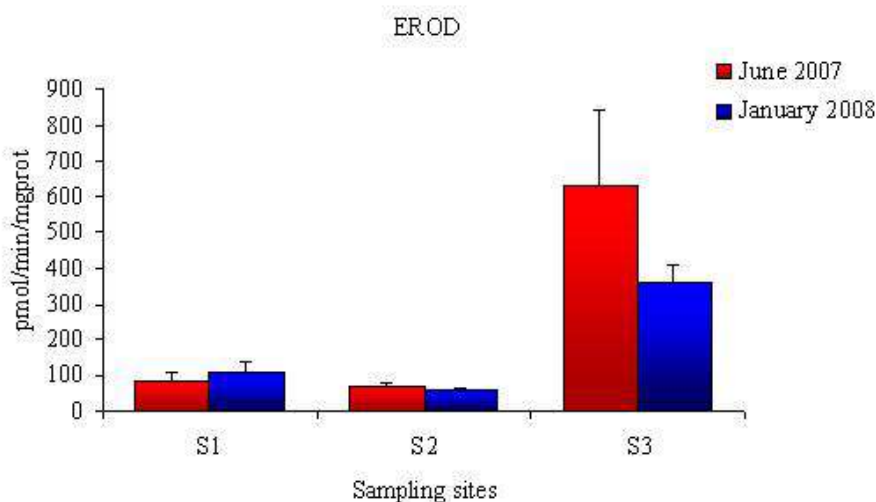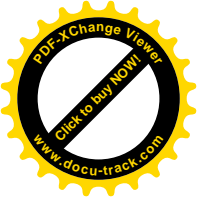
2085

Figure 2: EROD activity levels (pmol min^{-1} mg prot^{-1} ± s.d.) in *M. barbatus* in three different sites and two sampling periods.

spectrophotometric method described by Habig et al. [38] modified for microplate readers. AChE activity was measured on microplate by the method of Ellman et al. [39]. Total proteins were measured according to Bradford [40] and total proteins concentration was expressed in mg·ml^{-1}.

2.4 Total RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

The expression of the vitellogenin gene was qualitatively investigated on 45 and 14 male specimens from the summer and winter surveys, respectively. In particular, in June 16 from site S1 (size range 13.5-22 cm), 21 from site S2 (size range 15-22), and 8 from the site S3 (size range 15.5-20 cm), and in January 8 from the site S1 (size range 12-19.5 cm), 3 from site S2 (size range 16-19 cm) and 3 from site 3 (size range 12.5-16.5 cm) were collected. The small number of individuals analyzed for the winter survey was caused by the loss of some samples. Six females, one per survey and site, were analyzed as positive control. Total RNA from liver tissue was extracted in Trizol reagent, following the procedure recommended by the manufacturer (Invitrogen). The total RNA was digested with RNase free DNases to remove endogenous DNA contamination. Primer pair sequences, their amplicon size and annealing temperatures are shown in Table 1. All primer pairs gave a single band pattern for the expected amplicon size in all reactions, and no amplification occurred in RT reactions without enzymes.
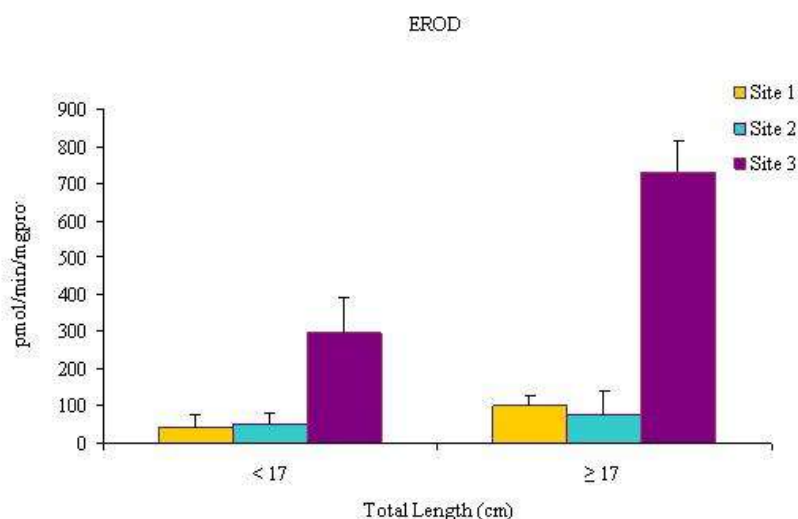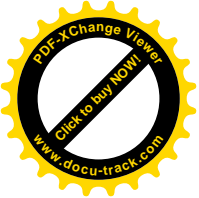
Figure 3: EROD activity levels (pmol min^{-1} mg prot^{-1} $\pm$ s.d.) in different size classes of *M. barbatus*.

1 µg of total RNA from each liver sample was used to synthesize cDNA. The fragments of genes of interest, vitellogenin and β-actin (used as housekeeping gene), were subsequently amplified in Thermal cycler MX3000 (Statagene). PCR was carried out in a 20 µl reaction mixture and conducted at 95°C for 15 min, 32 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 1 min, and terminated for 10 min at 72 °C. The PCR products were visualized by electrophoresis in agarose with ethidium bromide staining.

2.5   Histological analysis

Gonadal samples were dehydrated in a graded series of ethanol, cleared in xy-lene, embedded in resin, cut in 5 µm-thick sections and stained with haematoxylin-eosin. Testes were staged for maturity as follows: immature/resting (only spermatogonia) (stage I); developing (spermatocytes and spermatids, no spermatozoa) (stage II); maturing (all spermatogenic stages, including spermatozoa, in the seminiferous lobules (stage III); running (spermatozoa in the main sperm duct) (stage IV); spent-recovering (residual spermatozoa and spermatogonia) (stage V). The gonads were also examined for histopathological alterations, such as necrosis, degeneration, feminization, and/or delayed or arrested gamete development.
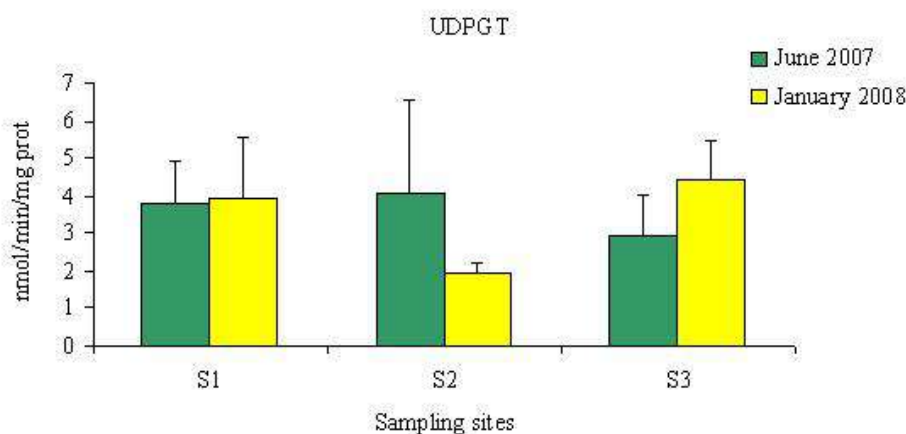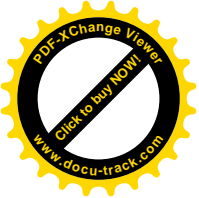
Figure 4: UDPGT activity levels (nmol min^{-1} mg prot^{-1} ± s.d.) in *M. barbatus* in three different sites and two sampling periods.

2.6 Statistical analysis

Biochemical activities were determined individually in 3-8 specimens per station, and the data are reported as mean ± S.D. The statistical analysis of data was done using one-way analysis of variance (ANOVA). The homogeneity of variances and the normality of data were checked using respectively Levene's test and Kolmogorov-Smirnov test, respectively. A p value ≤ 0.05 was considered as statistically significant. Data on EROD, GST, UDPGT and AChE were compared between survey, sites and size classes.

3 Results

7-ethoxyresorufin-O-deethylase activities for the three sampling sites in both survey (June 2007 and January 2008), are shown in Figure 2. These were significantly higher (p<0.001) in the samples from site 3 than in the other sample sites, both in summer and winter survey. There were no significant differences between sites 1 and 2 (p=0.97). No significant differences were noted in EROD activity between the summer and winter sampling, in either site 1 (p=0.34), 2 (p=0.42), or 3 (p=0.50). Comparing enzyme activities between two size classes (≤ 17 cm and ≥ 17 cm), corresponding to younger and older than 1 year, respectively (Project CAMP-BIOL, 2006), it was evidenced that larger individuals showed a greater induction of EROD activity (Figure 3).

Particularly, there was a significant difference between size (p ≤ 0.001) in sites 1 and 3, but not in site 2 (p=0.50). Comparing the two size classes in the two sampling periods and different sites investigated, there was no significant difference in summer for site 1 (p=0.22) and site 2 (p=0.74), while in site 3, the two size classes were significantly different (p ≤ 0.05). In twinter sur-
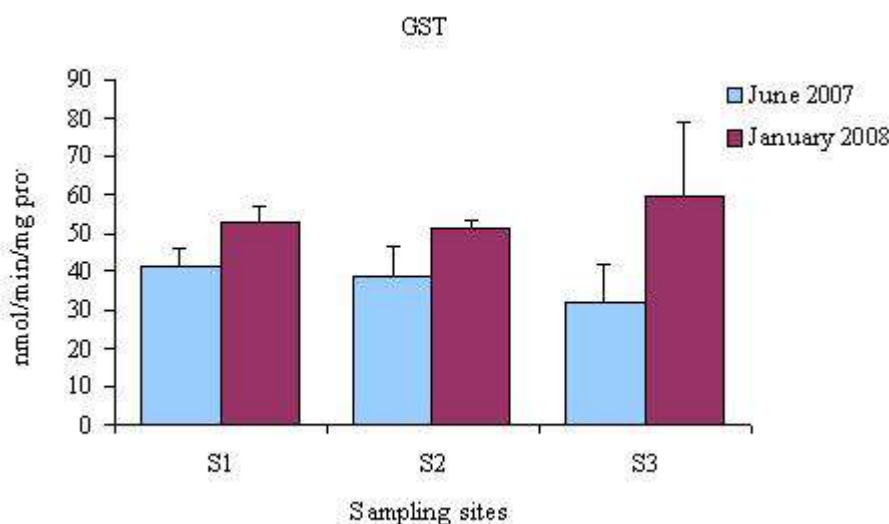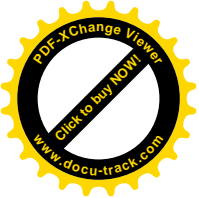
Figure 5: GST activity levels (nmol min^{-1} mg prot^{-1} ± s.d.) in *M. barbatus* in three different sites and two sampling periods.

vey EROD activities were significantly different between sizes in site 1 (p ≤ 0.01) but not in site 2 (p=0.06). The number of samples in site 3 was insufficient for comparison.

The results of UDPGT and GST activities are presented in Figures 4 and 5, respectively. The UDPGT activities ranged between 2 and 4.4 nmol min^{-1} mg prot^{-1}, and they did not show significant differeces between sampling sites (S1: p=0.84, S2: p=0.55, S3: p=0.72). There were no significant differences between the levels of UDPGT activity in the summer and winter sample period, either in site 1 (p=0.85), 2 (p=0.19) or 3 (p=0.10). Comparing the different size classes, no significant difference was found in any of the three sites (S1: p=0.84, S2: p=0.55; S3: p=0.72). Comparing the UDPGT activity for the two size classes in the two sampling periods and in different sites, there was no significant difference either in the first year (S1: p=0.66, S2: p=0.52, S3: p=0.62), nor in the second (S1: p=0.90, S2: p=0.22, S3: insufficient number of samples for comparison).

The GST activities ranged between 32.2 to 59.4 nmol min^{-1} mg prot^{-1} and revealed significant seasonal differences (p<0.001), but no differences between sites (p=0.64). No significant difference between size classes was found in any of the three sites (S1: p=0,31; S2: p=0,31; S3: p=0,62). Comparing the GST activity for size classes in the two sampling periods and in different sites, there was no significant difference either in the first year (S1: p=0.23, S2: p=0.10, S3: p=0.30), nor in the second (S1: p=0.87, S2: p=0.26, S3: insufficient number of samples for comparison).
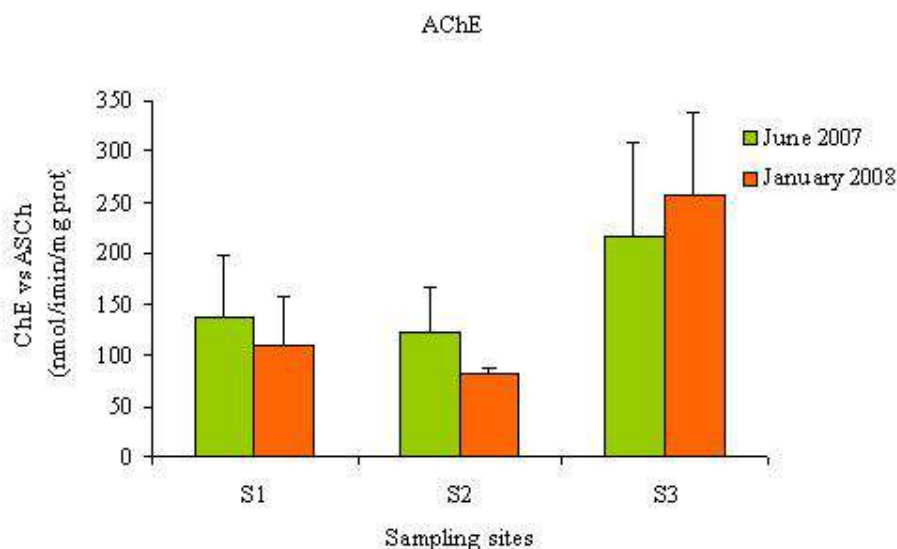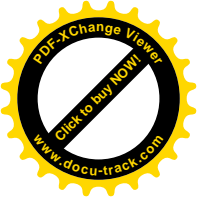
Figure 6: ChE vs ASCh activity levels (nmol min^{-1} mg prot^{-1} $\pm$ s.d.) in *M. barbatus* expressed in three different sites and two sampling periods.

The results of the analysis of acetylcholinesterase activity of *M. barbatus* are shown in Figure 6. Inhibition of AChE was significantly greater in site 1 and 2 (Gulf of Patti) both in first (p¡0.05) and in the second survey (p¡0.01) than in the site 3 (Milazzo refinery). No significant difference was observed between the AChE activities of the specimens collected from these two sites, either in the June (p=0.92) nor in January (p=0.70).

Regarding the comparison of data for the two surveys, there were no significant differences in this enzyme activity between summer and winter survey, either in site 1 (p=0.42) or site 2 (p=0.13) or site 3 (p=0.53).

Regarding the comparison between size classes, it wasn't significant difference in any of the three sites (S1: p=0.30; S2:

p=0.76; S3: p=0.46). Comparing the AChE activities for the two classes of size in the two sampling periods and in different sites, there was no significant difference either in the first (S1: p=0.64, S2: p=0.85, S3: p=0.68), nor in the second survey (S1: p=0.35, S2: p=0.14, S3: insufficient number of samples for comparison).

Vitellogenin gene was found to be expressed only in a specimen of 16 cm taken from site 3 in summer survey (Figure 7).

All the examined individuals from the three sites had testes at maturity stages III and IV, in both summer and winter samplings (Figures 8 and 9). None of the examined specimens showed any signs of histopathological alterations, such as necrosis, degeneration, feminization, and/or delayed or arrested gamete development.
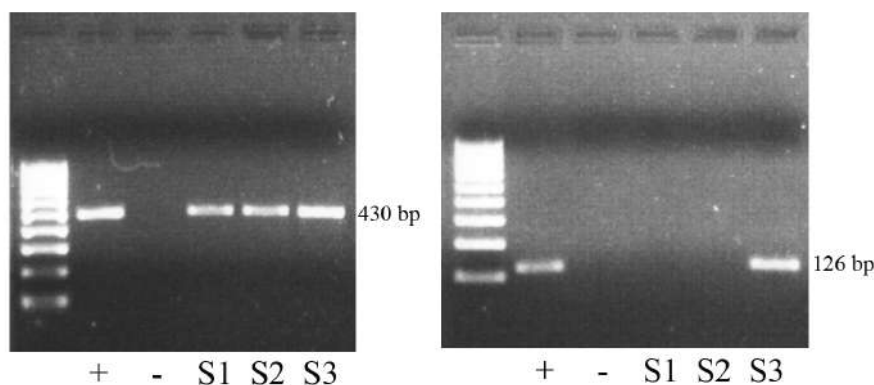
Figure 7: Vitellogenin gene expression in a male from S3 site (right). On the left, expression of the housekeeping gene (β-actin). + and - indicate respectively the positive and negative control. A marker of 100 bp was used.

4 Discussion and conclusions

The use of biological markers, measured at a molecular or cellular level, has been proposed in the last ten years as a sensitive early warning tool for the measurement of biological effects in environmental quality assessment [41]. The analysis of induction of cytochrome P450 enzyme activities in fish is one of the most effective biomarkers in the measurement of environmental quality, and several studies of environmental monitoring have found an induction of the activities of this multi-enzyme complex in relation to presence of anthropogenic sources of pollution, such as industries, urban sewage and harbours.

It is known that the mixed-function monooxygenase is induced by PAHs, but also from other compounds, such as polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans and PCB congeners in many species of fish [42, 43, 44, 45, 4, 46, 47].

In the present study, the highest EROD activity was measured in both summer and winter surveys in samples from site 3 (Milazzo), with values comparable to those reported by Porte et al. [28] in an area influenced by both urban and industrial wastes from Marseilles (about 650 pmol/min/mgprot). In fact, the Milazzo area is known as one of the most contaminated in the Mediterranean sea [24], being characterized by continuous discharges of pollutants deriving from oil processing., as well as from ship traffic, manoeuvring for loading and unloading of oil tankers and discharging of ballast waters. Moreover, a little further east of the oil refinery, the river Niceto flows, another possible source of contaminants inducing CYP450. Contamination by PAHs and heavy metals has subsequently been reported in sediments from the same area [48].
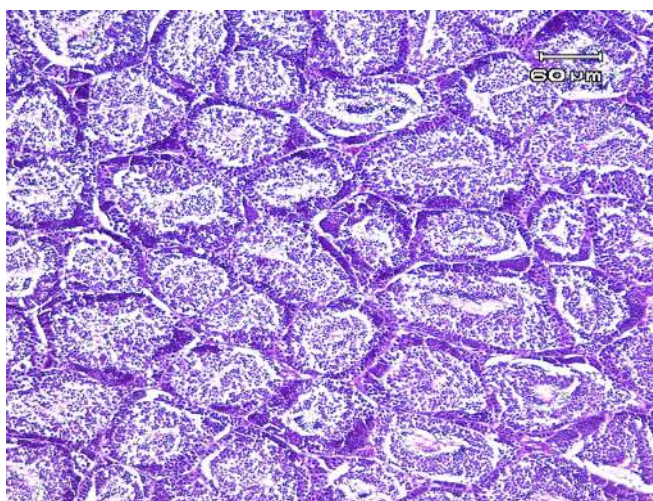
Figure 8: Histological section of a *M. barbatus* testis at stage III (maturing).

Sites 1 and 2 showed EROD activities significantly lower than site 3, but they were however moderately impacted, because the values in these sites are still high, compared to those reported in the literature from Burgeot, [24] (4.9-19.4 pmol/min/mg prot) and Corsi [25] (2.5-17.5 pmol/min/mg prot) for areas not industrialized. There were no significant differences between these two sites, probably because, although distant from each other (7 Km), both fall within the Gulf of Patti. This closed area, as reflected in the data provided by stations of the Air Weather Service of Messina, is characterized by winds coming mainly from the northwestern quadrant, which at low depth (up to 100 meters), are responsible of the South-East currents insisting on the coast. This causes a movement of water within the Gulf of Patti that goes from site 1 to site 2.

It is known that gonad activity may influence P450 cytochrome activity in females, as demonstrated by EROD activity decreasing [49], or being totally inhibited [50] during the spawning season. For this reason, enzyme activities were assayed in males, and, as expected, they did not show any significant seasonal differences. On the other hand, the size of the fish seems to have an influence on EROD activity, with significantly higher values in larger specimens (¿17 cm). These are older individuals (¿ 1 year), which may have been exposed to contaminants for a longer time, with consequent increase of detoxificating activity.

GST and UDPGT enzymes, involved in phase II detoxification process, are sensitive to exposure to PAHs [51, 52]. Although GST and UDPGT activities are used as biomarkers in environmental monitoring programs, these indicators are less sensitive than the enzyme activity of phase I. From the results obtained in this study the GST and UDPGT activity appeared lower than those reported in the literature by Regoli et al. [21] and Burgeot et al. [24]. In particular, UDPGT did not change
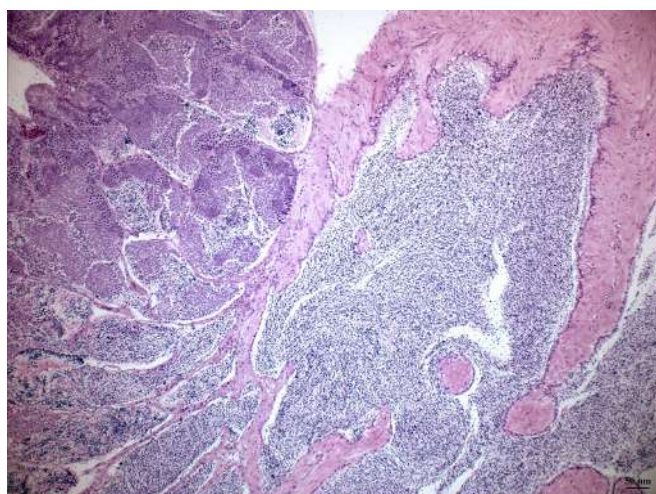
Figure 9: Histological section of a *M. barbatus* testis at stage IV (running).

significantly either between sites or seasons, unlike GST, which appeared to be affected by seasonality.

A significant inhibition of cholinergic enzyme AChE was recorded in brain tissue of red mullets in site 3, with values around 250 nmol/min/mg prot. AChE activity showed no significant differences between sites 1 and 2, where there have been similar values to those reported by Lionetto et al. [26] (70-130 nmol/min/mg prot) in harbour areas. More than organophosphate and carbamate insecticides, the AChE can also be inhibited by heavy metals, which could explain the values so high (almost twice) of this enzyme activity in site 3 highly industrialized and adjacent to the harbour of Milazzo. The results may suggest the exposure of red mullets to the AChE inhibitor compounds in sediments in front of the refinery and the harbour area of Milazzo.
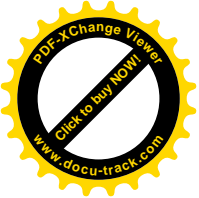
Most studies on the aquatic environment have evaluated the content of specific endocrine disruptors in surface waters [53,

54], in effluents from sewage treatment of civilian discharges [55, 56] and industrial discharges. Martin-Skilton et al. [14] investigated gonadal changes caused by endocrine disruption in *M. barbatus* in the North-West Mediterranean. These Authors reported delayed gamete maturation, intersexuality and fibrosis in fish from a highly impacted area, near the city of Marseille. These phenomena were more evident during the reproductive period (spring). In our study, however, although biomolecular analysis revealed the induction of vitellogenin gene in a single specimen from the Milazzo site, suggesting exposure to xenoestrogens, histology showed no pathological changes of the gonads, such as fibrosis or feminization, or inhibition of gamete maturation. Therefore, the reproductive potential of these specimens was not affected by exposure to contaminants. Histological analysis of female gonads is currently under way in order to validate further the results obtained so far.
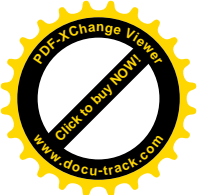
References

[1] A. Aguilar and A. Borrell. Abnormally high polychlorinated biphenyl levels in striped dolphins (Stenella coeruleoalba) affecte by the 1990-1992 Mediterranean epizootic. 1994.

[2] A. Borrell, A. Aguilar, and T. Pastor. Organochlorine compound levels in striped dolphins from the western Mediterranean during the period 1987-1993. 1996.

[3] M.C. Fossi. Strumenti di diagnosi e prognosi ambientali. 2000.

[4] A. Goksøyr and L. Förlin. The cytochrome P450 system in fish, aquatic toxicology and environmental monitoring. 1992.

[5] A. Goksøyr. Use of cytochrome P4501A (CYP1A) in fish as a biomarker of aquatic pollution. 1995.

[6] D.R. Livingstone. Cytochrome P450 in pollution monitoring. Use of cytochrome P4501A (CYP1A) as a biomarker of organic pollution in aquatic and other organisms. 1996.

[7] E. Stephensen, J. Svavarsson, J. Sturve, G. Ericson, M. Adolfsson-Erici, and L. Förlin. Biochemical indicators of pollution exposure in shorthorn sculpin (Myoxocephalus scorpius), caught in four harbours on the southwest coast of Iceland. 2000.

[8] M. Fulton and P.B. Key. Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. 2001.

[9] T.S. Gill, H. Tewari, and J. Pande. Use of the fish enzyme system in monitoring water quality: effects of mercury on tissue enzymes. 1990.

[10] A. Sturm, H.C. da Silvia de Assis, and P.D. Hansen. Cholinesterases of marine teleost fish: enzymological characterisation and potential use in the monitoring of neurotoxic contamination. 1999.

[11] L.L. Johnson, S.Y. Sol, D.P. Lomax, G.M. Nelson, C.A. Sloan, and E. Casillas. Fecundity and egg weight in English sole, Pleuronectes vetulus, from Puget Sound, Washington: influence of nutritional status and chemical contaminants. 1997.

[12] L.L. Johnson, D. Misitano, S.Y. Sol, G.M. Nelson, B. French, G.M. Ylitalo, and T. Hom. Contaminant effects on ovarian development and spawning success in rock sole from Puget Sound, Washington. a, 1998.

[13] S. Jobling, N. Bersford, M. Nolan, T. Rodgers-Gray, et al. Altered sexual maturation and gamete production in wild roach (Rutilus rutilus) living in rivers that receive treated sewage effluents. 2002.

[14] R. Martin-Skilton, R. Lavado, R. Thibaut, C. Minier, and C. Porte. Evidence of endocrine alteration in the red mullet, Mullus barbatus from the NW Mediterranean. 2006.

[15] D.E. Kime. Endocrine Disruption in Fish. 1998.

[16] G. De Metrio, A. Corriero, S. Desantis, D. Zubani, F. Cirillo, et al. Evidence of a high percentage of intersex in the Mediterranean swordfish (Xiphias gladius, L.). 2003.

[17] M. Penaz, Z. Svobodova, V. Barus, M. Prokes, and J. Drastichova. Endocrine disruption in a barbel, Barbus barbus population from the River Jihlava, Czech Republic. 2005.

[18] M.C. Fossi, S. Casini, L. Marsili, S. Ancora, G. Mori, G. Neri, T. Romeo, and A. Ausili. Evaluation of ecotoxicological effects of endocrine disrupters during a four-year survey of the Mediterranean population of swordfish (Xiphias gladius). 2004.

[19] D.E. Kime, J.P. Nash, and A.P. Scott. Vitellogenesis as a biomarker of reproductive disruption by xenobiotics. 1999.

[20] A. Canapa, M. Barucca, A. Celeste, E. Olmo, and F. Regoli. Preliminary investigations on vitellogenin m-RNA induction in some bioindicator Mediterranean fish species. 2002.

[21] F. Regoli, G. Frenzilli, R. Bocchetti, F. Annarumma, V. Scarcelli, D. Fattorini, and M. Nigro. Time-course variations of oxyradical metabolism, DNA integrity and lysosomal stability in mussels, Mytilus galloprovincialis, during a field translocation experiment. 2004.

[22] 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. 2000.

[23] T. Burgeot, G. Bocquènè, G. Pingray, D. Godefroy, J. Legrand, et al. Monitoring of biological effects of contamination in marine fish along French coasts by measurement of ethoxyresorufin-O-deethylase. 1994.

[24] T. Burgeot, G. Bocquene, C. Porte, J. Dimeet, R.M. Santella, et al. Bioindicators of pollutant exposure in the northwestern Mediterranean Sea. 1996.

[25] I. Corsi, M. Mariottini, C. Sensini, V. Menchi, C. Balocchi, and S. Focardi. Monitoring a marine coastal area: use of Mytilus galloprovincialis and Mullus barbatus as bioindicators. 2002.
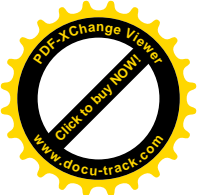
[26] M.G. Lionetto, R. Caricato, M.E. Giordano, M.F. Pascariello, L. Marinosci, and T. Schettino. Integrated use of biomarkers (acetylcholinesterase and antioxidant enzymes activities) in Mytilus galloprovincialis and Mullus barbatus in an Italian coastal marine area. 2003.

[27] C. Porte and J. Albaigés. Bioaccumulation patterns of hydrocarbons and polychlorinated biphenyls in bivalves, crustaceans and fishes. 1993.

[28] C. Porte, E. Escartin, L.M. García de la Parra, X.J. Biosca, and L. Albaigés. Assessment of coastal pollution by combined determination of chemical and biochemical markers in Mullus barbatus. 2002.

[29] UNEP/WHO. Identification of priority pollution hot spots and sensitive areas in the Mediterranean. 1999.

[30] P. Bougis. Reserches biometriques sur les rougets (Mullus barbatus L., Mullus surmuletus L.). 1952.

[31] Z. Haidar. Ecologie du rouget barbet (Mullus barbatus L.) en Adriatique Orientale. 1958.

[32] C. Leonzio, E. Bacci, S. Focardi, and A. Renzoni. Heavy metals in organisms from the northen Tyrrhenian Sea. 1981.

[33] V. Filipović Marijić and B. Raspor. Metallothionein in intestine of red mullet, Mullus barbatus as a biomarker of copper exposure in the coastal marine areas. 2007.

[34] I. Corsi, M. Mariottini, C. Sensini, L. Lancini, and S. Focardi. Cytochrome P450, acetylcolinesterase and gonadal histology for evaluating contaminant exposure levels in fishes from a highly eutrophic brackish eco system: the Orbetello Lagoon, Italy. 2003.

[35] M.D. Burke and R.T. Mayer. Ethoxyresorufin: Direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. 1974.

[36] A. Aitio. UDP glucuronyltransferase of human placenta. 1974.

[37] A. Collier, M. Tingle, J. Keelan, J. Paxton, and M. Mitchell. A highly sensitive fluorescent microplate method for the determination of UDP-glucuronosyl transferase activity in tissues and placental cell lines. 2000.

[38] W.H. Habig, M.J. Pabst, and W.B. Jakoby. Glutathione S-Transferases: the first enzymatic step in mercapturic acid formation. 1974.

[39] G.L. Ellman, K.D. Courtney, V. jr Andres, and R.M. Featherstone. A new and rapid colorimetric determination of acetylcholinesterase activity. 1961.
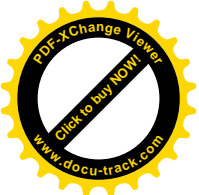
[40] M.M. Bradford. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. 1976.

[41] F. McCarthy and L.R. Shugart. Biomarkers of Environmental Contamination. 1990.

[42] J.F. Payne and W.R. Penrose. Induction of aryl hydrocarbon (benzo(a)pyrene) hydroxylase in fish by petroleum. 1975.

[43] J.R. Bend. Estimation of induction of enzymes that metabolize xenobiotics in vitro and in vivo. 1994.

[44] C.N. Statham, C.R. Elcombe, S.P. Szyjka, and J.J. Lech. Effects of polycyclic hydrocarbons on hepatic microsomal enzymes and disposition of methylnaphtalene in rainbow trout in vivo. 1978.

[45] J.J. Stegeman and J.J. Lech. Cytochrome P450 monooxygenase systems in aquatic species: carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. 1991.

[46] A.B. Okey. Enzyme induction in the cytochrome P-450 system. 1990.

[47] J.J. Stegeman and D.R. Livingstone. Form and functions of cytochrome P450. 1998.

[48] ARPA Sicilia. Monitoraggio delle Acque Marino-Costiere 2003-2004. 2004.

[49] L. Förlin and C. Haux. Sex differences in hepatic cytochrome P-450 monooxygenase activities in rainbow trout during an annual reproductive cycle. 1990.

[50] L. Förlin and T. Hansson. Effects of oestradiol-17β and hypophysectomy on hepatic mixed function oxidases in rainbow trout. 1982.

[51] R. Van der Oost, J. Beyer, and N.P.E. Vermeulen. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. 2003.

[52] D.R. Livingstone and P.S. Goldfarb. Biomonitoring in the acquatic environment: use of cytochrome P4501A and other molecular biomarkers in fish and mussels. 1998.

[53] N. Garcia-Reyero, E. Grau, M. Castillo, M.J. Lopez de Alda, D. Barcelo, and B. Pina. Monitoring of endocrine disruptors in surface waters by the yeast recombinant assay. 2001.

[54] H.E. Witters, C. Vangenechten, and P. Berckmans. Detection of estrogenic activity in Flemish surface waters using an in vitro recombinant assay with yeast cells. 2001.

[55] H.M. Kuch and K. Ballschmiter. Determination of endogenous and exogenous estrogens in effluents from sewage treatment plants at the ng/L-level. 2000.

[56] F. Tilton, W.H. Benson, and D. Schlenk. Evaluation of estrogenic activity from a municipal wastewater treatment plant with predominantly domestic input. 2002.