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# A multibiomarker approach in *Coris julis* living in a natural environment

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ABSTRACT

To monitor the health of aquatic organisms, biomarkers have been used as effective tools in assessing environmental risk. In this study was examined the teleost *Coris julis*, sampled in two marine sites in Messina (Italy) at different pollution degree, Milazzo, characterized by a strong anthropogenic impact, and Marinello, the natural reserve. *C. julis* is a species particularly suitable to biomonitoring because its feeding habits favor bio-accumulation of xenobiotics.

The following biomarkers were used to estimate the impact of highly persistent pollutants: cellular localization of cytochrome P4501A (CYP1A) and glutathione-S-transferase (GST) in the liver, their hepatic expression at the mRNA level, the enzymatic activity (EROD and BPMO), the micronucleus and comet assays in the blood, esterases (AChE in the brain and BChE in the blood) activity and evaluation of PAH metabolites in the bile.

The present findings provide evidence of statistically significant differences in parameters between individuals collected in two sites.

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## 1. Introduction

Evaluating the impact of anthropogenic contamination on a population can be difficult for a variety of reasons, including the different bioavailability of contaminants in relation to their location and the different biochemical and toxicological interactions that may exist (McCarthy and Shugart, 1990).

Aquatic environments, especially marine coastal areas and brackish inland waters, are subjected to strong impact by a variety of human activities. Faced with the growing demand to reconcile development needs with conservation, it is important to extend the use of techniques based on biological responses, as a tool for assessing the quality of the environment.

The benthic organism used as bioindicator in this study was *Coris julis* (Osteichtyes, Perciformes, the rainbow wrasse), which belongs to the family Labridae. This species is particularly suitable for this study because of its sedentary lifestyle and feeding habits favor bio-accumulation of xenobiotic compounds (Chiea et al., 2002; Fasulo et al., in press). *C. julis* feeds on zoobenthic organisms such as molluscs and benthic crustaceans (Sureda et al., 2006). It plays a key role in the food chain of the study area and thus can provide information about this environment.

*C. julis* was collected from two coastal areas of the northwest Mediterranean. The first is adjacent to a large industrial zone (Milazzo, Messina, South Italy), and the second lies in front of the natural reserve of Marinello, Messina (South Italy, Sicily Region, 2002).

The Milazzo area has been described as an industrial polluted zone of the north-eastern Sicily and its environmental risk have been highlighted (Dongarrà et al., 2003; Caruso et al., 2004; Yakimov et al., 2005). The area is strongly affected by anthropogenic activities, and it is an object of interest to the Italian government, which has declared it to be a "high environmental risk area" (Di Battista et al., 2005).

The Gulf of Milazzo is subjected to contamination by hydrocarbons caused by marine oil transport (Caruso et al., 2004), pipe/tanker accidents, dumping of tanker ballast water and petroleum run-off from the land (Yakimov et al., 2005).

This study highlights the importance of using a molecular, enzymatic and genotoxic biomarkers battery, as CYP1A, GST, micronucleus and Comet assay, esterases and PAHs metabolites, to assess contaminant exposure and its effects.

CYP1A belongs to a subfamily of the cytochrome P450-dependent monooxygenase enzymes (CYPs), and it is responsible for phase I of the metabolism of xenobiotics including dioxins, furans, polychlorinated biphenyls, polyaromatic hydrocarbons (PAHs) and DDT (Stegeman and Hahn, 1994; Goksøyr, 1995; Parkinson, 1995; Husøy et al., 1996; Jeong and Kim, 2002).

Phase I and II enzymes may also transform lipophilic compounds in bioactivated forms (McKinney et al., 2004). CYP1A, through this metabolic process, converts compounds from a fairly

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hydrophobic to more hydrophilic form followed by excretion of the modified chemical.

In fish species, the high sensitivity of two enzyme reactions catalyzed by specific CYP1A-isoforms, EROD (7-ethoxyresorufin-O-deethylase) and BPMO (benzo[a]pyrene monooxygenase), to exposure to toxic pollutants has been demonstrated in various studies, and their induction is currently used in many biomonitoring projects for the assessment of water quality and chemical pollution (Van der Oost et al., 2003).

GSTs are a family of multifunctional proteins involved in the cellular detoxification of xenobiotic compounds: they play a fundamental role in protection against endogenous and exogenous toxic chemicals (Sheehan et al., 2001). GSTs belong to the phase II enzyme family and they catalyze the conjugation of electrophilic metabolites with carrier molecules such as glutathione and glucuronic acid.

Vertebrates have two types of cholinesterase (ChEs) that differ in their substrate specificity: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). AChE hydrolyzes acetylcholine faster compared to other choline esters and is much less active towards butyrylcholine. In contrast, BChE is highly efficient at hydrolyzing both butyrylcholine and acetylcholine. In fishes, AChE is predominant in brain and muscle tissues, whereas BChE is present mostly in the liver and plasma (Habig and Di Giulio, 1991). ChEs inhibition is widely used as a biomarker due to its specificity to organophosphorus and carbamate compounds (Thompson, 1991; Galgani and Bocquene, 2000). In this study, the brain was used to determine the AChE activity, while the blood to BChE. Additionally, other studies have shown that ChEs are altered by PAHs, some heavy metals, and surfactants (Gill et al., 1990; Payne et al., 1996; Guilhermino et al., 2000; Moreira et al., 2004; Moreira and Guilhermino, 2005).

In this study, PAH metabolites in the bile were evaluated as well. PAH metabolites are usually determined in fish bile, where they are concentrated and stored prior to excretion. Biliary PAH metabolite analysis provides information about the actual exposure of fish to PAH compounds and reveals the state and suitability of the marine environment for fish (Vuorinen et al., 2006).

Micronuclei are formed by chromosome fragments or whole chromosomes, that lag at cell division due to the lack of a centromere, or to damage or a defect in cytokinesis (Heddle et al., 1991). In recent years, several researchers have used the formation of morphological nuclear alterations in erythrocytes of fishes as possible indicators of genotoxicity (Çavaş & Ergene-Gözükara, 2005a, 2005b; Da Silva Souza and Fontanetti, 2006; Ergene et al., 2007). The Comet assay, also known as single cell gel electrophoresis, is a microgel electrophoresis technique that detects DNA damage in individual cells (Tice et al., 2000).

The fish liver is a key organ that controls many life functions and plays a prominent role in the general anabolism and catabolism of an individual as well as in the metabolism of xenobiotics. Thus, the fish liver can be a good indicator of the health status of fish (Bowser et al., 1990; Vethaak and Rheinallt, 1990; Biagianti-Risbourg, 1992).

The aim of this study was to assess the responses of *C. julis* to persistent organic contaminants, through the use of the battery of biomarkers mentioned above for biomonitoring selected marine environments, for which samples analyzed were water and sediments.

## 2. Materials and methods

### 2.1. Sampling sites

The Gulf of Milazzo is a wide natural bay characterized by low water exchange that runs along 15 km of the north-eastern coast of Sicily. Milazzo is characterized by heavy industrialization, dense urbanization and tanker traffic from ships transporting crude and refined oil to and from the refinery located at this site (Yakimov et al., 2005). In contrast, the lagoon and coastal system of Marinello constitutes a wildlife reserve in the Sicilian region that covers an area of 401.25 ha: 248.13 ha are an integral reserve and 153.12 ha are a pre-reserve. This reserve is unique in that it contains many different environments within a small area.

### 2.2. Sampling and analysis of water and sediments

Surface and bottom water samples were collected in polythene bottles, and carried to the laboratory under refrigeration. Temperature, pH, conductivity and dissolved oxygen (DO) were measured in the field by a portable instrument (Multi 340i/SET, WTW Wissenschaftlich, Weilheim, Germany). Sediment samples were collected with a stainless steel grab sampler. The top 5 cm oxic layer of the sediment was scooped with a plastic spoon, stored in double-layer polythene bag, and kept at 4 °C for physicochemical analysis.

The water and sediment samples were collected every two months. In the laboratory, the water samples were analyzed for various physicochemical parameters following standard methods (Grasshoff et al., 1983; APHA, 1995). Nutrients were estimated by colorimetric methods from samples that were filtered through 0.45 μm Millipore membrane filter paper. All colorimetric estimations were performed using a spectrophotometer (Filterphotometer PF-11 MN, Macherey-Nagel GmbH and Co. KG–Düren, Germany).

The extraction of PAHs from sediments was conducted using conventional liquid solvent extraction techniques such as the Soxhlet extraction (Schantz et al., 1990). The extracts were filtered through a pre-cleaned Pasteur pipette filled with solvent-rinsed glass wool and pre-cleaned anhydrous $Na_2SO_4$, previously rinsed with dichloromethane and concentrated in a rotary evaporator with thermostatic bath at $T$=35 ((0.5) °C. The final volume was around 2 ml. The last stage in the procedure involved drying the solution containing PAHs under a weak nitrogen flow at room temperature. The dry residue was dissolved in 1 ml solution containing the following perdeuterated internal standard in cyclohexane (0.2 mg/L each): acenaphtene d10, phenanthrene d10, chrysene d12 and perylene d12.

Qualitative and quantitative determinations were carried out using a gas chromatograph (Shimadzu mod. GC-17 A, Milano, Italy) coupled with a mass spectrometer (Shimadzu, quadrupole detector mod. GCMS-QP5000) equipped with an acquisition data system (Shimadzu, CLASS 5000).

### 2.3. Sampling of C. julis

The sampling of *C. julis* was conducted monthly from April to September 2005. The specimens were collected in both areas at a depth of about 20 m, using a bow-net placed on the seabed and recovered after 30 min. All organisms collected were carried to the laboratory alive in a large container filled with oxygenated seawater, and then they were anesthetized with 1–2 g/l of MS222 (ethyl-ester-3-amino-benzoic acid, Sigma, Saint Louis, Missouri, US) and sacrified by decapitation.

Thirty specimens from Milazzo and 30 from Marinello were sampled each month. In total, from each site 180 individuals of the same size and weight class (mean size of $12 \pm 1$ cm length and $10 \pm 1$ g weight), and same sex (females) and sexually mature, were selected to standardize the sampling. Each month 10 of the 30 specimens were used for histology and immunohistochemistry, 10 for molecular analysis and the last 10 for analysis of enzyme activities.

The fish-killing method used in this study followed the guidelines of animal care and experimentation in compliance with the Italian National Bioethics Committee (INBC).

Liver and brain samples were preserved at −80 °C.

Liver samples were fixed in a solution of 4% paraformaldehyde in phosphate buffered saline (PBS) 0.1 M, pH 7.4, dehydrated in increasing ethanol concentrations and embedded in Paraplast (Bio-Optica, Milano, Italy).

Blood samples (1 ml) from fasting fish were drawn from the caudal vein into heparin vials and stored at −80 °C until required for analysis. For micronuclear assay 40 μl of blood was smeared on to pre-cleaned slides.

Bile was collected by piercing the exposed gall bladder wall with a needle fitted to a 1 ml disposable syringe, drawing bile into the syringe and emptying it into a 1 ml dark glass vial, and stored at −80 °C.

### 2.4. Histology and immunohistochemistry

Sections (5 μm thick) were processed for morphological staining using haematoxylin/eosin (H/E, Bio-Optica) (Mazzi, 1977).

Sections (5 μm thick) were prepared from paraffin-embedded tissues. The sections were treated using the indirect immunofluorescence method (Mauceri et al., 1999). Non specific binding sites for immunoglobulins were blocked by incubations for 1 h with normal goat serum (NGS) in PBS (1:5). The sections were incubated overnight in a humid chamber at 4 °C with the primary rabbit polyclonal antibody anti-CYTP450 1A1 (Abcam, Cambridge, UK) diluted 1:100

and with a rabbit polyclonal antibody anti-GST (Sigma, Saint Louis, Missouri, US) diluted 1:50.

After a rinse in PBS for 10 min, the sections were incubated for 2 h at room temperature with Fluorescein Isothiocyanate (FITC) conjugated goat anti rabbit IgG (Sigma).

Negative controls for immunohistochemical labeling were performed by substitution of non-immune sera for the primary or secondary antisera. Specificity of the labeling of some peptides was verified by incubating sections with antiserum preabsorbed with the respective antigen (10–100 g/ml). The preabsorption procedures were carried out overnight at 4 °C.

All observations were made with a motorized Zeiss Axio Imager Z1 microscope (Carl Zeiss AG, Werk Göttingen, Germany), equipped for the acquisition of images taken with the AxioCam camera (Zeiss, Jena, Germany).

### 2.5. RNA extraction and polymerase chain reaction (PCR)

Total RNA was extracted from the liver of specimens using TRIzol LS reagent (Invitrogen, Carlsbed, California, US) (Chomczynski and Sacchi, 1987). The RNA content was quantified using UV spectrophotometer (UV Mini 1240 Shimadzu, Milano, Italy).

The cDNA was synthesized using 4 μg of total RNA and oligo (dt)20 primer (150 pmol/μl) (Invitrogen), with MMLV reverse transcriptase (Invitrogen) as prescribed by the manufacturer's instructions. 2 μl of the resulting cDNA were amplified in the PCR reaction.

The sequences of primers were based on the CYP4501A and GST conserved regions known for Teleosts and were as follows: CYP1A sense primer: 5′-GATCA-CTGTGAGGACAGGAA-3′ and CYP1A1 antisense primer: 5′-TGCCACTGATTGATG-AAGAC-3′; GST sense primer: 5′-TGCTGTGGGGCTCCGGC-3′ and GST antisense primer: 5′-CTCCAGCCAGGTAGGAGGCC-3′.

The actin gene of each examined organism was used as positive control; the gene was amplified using sequence primers based on the actin cDNA sequence of *C. julis* to obtain a fragment of 400 bp sequence.

PCR was prepared using 2.5 μl of 10 × buffer, 0.13 μl of 5 U/μl Poly Taq polymerase (Invitrogen), 0.8 μl of 50 mM $MgCl_2$, primers (50 μM each), 1 μl of cDNA template, 0.5 μl of 10 mM dNTPs, and Milli-Q water (Millipore, Vimodrone MI, Italy). The total reaction was performed in a 25 μl volume. The program used to amplify fragments of cytochrome P450 1 A was 95 °C for 2 min and 35 cycles at 95 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 5 min.

To amplify fragment of GST the annealing temperature was 56 °C for 30 s, and the extension temperature was 72 °C for 1.5 min.

To perform the reaction Ep-Gradient the Mastercycler (Eppendorf, Milano, Italy) was used.

### 2.6. Cloning and sequencing of PCR products

The PCR products amplified from the liver of *C. julis*, and not yet present in the Genbank database, were separated on 1% agarose gels and purified from the gel using QIAquick (Qiagen, Milano, Italy) following the manufacturer's protocol. The products were ligated into the plasmid pGEM-T Easy Vector (Promega, Madison WI, US).

Ligations were carried out in a 10 μl reaction volume using 1 μl T4 DNA Ligase (NEB, Celbio s.p.a., Pero MI, Italy), 1 μl buffer 10 ×, 50 ng vector and 30 ng fragment. The reaction mixture was incubated overnight at 4 °C. The products of ligation were transformed into *E. coli* JM109 by heat shock at 42 °C for 50 s following the exact protocol of the pGEM-T Easy Vector (Promega). Transformed cells were plated on LB plates supplemented with ampycilline (100 mg/ml), X-Gal (80 mg/ml) and IPTG (0.5 mM). Plasmid DNA was purified using the GenElute Plasmid miniprep kit (Sigma, Saint Louis, Missouri, US), and sequenced, using universal M13fw and M13rv primers and the ABI PRISM BigDye Terminator 3.1 Cycle Sequencing kit (PE Applied Bio-system, Monza MI, Italy).

### 2.7. Enzymatic activities

#### 2.7.1. EROD and BPMO activities

Livers were homogenized in 0.1 M sucrose buffer (pH=7.5) at a tissue weight/buffer volume ratio of 1:5. Homogenates were then centrifuged at 9000*g* for 20 min at 4 °C to obtain a post-mitochondrial supernatant (PMS), which was used as a source of enzyme for the monooxygenase assay as proposed by O'Hare et al. (1995).

EROD activity was determined following Lubet et al. (1985) and was expressed as pmol resorufin/min/mg microsomal protein.

A final volume of 2.5 ml containing 50 mM Tris–HCl buffer at pH=7.5 and 25 mM MgCl2 was used, and the ethoxyresorufin was incubated at 20 °C. The reaction began with the addition of NADPH (10 mM), and the activity was followed by fluorescence measurements at an excitation wavelength of 522 nm and an emission wavelength of 586 nm.

BPMO activity was measured following Kurelec et al. (1977) using 100 μl of PMS and incubating the reaction mixture for 1 h and the activity was expressed as F.U./h/mg microsomal protein (F.U.=fluorescence unit). Fluorescence measurements were performed to an excitation wavelength of 396 nm and an emission wavelength of 522 nm.

Total protein content in PMS fraction were measured by the Bio-Rad Protein Assay (Bio-Rad, Segrate MI, Italy) using BSA as a reference standard.

Fluorescent aromatic compounds (FACs) in the bile were determined in fish exposed to refinery effluent by fixed wavelength fluorescence measurements at specified excitation/emission wavelength pairs for selected PAH metabolites (Krahn et al., 1987, 1993; Ariese et al. 1993).

The spectrofluorimetric assays were carried out using a Perkin Elmer LS 55 luminescence spectrometer (Perkin Elmer LAS, Beaconsfield, UK).

#### 2.7.2. Esterase activities

Esterase activity was evaluated, respectively, in the brain for AChE and in the blood for BChE, as known from the bibliography that is more expressed in those tissues (Chuiko, 2000; Alpuche-Gual et al., 2008).

To measure AChE activity brain tissue was homogenized in 0,1 M TRIS/HCl 0.1% triton buffer (pH 8).

Homogenates were centrifuged at 1000*g* for 10 min at 4 °C to obtain a supernatant, which was used as a source of the enzyme.

AChE activity was determined according to the method of Westlake et al. (1981).

To measure BChE activity, blood samples were centrifuged at 1000*g* for 5 min and the supernatants were recovered for analysis. Serum BChE activity was determined colorimetrically by the Ellman method (1961).

The kinetics of the reactions for AChE and BChE were recorded for 5 min at 410 nm wavelength. Enzyme activity was expressed as μmol substrate/min/mg protein.

Measurements were conducted using a UV Mini 1240 (Shimadzu, Milano, Italy).

### 2.8. Genotoxicity analysis

#### 2.8.1. Micronuclear assay

The slides with the blood smear were fixed in pure methanol for 10 min, hydrated and then stained with 10% Giemsa solution for 15 min. A total of 2000 erythrocytes were examined for each specimen under the light microscope (Zeiss Axio Imager Z1), to determine the presence or absence of micronuclei or nuclear abnormalities.

#### 2.8.2. Comet assay

Due to the high quantity of alkali-labile sites in highly condensed chromatin of fish erythrocytes (Singh et al., 1989; Frenzilli et al., 1999), the assay was conducted at pH > 13 to detect single and double strand breaks. The alkaline Comet assay was performed as described by Singh et al. (1988) with some modifications. Blood samples were centrifuged at 2000 rpm for 5 min and pellets were resuspended in Phosphate Buffered Saline (PBS). 10 μl of the diluted sample were mixed with 65 μl of 0.7% low-melting-point (LMP) agarose then the 75 μl mixture was layered on the precoated slides on 1% normal melting point agarose. The slides were covered with a cover slip, and were left for 5 min in a refrigerator to solidify. The cover slip was gently removed and 75 μl of 0.7% low melting agarose were added and another cover slip was placed on top. The samples were left for another 5 min in the refrigerator. After removing the cover slip, the slides were placed in lysis buffer (2.5 M NaCl, 100 mM EDTA-Na, 10 mM Tris–HCl) for 2 h at 4 °C. After lysis the slides were placed in an electrophoresis box for 20 min in running buffer, to allow the unwinding to occur.

Electrophoresis was performed using the same running buffer at 20 V and 240 mA for 20 min. The slides were then neutralized with Tris buffer 0.4 M pH7.5 and stained with ethidium bromide (2.5 μl/ml).

The slides were examined with the Zeiss Axio Imager Z1 fluorescence microscope. To determine whether visual scoring correlated with computerized image analysis the same cells were also scored for DNA damage using the Comet assay IV software (Perceptive instruments, Suffolk, UK).

To quantify the induced DNA damage, we considered two parameters: the tail length and the tail moment (TM). TM is a measure of the migrated DNA in the tail multiplied by the tail length (Olive et al., 1990).

### 2.9. Statistical analysis

Immunoreactive cell quantification was performed by counting the positive cells using Axio Vision Release 4.5 software (Zeiss, Göttingen, Germany). The intensities of bands of CYP1A and GST were measured with Quantity One software (Bio-Rad, Marnes-la-Coquette, France), and FL WINLAB 4.00.02 software (Perkin Elmer, Norwalk, Conn., US) was used for EROD, BPMO and PAHs spectrofluorimetric analysis. All values obtained were analyzed statistically with Graph Pad software (Instat, La Jolla, CA, US) using one-way analysis of variance (ANOVA).

The significance level adopted throughout the study was $P<0.0001$ to immunohistochemical analysis, $P\leq 0.0005$ to molecular analysis, $P<0.0001$ to enzymatic activities and $P<0.05$ to comet assay.

## 3. Results

### 3.1. Analysis of water and sediments

During summer in Milazzo the highest water temperature at the surface is 26 °C and at 20 m deep is 16 °C; salinity is 39°/00 PSU (Practical Salinity Units) (Table 1). In Marinello, the highest surface water temperature is 24 °C and at 20 m is 16 °C; salinity is 37°/00 PSU (Table 1).

The mean of the concentration of benzo(a)pyrene, benzo(g,h,i)perylene, indeno(1,2,3-cd)pyrene, benzo(k)fluoranthene and benzo(b)fluoranthene in the sediments were present in both areas, but concentrations at the Milazzo sampling station were higher (Table 2).

**Table 1**
Physicochemical parameters of the Marinello and Milazzo environments during the summer season.

| Sampling area | Milazzo | Marinello |
|---|---|---|
| **Temperature °C** | 25.8 | 24 |
| **Conductivity (mS/cm)** | 56.6 | 57 |
| **Salinity (PSU)** | 39‰ | 37‰ |
| **Oxygen (mg/l)** | 5.0 | 6.15 |
| **pH** | 8.13 | 8.09 |
| **Ammonium 10 (mg/l)** | 0.1 | 0.6 |
| **Free chlorine (mg/l)** | 0.03 | 0.03 |
| **Total chlorine (mg/l)** | <0.1 | 0.07 |
| **Fluorine 2 (mg/l)** | <0.1 | <0.1 |
| **Total phosphate (mg/l)** | <0.1 | 0.1 |
| **Nitrites 2 (mg/l)** | <0.01 | <0.01 |
| **Nitrites (mg/l)** | <0.01 | <0.01 |
| **Orto phosphate (mg/l)** | <0.01 | 0.2 |
| **Potassium (mg/l)** | 27 | 27 |

**Table 2**
Concentration of PAHs expressed in μg/Kg of dry sample in sediments in Marinello and Milazzo during the summer season.

| | Milazzo | Marinello |
|---|---|---|
| ***acenaphtylene*** | 11.3 | 0 |
| ***acenaphthene*** | 10.4 | 4.5 |
| ***fluorine*** | 2.9 | 2.1 |
| ***phenanthrene*** | 12.2 | 5.9 |
| ***anthracene*** | 3 | 0.7 |
| ***fluoranthene*** | 26.7 | 12.6 |
| ***pyrene*** | 22.8 | 10.7 |
| ***1 methyl pyrene*** | 1 | 0.7 |
| ***benzo(a)anthracene*** | 26.9 | 9.3 |
| ***chrysene*** | 13.7 | 8.1 |
| ***benzo(b)fluoranthene*** | 20.9 | 7.1 |
| ***benzo(k)fluoranthene*** | 8.2 | 4.9 |
| ***benzo(a)pyrene*** | 17.1 | 6.5 |
| ***perylene*** | 0.4 | 0.6 |
| ***indeno(1,2,3-cd)pyrene*** | 5.9 | 3.7 |
| ***dibenzo(a,h)anthracene*** | 2.9 | 2 |
| ***benzo(g,h,i)perylene*** | 6.6 | 5.7 |

### 3.2. Histology and immunohistochemical analysis

Histomorphological analysis of liver tissue using dichromate staining revealed hepatic parenchyma with a homogeneous structure consisting of a system of cords of hepatocytes, in the specimens of *C. julis* collected from the control site. The biliary canals are located in a central position in each cell cord forming the bile ducts (Fig. 1A). In contrast, specimens sampled from Milazzo exhibited some alterations in tissues, dramatically increased vacuolation in the hepatocytes and melano-macrophage containing melanin were present (Fig. 1B). These macrophages are pigmented cells that can appear isolated or arranged in clusters to form melano-macrophage centers (MMCs). MMCs in *C. julis* were present as granular pigmented material (from yellow to dark brown in the specimens from Marinello and Milazzo, respectively) when stained with haematoxilin/eosin.

The immunohistochemical investigation of the liver of *C. julis* sampled from Milazzo revealed a significant anti-CYP1A1 immunopositivity in the perinuclear cytoplasm and diffuse staining throughout the peripheral cytoplasm. In contrast, intracellular spherical to ovoid areas of low reactivity that corresponded to nuclei were present (Lester et al., 1993) (Fig. 2A, B). Anti-GST immunoreactivite cells present in the samples from Milazzo confirm the results previously described for CYP1A1 (Fig. 3A, B).

Statistical analyzes of the mean of immunoreactive cells are represented in Figs. 2C and 3C ($P<0.0001$).

### 3.3. Amplification and sequencing of CYP1A- and GST-specific complementary DNA

RT–PCR products were characterized by electrophoresis on SYBR safe-stained agarose gel. Bands of 402 bp for CYP1A and 483 bp for GST were visualized. The data were normalized with the expression of cytoplasmic actin, which was expressed at basal levels both in control animals and in those from Milazzo. CYP1A and GST genes showed a physiological level of expression in the livers of control animals, in contrast were highly expressed in animals from Milazzo (Fig. 4A, B). CYP1A and GST band intensity differed significantly between Marinello and Milazzo individuals ($P=0.0005$ for CYP1A and $P<0.0001$ for GST) (Fig. 4C, D).

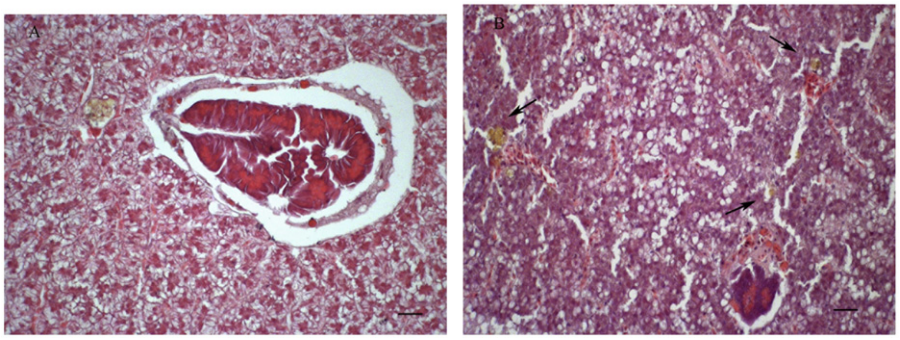


**Fig. 1.** Liver section of specimens collected from Marinello (A) showing hepatic cell cords located among sinusoids and bile duct compared with specimens from Milazzo characterized by damaged structure and melano-macrophage containing centers (B, arrows). Scale bar: 10 μm.

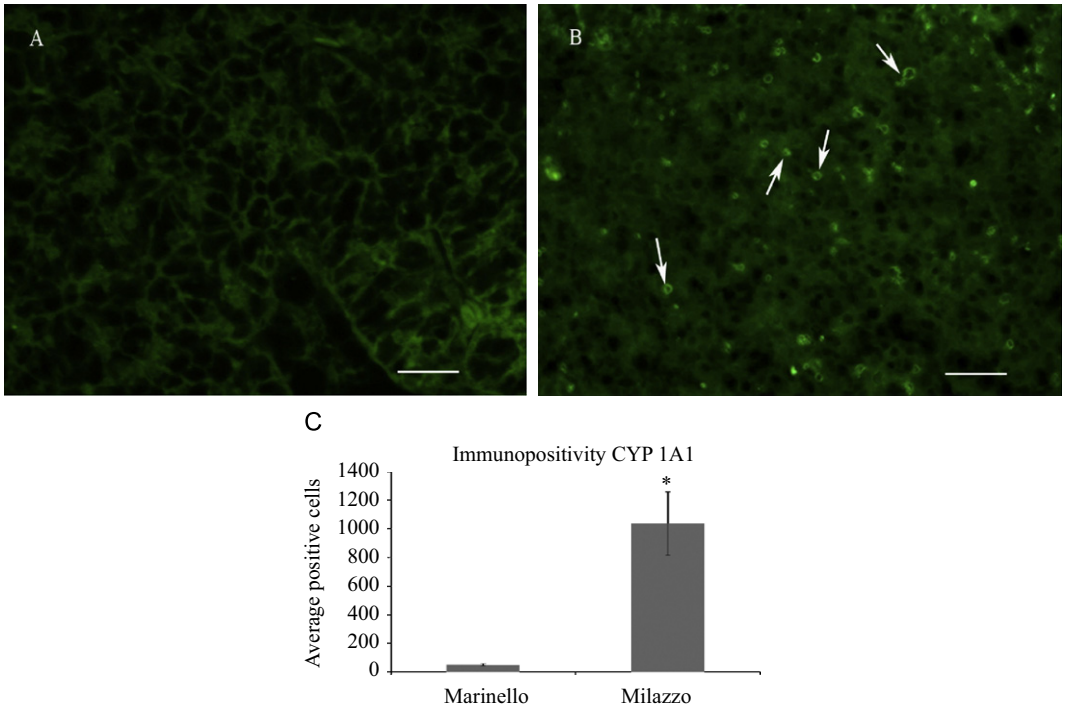


**Fig. 2.** Immunohistochemical labeling for CYP1A1 of hepatic tissue of specimens collected from Marinello (A) and Milazzo (B, arrows) respectively. Mean and standard deviation (SD) of immunopositive cells (C), (∗: $P < 0.0001$ extremely significant). Scale bar: 10 μm.

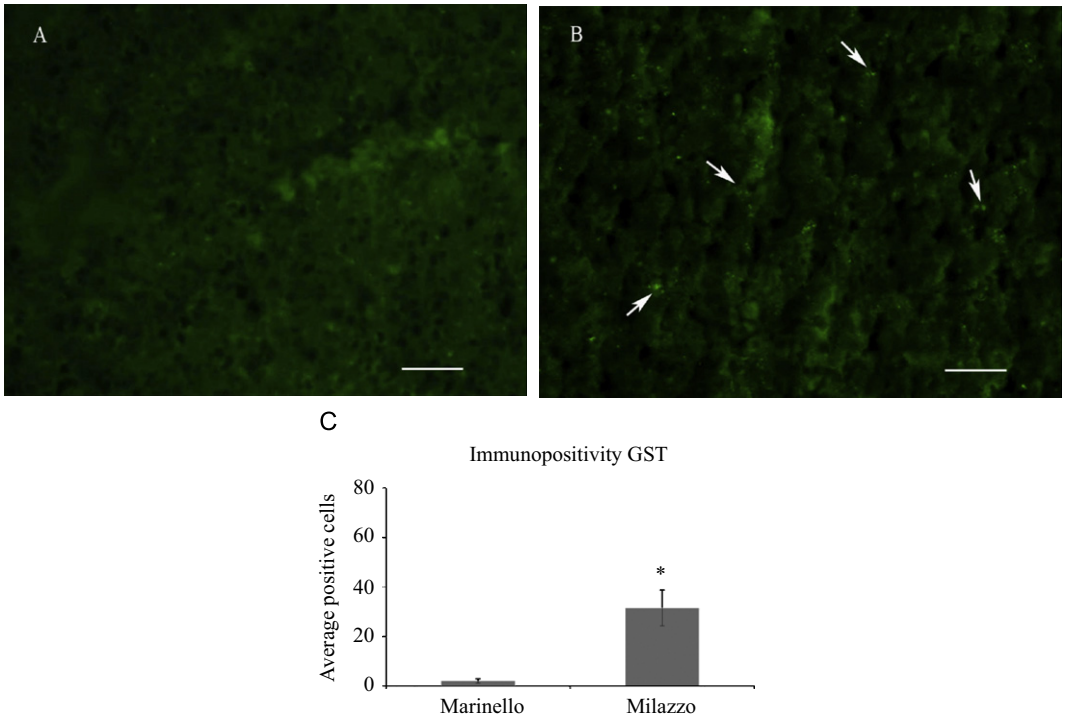


**Fig. 3.** Immunohistochemical labeling for GST of hepatic cells of specimens collected from Marinello (A) and Milazzo (B, arrows) respectively. Mean and standard deviation (SD) of immunopositive cells (C), (∗: $P < 0.0001$ extremely significant). Scale bar: 10 μm.

PCR products were cloned and sequenced, and the partial nucleotide sequence of the CYP1A cDNA was submitted to GenBank and dbEST (GenBank Accession N° FK669937). The nucleic acid sequences of CYP1A and GST were compared with homologous genes of other fish species. The highest degree of similarity was found with CYP1A sequences of *Ammodytes marinus* (87%), and the highest level of homology was found with GST sequences of *Epinephelus coioides* (86%).

### 3.4. Enzymatic activities

Each specimen of *C. julis* collected from Milazzo showed higher EROD and BPMO activities than those collected from Marinello.

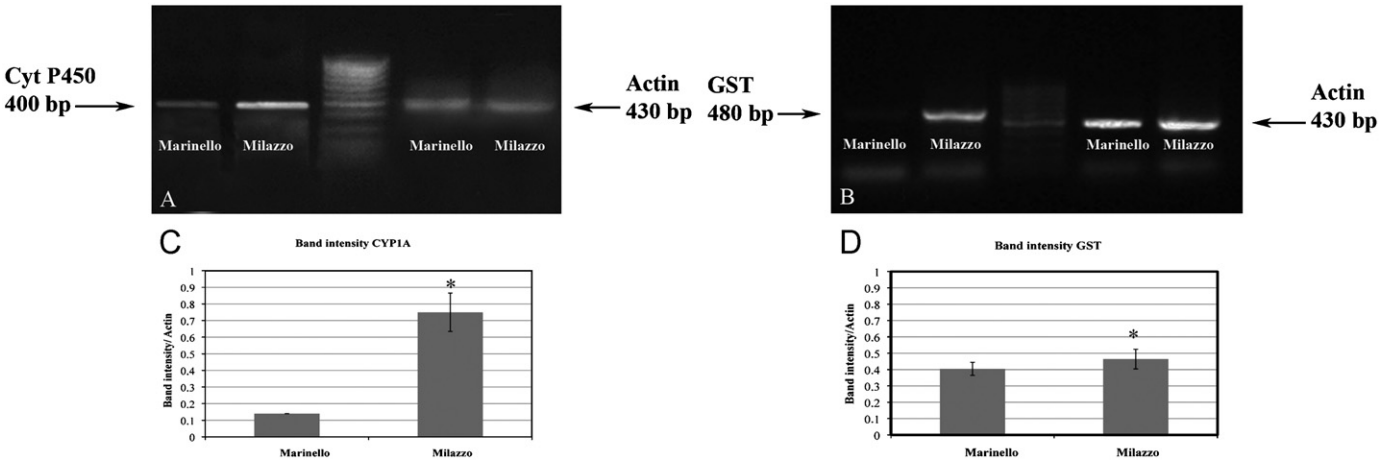


**Fig. 4.** (A) Amplification of CYP1A and actin cDNA in the liver of *C. julis* of Marinello and Milazzo. (B) Amplification of GST and actin cDNA in the liver of *C. julis* of Marinello and Milazzo. (C and D): mean and standard deviation (SD) calculated from the ratio between CYP1A or GST and actin band intensity (∗: significantly different $P=0.0005$ for CYP1A and $P<0.0001$ for GST).

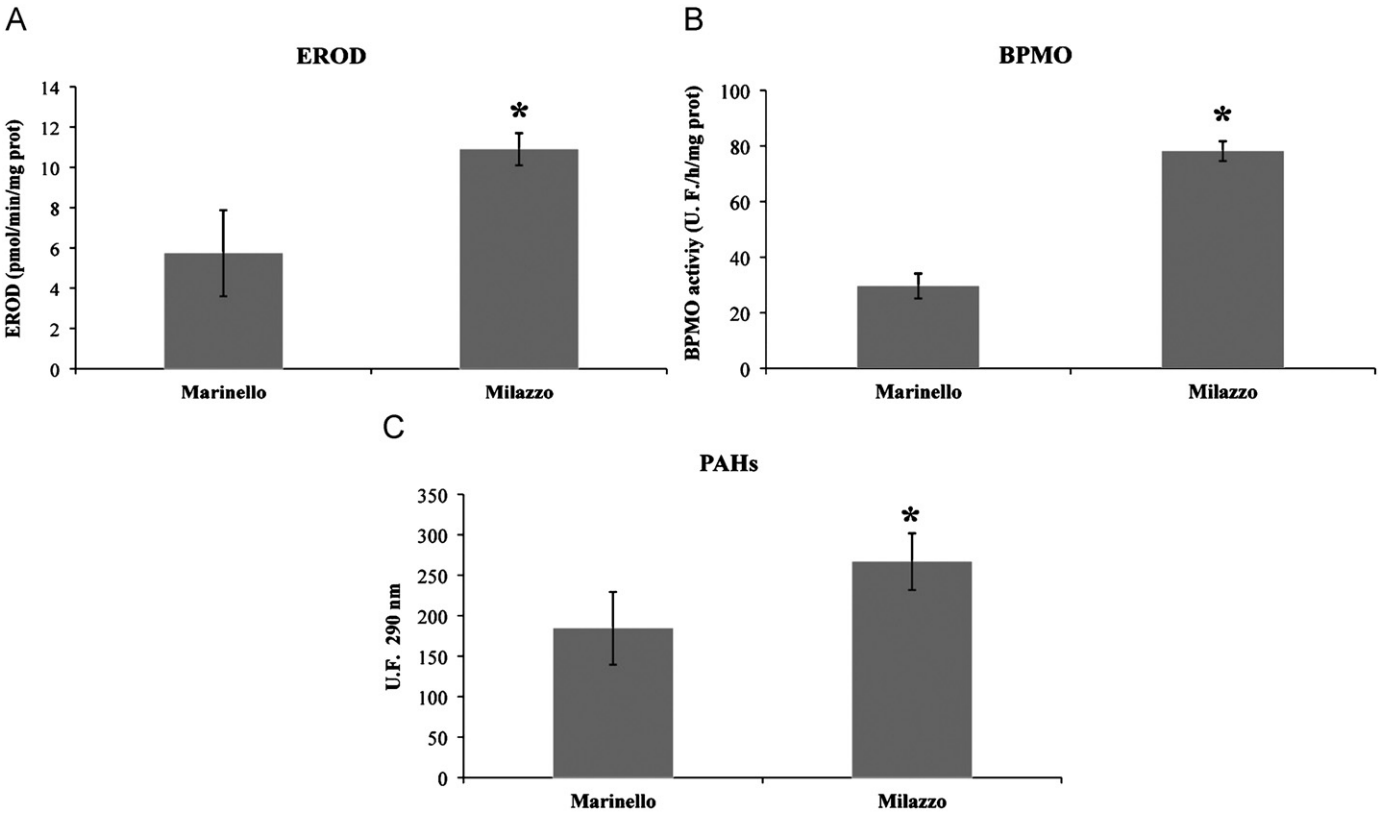


**Fig. 5.** EROD activity expressed as pmol resor/min/mg prot (A), BPMO activity expressed as U.F./h/mg prot (B) and evaluation of PAH metabolites in the bile expressed as U.F. at 290 nm (C) in specimens collected from Marinello and Milazzo, respectively. Values significantly different from the control ($P<0.0001$) are marked with asterisk.

Mean EROD activity was twice higher in the polluted area that of Marinello (5.7 pmol resor/min/mg prot) and mean BPMO activity was 78.1 U.F./h/mg prot versus 29.6 (Fig. 5A, B). The mean PAH concentration of the bile samples from Milazzo fish studied was 266.8 U.F. at 290 nm comparated to 184.4 for the Marinello fish (Fig. 5C).

The obtained results were statistically significant ($P<0.0001$).

The fish collected from two sites differed greatly in their AChE (Fig. 6A) and BChE (Fig. 6B) activities. In the samples of *C. julis* collected from Milazzo, the mean values were 0.048 μmol/min/mg and 0.25 μmol/min/mg, respectively, compared to 0.21 μmol/min/mg, 0.91 μmol/min/mg in samples from Marinello. The difference was statistically meaningful ($P<0.0001$).

### 3.5. Genotoxicity analysis

In the samples from Milazzo, nuclear abnormalities were apparent (Fig. 7A), but no anomalies were detected in the nuclei erythrocytes of the Marinello specimens (Fig. 7B). Identified anomalies were blobbed, notched and lobed following the classification of Carrasco et al. (1990). These nuclear abnormalities are good indicators of genotoxic damage (Bombail et al., 2001), although their formation mechanism is not yet

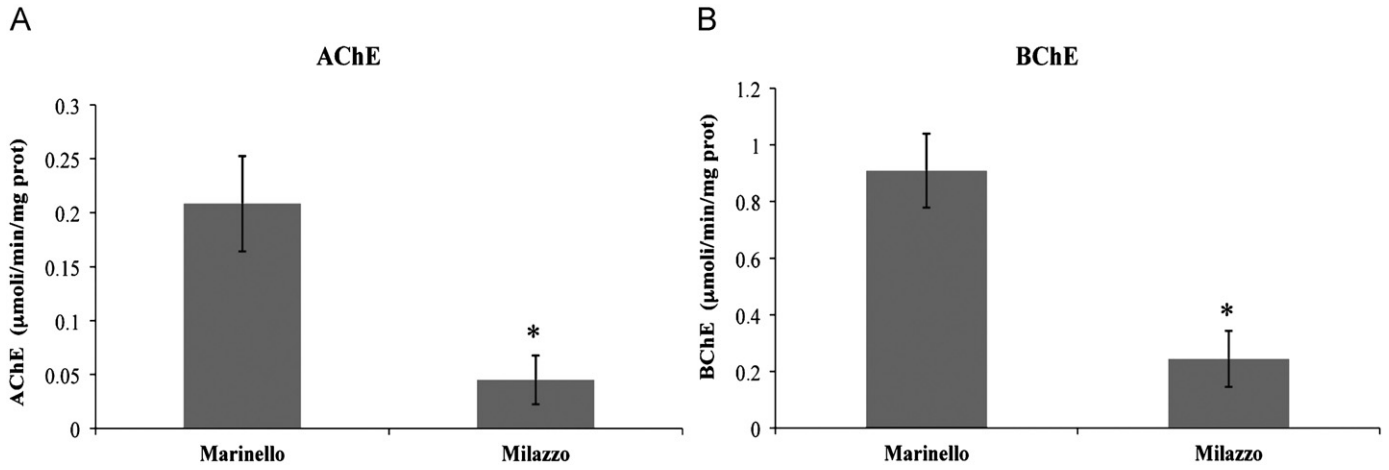


**Fig. 6.** AChE and BChE activities expressed as µmol/min/mg in *C. julis* collected from Marinello and Milazzo (∗: $P < 0.0001$ extremely significant).

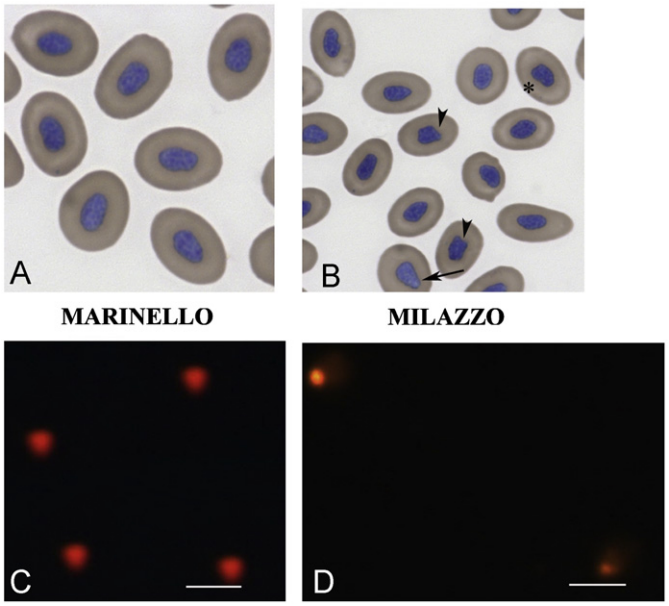


**Fig. 7.** Photomicrographs of erythrocytes of *C. julis* with (A) normal nuclei (100 ×) and (B) nuclear abnormalities (arrow=blobbed, arrowhead=notched and asterisk=lobed) (63 ×). Representations of normal erythrocytes of specimens collected from Marinello (C) and comet images from erythrocytes of specimens collected from Milazzo (D). Scale bar: 10 µm.

understood (Çavaş and Ergene-Gözükara, 2003). We observed a Frequency Nuclear Abnormalities (FNA) of 62.5‰ in the fish erythrocytes collected from Milazzo.

The highest level of DNA damage, by Comet assay, was observed in erythrocytes from fish sampled at Milazzo: the percentage of DNA that migrated in the comet tail was higher than in the controls. The ANOVA showed significant differences between the tail length ($P < 0.001$) and the TM ($P < 0.05$) values observed in all specimens collected from the two sites (Table 3).

Fishes sampled in the control site did not show significant damage to DNA (Fig. 7C), while a clear degree of DNA strandbreakage was found in the specimens exposed to contaminants (Fig. 7D).

## 4. Discussion

The analysis of PAHs from the superficial sediments revealed the presence of several chemicals in both the polluted and

**Table 3**
Mean values of tail lenght and tail moment in specimens collected from two different sites.

| Sampling sites | Tail length (µm) | Tail moment |
|---|---|---|
| Marinello | $7.6 \pm 0.37$ | $0.05 \pm 0.005$ |
| Milazzo | $23.34 \pm 3.05$ | $9 \pm 3.13$ |

references sites, but the concentrations were higher in Milazzo. PAH measurement in sediments allows us to define the origin of specific pollutants in the studied site. The Milazzo station displays a dual pattern of pollution: one due to petroleum, which originated from the neighboring oil refinery (Burgeot et al., 1996) and is particular to this environment, and the other one of industrial-human origin, which characterizes the Mediterranean Sea (Burgeot et al., 1996). Phenanthrene, fluoranthene and pyrene are representative of human pollution being of pyrolytic origin, but both sampling areas contained pollution due to the petroleum refinery, as illustred by the presence of petrogenic origin PAHs. Yakimov et al. (2005) analyzed organic contamination in superficial sediments in the same area and reported high concentrations of different hydrocarbons originating from human activities. Among the PAHs, the toxicants present in the sediment of the analyzed samples are considered by the Environment Ministry (Health Ministry, Decree n. 367 of 06/11/2003) to be very dangerous for human health.

In this study some tissue alterations have been found in the specimens of *C. julis* sampled from Milazzo compared to those from Marinello and the increase in MMCs in these individuals suggests the presence of dead cells or a high replacement of red blood cells.

The hepatic expression of CYP1A1 has been measured at the enzyme activity level (EROD and BPMO) and the mRNA level (estimated by means of RT–PCR). The hepatic expression of GST has been also measured at the mRNA level; and the immunohistochemical techniques were used to detect cellular localization of CYP1A1 and GST in the liver.

The liver of specimens from Marinello showed none or only weak immunoreactivity; on the contrary in the liver of fish from Milazzo had high anti-CYP1A1 and anti-GST immunostaining, which could indicate xenobiotic exposure resulting in an increased oxidative stress.

The presence of CYP1A1 and GST immunopositive cells and the CYP1A and GST mRNA expression in the liver of *C. julis* from Milazzo have demonstrated that the fish is well adapted to its

environment with detoxificant defenses participating in the adaptation (Husøy et al., 1996; Jeong and Kim, 2002).

The biomarker analysis data revealed a high induction of EROD and BPMO activities in the liver of the *C. julis* samples collected from the Milazzo area. Induction of the CYP4501A-dependent monooxygenases in the fish liver is indicative of contaminated environment because this organ is responsible for the biotrasformation of a number of xenobiotic compounds including dioxins, furans, PCBs, PAHs and DDT (Stegeman and Hahn, 1994; Goksøyr, 1995; Parkinson, 1995; Husøy et al., 1996; Fossi, 2000; Jeong and Kim, 2002). Thus the higher activity detected in the Milazzo specimens compared to that of individuals from the reference area presumably indicates exposure to polycyclic aromatic hydrocarbons, planar polychlorinated biphenyls, and polyhalogenated aromatic hydrocarbons. The PAH analyzes on bile samples confirmed the MFO data, showing a higher value in *C. julis* sampled in Milazzo compared to those from Marinello.

In this study, the activity of two esterases, AChE and BChE, were considered. The esterase activities were greatly inhibited in *C. julis* specimens collected from industrial area. AChE in the brain samples exhibited an inhibition of 77% and BChE in blood samples exhibited an inhibition of 72.5% relative to the reference area. These results could suggest the presence of inhibitory substance used in the agricultural activities of the biotope of sampled fish, such as organophosphorus (OPs) insecticides and carbamates (CBs). Several studies have shown that ChE activity represents a useful biomarker to detect exposure to, as well as effects of neurotoxic chemicals such as OPs and CBs under (Alpuche-Gual et al., 2008). In fact, the area around Milazzo is known for both its oil refineries and also the intensive agricultural activities conducted around the city. Our integrated approach confirmed that Milazzo, located in the northwestern part of the Mediterranean Sea, is impacted by industrial pollution and mainly by the products of the oil refineries (Yakimov et al., 2007).

To detect the mutagenic and genotoxic effects of chemicals in the environment, the micronucleus test and comet assays were used (Tucker and Preston, 1996; Kassie et al., 2000; Baršienė et al., 2006) on blood samples. Among the set of biomarkers, the Comet assay has been used for detecting DNA damage and the micronucleus test in erythrocytes for the assessment of genotoxicity in fish species. Recently, particular attention has been devoted to both tests in order to identify substances with genotoxic activity. The results, from the in situ study of *C. julis* from Milazzo area, of nuclear abnormalities that followed the general trend exhibited by the DNA migration in the Comet assay revealed a significantly higher level of DNA damage compared to the unpolluted site, indicating that genotoxic compounds are discharged into the Gulf of Milazzo. In this site the higher genotoxic impact can reflect the more elevated contamination by PAHs, OPs and CBs.

Results of the chromosomal aberration analysis presented in this study suggest that long-term exposure to genotoxic compounds could cause a significant increase in the level of DNA damage; the DNA damage detected herein could have been originated from DNA single strand breaks, DNA double strands break, DNA adduct formations, and DNA–DNA and DNA–protein cross-links (Mitchelmore and Chipman, 1998).

The ecotoxicological results obtained in this study, allow us to consider some potential ecological consequences of the pollution in the area of study. The ecotoxicological status of *C. julis* in Milazzo, as highlighted by the biomarkers analyzes, can lead to ecological changes in its population, in the food chain and in the entire ecosystem. The ecocytotoxicological alterations can involve many variations in the basal activities and thus in the life cycle of this species, typical of the biotope, even its disappearance and consequent damages in the food web (Chiea et al., 2002; Fasulo et al., in press).

## 5. Conclusion

In conclusion, the induction of CYP1A and GST in the liver of fish from Milazzo suggests that the defense system plays an important role in the fish response to the presence of PAHs. However, the presence of other inducers cannot be discounted, due to the intense urban and industrial activities in this area, as demonstrated by values of the esterase activities.

Micronucleus test and comet assay showed a clear genotoxic effect in this species and confirmed the status of medium-scale pollution in the studied area.

Use of different biomarkers in *C. julis* showed different toxic and adaptive responses, which are useful as biomarkers of stress and gradient pollution.

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