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Biochemical and bioaccumulation approaches for investigating marine pollution using Mediterranean rainbow wrasse, *Coris julis* (Linneaus 1798)

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ABSTRACT

A multibiomarkers approach was used in order to estimate and monitor marine pollution. *Coris julis* (Linneaus, 1758) was chosen as a sentinel organism, and the specimens were collected from three well-known sites along the Ionic coast of Sicily: the protected marine area (P.M.A) "Cyclop's Islands" of Acitrezza (CT), used as a control site, Riposto (CT), and the industrial site of Augusta (SR). Abiotic levels of contaminants were also detected. High levels of biotic and abiotic accumulation were found at the industrial site in which the presence of genotoxic and oxidative damage were also evidenced, measured by Micronuclei, Alkaline and Fpg-modified Comet assays. The protein expression analysis showed metallothioneins (MTs) as good tissue-specific markers of metal accumulation. Their levels were significantly higher in muscle than in liver tissue for all the sampling sites, with a positive correlation among tissue levels and the degree of pollution at the sites. Conversely, heat shock proteins 70 (HSP70) expression was higher in Augusta and Riposto than in the control site, but no significant difference was found between the examined tissues among all sites.

1. Introduction

Aquatic pollution, due to the growing levels of contaminants in the marine environment, represents a serious and global problem (Conti et al., 2012; Copat et al., 2012a, 2012b; De Andrade et al., 2004b; Sasaki et al., 1997). Bioconcentrations of highly persistent pollutants such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides and toxic metals in species of marine organisms, and/or biomagnification along the trophic chain, are considered to be one of the major threats to human and ecosystem health.

Recent literature data shows the advantage of utilizing a multidisciplinary approach in monitoring the acute and chronic adverse effects caused by pollution (Huang et al., 2011; Jebali et al., 2011; Knapen et al., 2007; Parolini et al., 2010; Sanchez et al., 2007; Smolkova et al., 2004). As a matter of fact, the use of a single-factorial approach to analyse the state of marine ecosystems underestimates the complexity of anthropogenic impact and generally leads to an unclear, distorted and incomplete knowledge of the consequences.

In fish, stress response can be considered an early pollutants-induced event that may elicit forms of cellular damage such as different types of DNA damage (adduct formation, strand breaks, changes in composition of DNA's minor base, increase in the level of DNA repair, oxidative DNA damage and apoptosis), and in particular oxidative DNA damage, which is often used as an indicator of the effects of pollutants in ecotoxicological studies (Vijayavel and Balasubramanian, 2008). A wide literature (Akcha et al., 2004; Fasulo et al., 2010; Frenzilli et al., 2004; Steinert et al., 1998) establishes the positive correlation between site contamination and DNA damage, confirming in particular that Comet and Micronuclei assays are two useful tools in determining the potential genotoxicity of water pollutants in monitoring programs, both in controlled and natural conditions (Buschini et al., 2004; Matsumoto et al., 2006; Nwani et al., 2010; Rocha et al., 2009; Russo et al., 2004). Furthermore, pollution modulates the expression of some stress-related proteins, such as metallothioneins (MTs) and heat shock proteins (HSPs) (Padmini and Usha Rani, 2008; Wang et al., 2007; Webb and Gagnon, 2009). MTs are cytosolic and/or nuclear cysteine-rich proteins, selectively linking their cysteine residues to Cu^{2+} and Zn^{2+} and other toxic metals (Hellou, 2011). Thus, these proteins, involved in the mechanisms of general responses to stress as well as in the tolerance and the detoxification of heavy metals, have been proposed as a sensitive

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biomarker in the assessment of potential effects induced by metal exposure (Flora et al., 2008; Ivankovic et al., 2005). HSPs, indeed, a family of ubiquitous proteins, are considered the first line of defence following exposure from high temperatures and many other stressors, including xenobiotics and contaminants. Therefore, they are commonly accepted as biochemical indicators of toxicity index, providing a measure of proteotoxicity of pollutants (Iwama et al., 1998; Kohler et al., 2007; Padmini and Usha Rani, 2008; Sanders and Martin, 1993).

The goal of our study was to select specific and good markers useful in marine monitoring programs. The impact of the different marine pollutants on *Coris julis* collected from different marine sites of the Ionic coast of Sicily was evaluated. This teleost was chosen as a model since it is widely distributed in the Mediterranean sea and considered to be a good bioindicator (Bonacci et al., 2003; Fasulo et al., 2010), including all criteria of fish bioindicators species (Whitfield and Elliot, 2002).

In particular, our experimental project focused on detecting different chemical parameters (PCBs, PAHs, organochlorine pesticides and toxic metals in water and sediments, the muscle bioaccumulation levels) as well as some biological markers to evaluate early adverse effects of contamination: (i) DNA status by both Micronuclei test and Comet assays, focusing on oxidative DNA damage in blood and hepatic cells by Fpg-modified version of Comet assay; (ii) the levels of HSP70 and MTs in the liver and muscles.

2. Materials and methods

2.1. Study areas and tissue sampling

Field sampling was conducted at three different sites along the Ionic coast of Sicily: (1) the protected marine area (P.M.A.) "Cyclop's Islands" of Acitrezza (CT) (37° 33′46.63″ N-15° 09′ 44.19″ E), which is only a hundred meters from the coast, and includes a small harbour and numerous freshwater springs, which come from the rural and volcanic hills (2) the rural and volcanic area of Riposto (37° 43′ 24.72″ N-15° 12′ 43.46″ E), in which is situated the biggest touristic harbour of Sicily; 3) Augusta harbour (37°12′40.34″ N-15°13′34.08″ E), a site that hosts one of the biggest petrochemical plants in Italy and is on the list of Italian sites at elevated environmental risk (Fig. 1).

Sea water temperatures during sampling procedure ranged between 18 and 21 °C at the Riposto and P.M.A sites, and between 19 and 21 °C at the Augusta site.

A total of 90 females of *C. julis* 4, 5 years old (average length of 15 cm) were collected between May and June 2010 ($n=30$ for each site). Fishes were promptly transported to the laboratory for analysis in containers of 25 l filled with sea water taken at the same time of sampling. In each container were placed only 5 fish to avoid stress conditions and the travel time ranged from a minimum of 15 min (PMA) to a maximum of 35 min (Augusta).

Blood samples were collected from the caudal vein with an heparinised syringe. Then the specimens were sacrificed by a blow to the head and the liver and muscle fillets were excised. Samples for the immunoblotting and Comet assays analyses were processed immediately according to each analysis protocol; for the chemical and micronuclei analyses, samples were stored at −80 °C until analysis.

2.2. Chemical analyses of water and sediment

Water and sediment samples were collected in the three sampling sites, during the collection of fish. Water was filtered on-site through Whatman filter papers (porosity 0.45 μm). Sediment samples, characterized by a mixed granulometry of sand and gravel, were taken using a grab technique to define the characteristics of the area at the time of collection. The larger fractions of samples were removed. Sediment and water samples were collected in specific containers: teflon containers, previously treated with 1 M HNO_3 for 12 h and then washed with double-distilled water (Merck), were used for heavy metals analysis, whereas amber glass bottles fitted with teflon-lined screw caps were used for the analysis of organochlorine pesticides, PAHs and PCBs. Water samples were stabilized with the addition of 1 ml of HNO_3 65% for each 1-l sample for the analysis of chromium (Cr), mercury (Hg), lead (Pb), cadmium (Cd), arsenic (As) and PAHs, while for the analysis of chlorinated pesticides and PCBs the samples were stabilized with 1 ml of HCl 37% for each 1-l sample, and stored at −4 °C.

2.2.1. Heavy metals

Water analysis was performed following the UNI EN ISO 17294–2:2005 method for As, Cr, Cd and Pb, and with the UNI EN 1483:2008 method for Hg. Sediment analysis was performed with the 3051A and 6010C EPA methods. A total of 0.25 gr of each sediment sample were mineralized with 6 ml of HNO_3 65% (Carlo Erba Chemicals), 1 ml of H_2O_2 (Carlo Erba Chemicals), and 1 ml of $HClO_4$ 65% (Carlo Erba Chemicals) with a 30 min operation cycle at 200 °C. After mineralization, the samples were brought up to 40 ml by adding ultra pure water (Merck), then divided into 2 aliquots of 20 ml each, one for the study of Hg and the second for the study of the other metals. The sample used for the analysis of Hg was first oxidized with 5% potassium permanganate ($KMnO_4$), then neutralized with 1.5% hydroxylamine hydrochloride ($NH_2OH \cdot HCL$). An ICP-MS Elan DRC-e (Perkin Elmer) was used for Cr, Cd, As and Pb quantification in water and sediments. Hg was analysed with a Flow Injection Analysis System 100 (FIAS) (Perkin Elmer) using the cold vapour capture technique. Standards were prepared on the basis of multi-element reference solution ICP Standards (Merck).

2.2.2. Polycyclic aromatic hydrocarbons (PAHs)

Water samples were extracted and purified with Pressurized Solvent Extraction (PSE) on C18 Bond Elut of 12 ml, in accordance with the analytical method EPA 500.1. PAHs in sediments were extracted with an Accelerated Solvent Extraction (ASE)—Fast PSE (LabService) following the analytical method EPA 3545A. Extracts were concentrated in a Büchi Syncore and then purified with Gel Permeation Chromatography (GPC, LabService). PAHs analysis was carried out with an HPLC Perkin Elmer 200 with UV and FL detectors, using certified standard reference material 11647 PAH NIST.

2.2.3. Organochlorine pesticides

Water samples were extracted at neutral pH using the EPA 3520 method. Using the EPA 3550 method, 10 g aliquots of sediment samples were extracted. The extracts were purified with GPC (LabService), in accordance with the EPA 3640 method. The analysis of organochlorine pesticide concentrations in the extracts was carried out according to the EPA 8081A method, using Gas Chromatography (GC) 2010 AF Shimadzu apparatus and certified standard reference material multi-element Custom Pesticides (Restek).

2.2.4. Polychlorinated biphenyls (PCBs)

Extraction and purification steps were the same as those used for the organochlorine pesticide chemical analysis (EPA 3520 and EPA 3550). The extracts were then subjected to a sulphuric acid cleanup following the EPA 3665 method. After cleanup, the extracts were analysed with the EPA 8082 method using GC 2010 AF Shimadzu apparatus and certified standard reference material mono-element Custom PCB (AccuStandard).

2.3. Heavy metals accumulation in muscle

Using an heated mixture of strong acids, 1 g of muscle tissue per fish was mineralized in a microwave system Ethos TC (Milestone). The method for animal tissue requires a digestion solution prepared with 6 ml of HNO_3 65% (Carlo Erba Chemicals) and 2 ml of H_2O_2 30% (Carlo Erba Chemicals) with a 50 min. operation cycle at 200 °C. After mineralization, the samples were brought up to 20 ml by adding ultra pure water (Merck), then they were divided into two aliquots of 10 ml each: one for Hg measurement and the second for the other metals. The sample for Hg analysis was oxidized with potassium permanganate 5% ($KMnO_4$), to obtain the conversion of organic Hg into inorganic Hg, then neutralized with hydroxylamine hydrochloride ($NH_2OH \cdot HCl$) 1.5%. An ICP-MS Elan-DRC-e (Perkin Elmer) was used for the quantification of As, Cd, Cr, Pb and Zn. Hg was analysed with a FIAS 100 (Perkin Elmer) using the cold vapour capture technique. Standards for the instrument calibration were prepared on the basis of the mono-element certified reference solution AAS Standard (Merck).

2.4. Immunoblotting

HSP70 and MTs determination in muscle and liver was carried out by Western blotting as previously reported in Tigano et al. (2009). Briefly, the fish tissues were weighed, homogenized 1:10 (w/v) in a lysis buffer (Tris–HCl 40 mM, EDTA 25 mM, 0.2% SDS, pH 7.4) containing 1/100 (v/v) protease inhibitors (Sigma) and centrifuged. Total protein concentration in the supernatant was determined according to the Bradford method (Bradford, 1976). Thirty micrograms of protein/lane were analysed by minigel SDS-PAGE (8% for HSP70; 12% for MTs) and transferred to a nitrocellulose membrane using Transblot (Biorad). The HSP 70 and MTs levels were measured by incubating nitrocellulose membranes overnight at 4 °C with mouse monoclonal primary antibodies specific for fish epitope anti-HSP70 (1:1000, cat. N. C92F3A-5 - Abcam) and anti-MTs (1:500, cat. N. 56262-QED Bioscience Inc.) respectively.

The complex protein-primary antibody was detected using a HRP-conjugated Ig-G anti-mouse secondary antibody (1:3000 Santa Cruz) by the

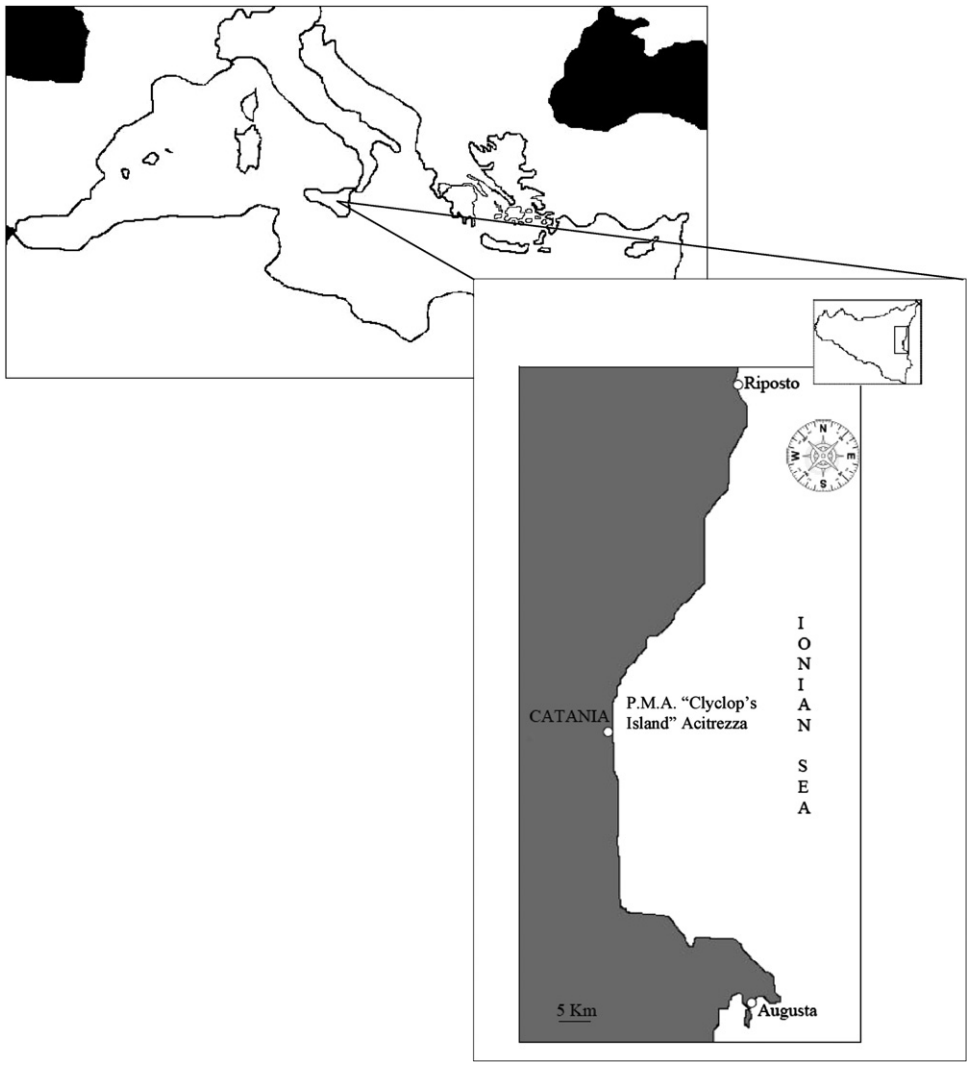

Fig. 1. Sampling sites along the Ionic coast of Sicily.

chemiluminescent method. Quantitative measurements were performed by densitometry analysis of the X-ray films with the Scion Image (4.03) program, expressing values as arbitrary densitometric units (A.D.U.). The results represent the mean of three independent experiments performed in triplicate.

2.5. Comet assay

The test was carried out on whole blood and/or on isolated hepatic nuclei following our standardized protocol (Tigano et al., 2009). Prior to the assay, the viability of both erythrocytes and hepatic nuclei was assessed with trypan blue and a cell viability of 85% was considered to be suitable for the comet assay. Fpg-Comet assay protocol (Trevigen Fpg-flare kit ®) was performed as previously described in Tigano et al. (2009) in order to measure both total DNA fragmentation and 8-dOH guanine by using Fpg enzyme, which specifically recognizes and cleaves the purine oxidate bases in DNA generating strand breaks as a result of its endonuclease activity (Collins et al., 1996). Electrophoresis conducted under alkaline conditions (pH 12.1) enables the detection of single/double strand breaks, abasic sites and others types of damage. For each sample, two slides with the agarose embedded cells or nuclei (minigel) were needed and incubated for 40' in a humidity chamber, covering each minigel with 100 μl of working Fpg enzyme solution (dilution 1:1000) or with 100 μl of Fpg Flare reaction buffer alone (kit), considered as the negative control sample. The samples were analysed using a fluorescence microscope (Leica) equipped with a camera and the CASP (1.2.2) image analysis software. Each phase was performed in the dark and all analyses were carried out in triplicate, scoring 50 randomly selected nucleoids per sample and calculating the percentage of DNA present in the tail of comet (%TDNA) using the ESCODD guidelines (ESCODD, 2003). In order to relate DNA damage to break frequencies, the %TDNA values were converted to DNA break/10^9 Da/Gy standard units. The reference values of TDNA% were assessed in separate experiments through an indirect calibration obtained following exposure of *C. julis* erythrocytes to X-rays according to Collins et al. (1996). In the case of 8-Oxo-dG measurement, the %TDNA data was also expressed as a number of oxidised bases, breaks/10^6 bp (altered bases/10^6 unaltered bases) (Griffiths et al., 2002).

2.6. Micronuclei assay

A drop of blood from caudal vessels was directly smeared on slides. The slides were then air-dried, fixed in methanol for 10 min and stained with a 10% GIEMSA solution for 15 min. For each slide, 2000 erythrocytes were scored using a light microscope. Only the cells clearly isolated from the surrounding cells were scored. Round or ovoid-shaped non-refractory particles similar to chromatin in colour and structure, with a diameter of 1/3–1/50 of the main nucleus and clearly detached from it were interpreted as micronuclei, while nuclear abnormalities were classified as suggested by Carrasco et al. (1990).

2.7. Statistical analysis

One-way analysis of variance (ANOVA) was used to test differences in muscle metal concentrations and blood micronuclei scores among sites, and a two-way ANOVA was used to test for differences between sites and tissues for HSP70, MTs, and DNA damage levels.

Furthermore, Spearman test was applied to test the correlation between each of the fish biochemical parameters and muscle metals bioaccumulation to evaluate the sensitivity of each biomarker to these pollutants. All analyses were computed using the software SYSTAT version 9, Systat Inc., Evanston, IL, USA. We applied $p=0.05$as the minimum level of significance.

3. Results

3.1. Chemical analyses of water and sediment

Our results were compared to quality standards for toxic substances in aquatic environments set by the Italian Environment and Land Ministry (D.M. 367/2003; D. Lgs 152/2006).

The analysis of the samples from Augusta showed levels above the limits set by Italian law for total PAHs (1.301 μg/l) and Cd (5.065 μg/l) in water and total PAHs (2.420 mg/kg), benzo(*k*)-fluoranthene (0.098 mg/kg), benzo(a)pirene (0.457 mg/kg), Hg (1.789 mg/kg) and PCBs (0.067 mg/kg) in sediments. In contrast, the chemical analysis at the Riposto and P.M.A sites revealed low concentrations of contaminants both in water and sediments (by referring to Appendix A for complete list of analyzed compounds' results).

3.2. Bioaccumulation of heavy metals in muscle

One-way ANOVA testing showed a highly significant difference for Cd ($p<0.05$), Cr, Cu and Hg ($p<0.0001$) among the sites. In particular, mean values of Cd, Cr and Cu showed an increase trend P.M.A. < Riposto < Augusta, instead Hg had the trend Augusta > Riposto = P.M.A. Furthermore, Hg concentrations were above limits set by law (D.M. 1881/2006) in 10% of the total analysed fish sampled in Augusta (Table 1).

Spearman test evidenced that Cr concentration has a positive correlation with all biomarkers analysed ($p<0.001$) except for liver MTs ($p=0.053$) and muscle HSP70 expression ($p=0.115$). The same happened for Hg ($p<0.001$) except for liver MTs ($p=0.365$) and HSP70 expression in both tissues analysed (muscle $p=0.415$, liver $p=0.379$).

3.3. Immunoblotting

Levels of MTs and HSP70, expressed as arbitrary densitometric units (A.D.U.), were reported in Figs. 2 and 3, respectively. Two-way ANOVA analysis revealed that MTs expression was significantly different between tissues ($p<0.0001$), being higher in muscle than in liver and between sites ($p<0.0001$), but there was also a statistical significance in the interaction term between site and tissue ($p=0.02$), meaning that MTs had a different classification at the 3 sites in each tissue. In particular, muscular MTs levels of *C. julis* from Augusta were the highest and the lowest MTs levels were found in both tissues of the P.M.A. fish when compared with the samples of other sites. Conversely, the HSP70 two-way ANOVA analysis, showed a significant difference between sites ($p<0.001$), but the expression was similar between tissue ($p=0.139$), and the interaction term between sites and tissue was not statistically significant ($p=0.797$). Again, the mean values measured in both tissues at the P.M.A. were the lowest.

3.4. Comet assay

The averages of %TDNA in the comet tail for both whole blood and hepatic nuclei samples were shown in Fig. 4. Total DNA damage (FULL COLUMN: Buffer damage + Oxidative damage) was significantly higher in all the Augusta samples, compared with the bioindicators of other sites. In particular, the total DNA fragmentation was higher in the hepatic nuclei (Augusta, 78%; Riposto, 48%; P.M.A. 24%) than in blood cells (Augusta, 66%; Riposto 33%; P.M.A. 20%), as it also results when the damage is expressed as DNA break/10^9 Da (Table 2). Two-way analysis confirmed that the total DNA damage was significantly different across site ($p<0.0001$) and tissue ($p<0.0001$), but the interaction term between the two variables was not statistically significant ($p=0.15$) (Fig. 4).

The degree of oxidative damage measured by the Fpg-Comet assay resulted to be very similar in all the samples of the studied areas (hepatic nuclei: Augusta 23.54%, Riposto 25.24%, P.M.A. 27,02 of total TDNA, respectively; blood cells: Augusta 24.5%, Riposto 26.2%, P.M.A. 21%) when the data was expressed as a percentage of ratio oxidative damage/total DNA damage. In contrast, when the analysis of data was performed considering the number of FPG-sites as 8-Oxo-dG/10^6 Gua, significant differences were observed in oxidative damage between Augusta and the other two sites in both tissues examined, as reported in Table 2. The levels of oxidative damage resulted to be the highest in specimens collected in Augusta and the lowest in specimens collected in the P.M.A. Two-way analysis revealed a significant difference in oxidative damage between hepatic nuclei and blood cells ($p<0.0001$) and among sites ($p<0.001$). Conversely to the total DNA damage, the interaction term between site and tissue for oxidative damage was statistically significant ($p<0.0001$) (Fig. 4 and Table 2).

3.5. Micronuclei assay

True micronuclei (fully detached, with a diameter of less than one-third of the main nuclei) were found to be rare. Other nuclear abnormalities, in particular "notched" nuclei, were common and represented the largest majority of nuclear alterations. A higher percentage of micronuclei and nuclear abnormalities in samples from Augusta (70%) compared with specimens from Riposto (20%) and the P.M.A (10%) were found. Two-way analysis of variance showed indeed a significant difference of micronuclei and nuclear abnormalities between sites ($p<0.001$) (Fig. 5).

4. Discussion

This work studied *C. julis* (Linneaus, 1758) as a bioindicator and relied on a multibiochemical approach in order to represent a panel of markers useful in environmental biomonitoring programs.

Our research was performed at three different sites along the Sicilian coast by measuring background contamination in abiotic samples, metals bioaccumulation in muscle, MTs and HSP70 expression in liver and muscle, as well as DNA damage.

The chemical analysis of contaminants in water and sediments of marine ecosystems is a preliminary investigation for assessing anthropogenic impacts (Flammarion et al., 2002). In our research, Augusta, one of the largest industrial sites in Italy, resulted to be the most contaminated with the highest levels of PAHs, PCBs, pesticides and heavy metals in water and sediments, sometimes over the limits set by Italian legislation. However, traces of PAHs

Table 1
Bioaccumulation of heavy metals in muscle.

	Pb	Cd*	Cr***	Cu***	As	Zn	Hg***
P.M.A.							
Mean	0.032	0.002	0.239	0.340	11,79	7.902	0.021
Sd	0.022	0.001	0.107	0.138	3.433	2.851	0.016
RIPOSTO							
Mean	0.068	0.003	0.387	0.485	3.701	6.166	0.017
Sd	0.067	0.002	0.171	0.250	2.368	1.287	0.032
AUGUSTA							
Mean	0.095	0.011	0.724	2.670	6.765	7.015	0.450
Sd	0.159	0.016	0.324	2.672	2.824	6.363	0.482

The values are: the mean concentrations of heavy metals (mg/kg wet *w.*) in the muscle of *C. julis* (*N*=30) and standard deviation (Sd). One-way Anova analysis.

* $p<0.05$.

*** $p<0.0001$.

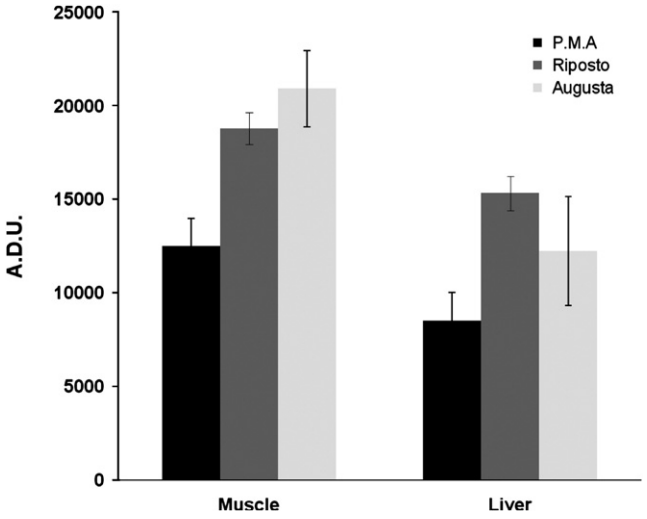

Fig. 2. Metallothionein expression in muscle and liver of *C. julis*. The values, expressed as arbitrary densiometric units (A.D.U.), are the mean ± SD of three independent experiments performed in triplicate. Two way analysis by sites $p < 0.0001$, and tissue $p < 0.0001$, interaction term $p = 0.02$.

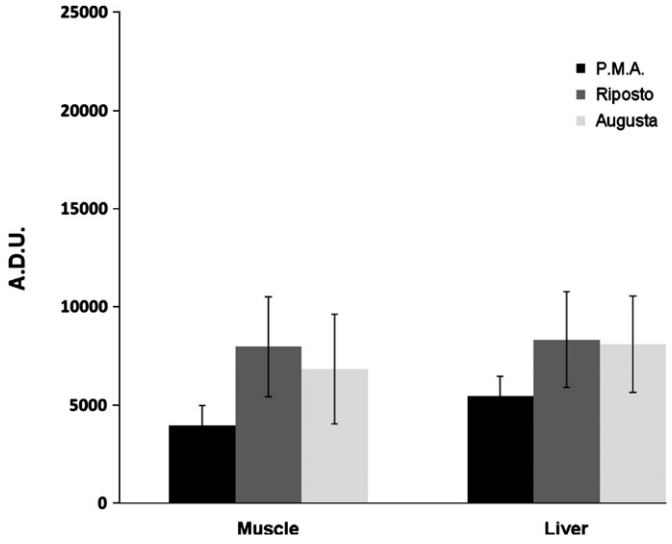

Fig. 3. HSP70 expression in muscle and liver of *C. julis*. The values, expressed as arbitrary densiometric units (A.D.U.), are the mean ± SD of three independent experiments performed in triplicate. Two way analysis by sites $p < 0.0001$, and tissue $p = 0.139$, interaction term $p = 0.797$.

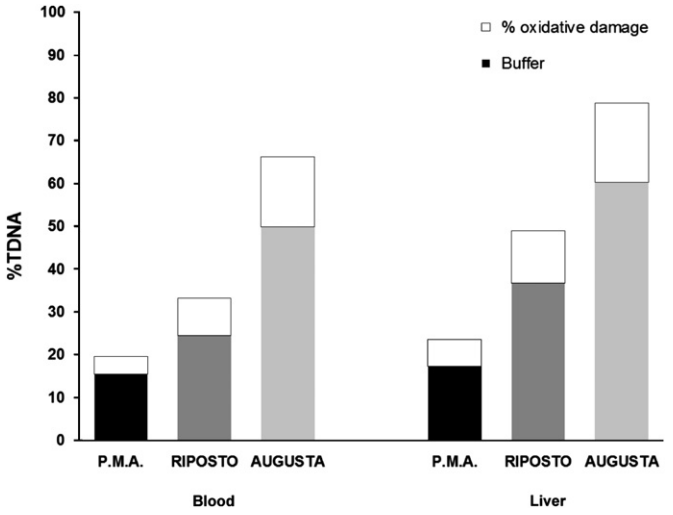

Fig. 4. Results of the Comet assay for blood and liver. Values are expressed as %TDNA (percent of DNA in the□ comet tail). Total DNA □ damage: Two way analysis by site $p < 0.0001$ and tissue $p < 0.0001$, interaction term $p = 0.15$ % oxidative damage: Two way analysis by site $p < 0.0001$ and tissue $p < 0.0001$, interaction term $p < 0.0001$ + : total DNA damage; percentage of oxidative damage in respect to the total DNA damage.

and metals at Riposto and the P.M.A. sites were also detected, especially in sediments.

This data is in agreement with the higher bioaccumulation levels of metals analysed in the muscle of *C. julis* (Linneaus, 1758) specimens collected at Augusta, where only Hg was over the limits set by EC Regulation 1881/2006 in 10% of cases. Although the higher load of contaminants and exposure biomarkers were found at the Augusta site, among muscle metals detected, only Cr and Hg revealed a strong positive correlation with almost all of the biomarkers analysed.

Particularly evident is the correlation between MTs expression and degree of pollution, confirming that their expression levels are influenced by various metals (Berthet et al., 2005; Hamza-Chaffai et al., 2000; Rotchell et al., 2001; Sinaie et al., 2010). Furthermore, confounding factors such as seasonal variation, age and reproductive status, water temperature, types of tissue and individual variation, could all contribute to MTs expression modulation, interacting with each other or acting all together (Shariati and Shariati, 2011).

Among these factors, temperature is well known to modulate the aquatics specimens' sensitivity to metals by affecting physiological tolerance, energy demand and oxygen supply and/or mitochondrial biogenesis (Sokolova and Lanning, 2008). However, no variation in temperature was found among the three sites. So the quantitative difference observed in MTs tissues expression may reflect metal loading at a single point in time (Knapen et al., 2007).

In agreement with van der Oost et al. (2003), the variation in MTs expression among tissues evidences the more discriminative power of muscle in respect to liver. These differences could be due to: (i) the lower metal-binding capacity (metal uptake and/or accumulation) of the liver in respect to the muscle, (ii) a distorted mechanism of membrane transport, (iii) an altered mitochondrial biogenesis with consequent reduced energy power or oxygen supply, all induced by metal intoxication.

Moreover, it may be hypothesized that the presence of organic contaminants reduces MTs synthesis by increasing the demand for cysteine residues for the synthesis of GSH, a very important cellular antioxidant molecule (van der Oost et al., 2003).

For the expression of HSP70, no difference has been found between muscle and liver among sites. In our opinion, the observed un-modulated expression of HSP70 at the Augusta site compared to Riposto results by the suppression of the cytoprotective upregulation of molecular chaperone due to either simultaneous exposure to different stressors or high concentrations of pollutants at this site (Ivanina et al., 2009). This is well-known as hormetic adaptive response, which is characterized by a low-dose stimulation and a high-dose inhibition, since a living organism is not a passive entity but, rather, a dynamic unit that will respond to damage signals with a coordinated series of temporally mediated repair processes, which are adaptive in nature (Calabrese, 2008). The HSP70 decrease at high metal concentrations was also attributed to pathological damage and inhibition of protein synthesis in the cell (Clayton et al., 2000; Ivanina et al., 2009). As previously shown by other authors in fish that are overwhelmed by stress, HSP70 levels are suppressed (Kohler et al., 2001), and, one stressor may increase resistance to another

Table 2
Determination of Fpg- sensible sites assessed by FPG-Comet Assay at pH 12.1.

	P.M.A.		Riposto		Augusta	
	Blood	Liver	Blood	Liver	Blood	Liver
Buffer pH 12.1	15.4 ± 2.8	17.23 ± 3.1	24.4 ± 2.6	36.6 ± 4.6	49.9 ± 3.6	60.2 ± 4.7
Breaks/10^9 Dalton	0.43	0.53	0.74	1.43	1.74	2.17
FPG pH 12.1	19.5 ± 3.1	23.61 ± 4	33.2 ± 3.3	48.9 ± 4.3	66.2 ± 4.0	78.8 ± 5.2
Breaks/10^9 Da	0.62	0.74	1.08	1.77	2.57	3.41
Fpg-sites/10^9 Da	0.19	0.22	0.34	0.34	0.837	1.24
8 oxo-dGua/10^6 Gu	0.11	0.13	0.21	0.21	0.51	0.76

Values expressed as %TDNA (mean ± SD). Each value is averaged from three different experiments in which each sample was processed in duplicate. %TDNA are also converted in: (a)Breaks/10^9 Da using for calculation of DNA break frequencies the calibration curve reported by Collins et al. (1996); (b) 8oxo-dGua/10^6 Gu using as conversion factor 1 8-oxo-Gua per 10^9 Da is equivalent to 1 8-oxo-Gua per 0.61×10^6 Gua as reported by Griffiths et al. (2002).
% TDNA: Two way analysis by site $p < 0.0001$ and tissue $p < 0.0001$, interaction term $p = 0.15$% oxidative damage: Two way analysis by site $p < 0.0001$ and tissue $p < 0.0001$, interaction term $p < 0.0001$.

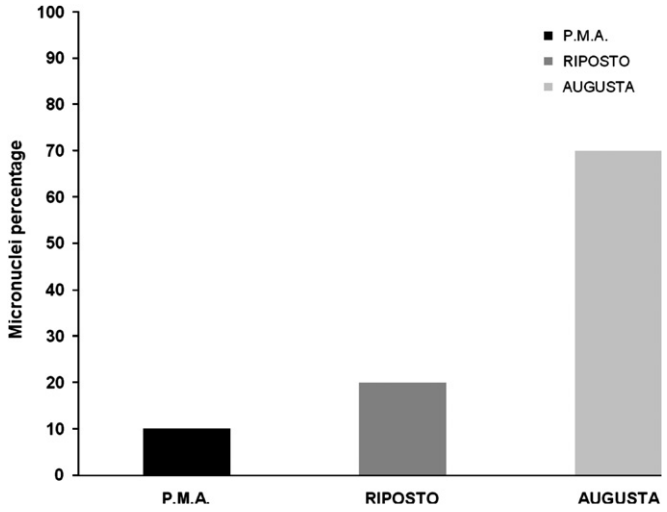

Fig. 5. Frequency of micronuclei and other nuclear abnormalities. One way analysis by sites $p < 0.0001$.

stressor, until it reaches a threshold above which the animal is unable to respond adequately or quickly to a stressful event (Basu et al., 2002).

Recently Fasulo et al. (2010) have defined the increase observed in both MTs and HSP70 expression in *C. julis* as a defensive process elaborated by stressed fish to maintain functions of the organs more exposed to the action of pollutants. This hypothesis can fully explain our results for HSP70 expression in *C. julis* from Riposto where HSP70 induction by low doses of contaminants was observed. In addition, no significant change in temperature was observed among the sampling sites such as to justify a temperature induced HSP70 modulation, so we conclude that the absence or the lowest concentrations of pollutants are responsible for the lowest HSP70 levels measured in the P.M.A.

Other authors (Webb and Gagnon, 2009) reported a high variation among individuals of *Acanthopagrus butcheri* in HSP70 levels in several tissue groups, and muscle provided the best discriminatory power to elucidate spatial variability.

Utilizing Comet assay to examine DNA damage provided both answers to the goal of the study and useful information about the importance of the method in evaluating the data. In fact, when the measurement of oxidative DNA damage was expressed as a percentage of total DNA damage, it represented about 23–25% of total damage and it did not vary among sites or tissues. Conversely when oxidative DNA damage was reported as 8-0xo-dG/10^6 Gua, significant differences were observed among sites: Augusta samples had the highest levels of oxidative damage while the lowest were found in the P.M.A.

The Augusta samples also showed a higher DNA fragmentation both in blood cells and in hepatic nuclei compared to Riposto and/or the P.M.A. samples, reflecting well with the observed levels of pollutants in water and sediments. Moreover, DNA fragmentation was higher in hepatic nuclei than in blood cells, with a trend already observed by us in *Parablennius sanguinolentus* (Tigano et al., 2009). Furthermore, high levels of DNA fragmentation were recorded in the liver of specimens of *Scophtalmus maximus* caught in an environment rich in PAHs and heavy metals (Hartl et al., 2007). Thus, it is likely that the liver bioaccumulates and biotransforms pollutants, causing genotoxic effects and probably enhancing oxidative damage (Ingelman-Sundberg and Hagbjork, 1982; Lemaire et al., 1994). In addition, it must be considered that circulating cells are usually less sensitive than hepatocytes or gill cells, since the erythrocytes have both a low metabolising capacity and high turnover rates. In our opinion, comet assay performed on fish-erythrocytes could be a excellent short-term assay for a rapid and in vivo evaluation of pollution in order to obtain a snapshot for a given point in time.

Comet assay data is largely confirmed by micronuclei analysis showing a high percentage of abnormalities in the Augusta samples compared to Riposto and/or the P.M.A. This data, in agreement with other authors, indicates that there is an increase in DNA damage in fish inhabiting polluted waters (de Andrade et al., 2004a; Kligermann, 1982).

5. Conclusion

Our data, confirming the use of *C. julis* as a good bioindicator in accordance with reported literature data (Bonacci et al., 2003; Fasulo et al., 2010; Sureda et al., 2006; Tigano et al., 2004), evidence that:

i) It is important to measure several biomarkers in order to correctly perform a biomonitoring program.
ii) There is a positive correlation at the Augusta site among concentrations of PAHs and PCBs, MTs levels and DNA damage.
iii) The Comet assay and MTs muscle expression in *C. julis*, represent a measurable biochemical response to water/sediment contamination, and may be considered valuable biomarkers in the assessment of pollution impact. However, tissue and specimen specificity need to be considered, together with variability in time and in the concentration of exposure.
iv) Attention should be paid in considering stress oxidative biomarkers such as oxidative DNA damage (8-hydroxyguanosine) and HSP70 levels as universal and absolute biomarkers because of hormetic effects and differences in response to acute or chronic pollution impact.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2012.09.012.

References

Akcha, F., Leday, G., Pfohl-Leszkowicz, A., 2004. Measurement of DNA adducts and strand breaks in dab (*Limanda limanda*) collected in the field: effects of biotic (age, sex) and abiotic (sampling site and period) factors on the extent of DNA damage. Mutat. Res. 552, 197–207.

Basu, N., Todgham, A.E., Ackerman, P.A., Bibeau, M.R., Nakano, K., Schulte, P.M., Iwama, G.K., 2002. Heat shock protein genes and their functional significance in fish. Gene 295, 173–183.

Berthet, B., Mouneyrac, C., Perez, T., Amiard-Triquet, C., 2005. Metallothionein concentration in sponges (Spongia officinalis) as a biomarker of metal contamination. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 141, 306–313.

Bonacci, S., Lancini, L., Iacocca, A., Corsi, I., Focardi, S., 2003. *C. julis* as a new Bioindicator Species For Pollution Monitoring In Marine Protected Areas: Application Of A Biomarker Approach, European Society for Comparative Physiology and Biochemistry Twentieth seconed Conference, Alessandria, p. 97.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Buschini, A., Martino, A., Gustavino, B., Monfrinotti, M., Poli, P., Rossi, C., Santoro, M., Dorr, A.J., Rizzoni, M., 2004. Comet assay and micronucleus test in circulating erythrocytes of Cyprinus carpio specimens exposed in situ to lake waters treated with disinfectants for potabilization. Mutat. Res. 557, 119–129.

Calabrese, E.J., 2008. Hormesis: why it is important to toxicology and toxicologists. Environ. Toxicol. Chem./SETAC 27, 1451–1474.

Carrasco, K.R., Tilbur, K.L., Myers, M.S., 1990. An assessment of piscine micronuclei test as an in situ biological indicator of chemical contaminant effect. Can. J. Fish. Aquat.Sci. 47, 2123–2136.

Clayton, M., Steinmann, R., Fent, K., 2000. Different expression patterns of heat shock proteins hsp 60 and hsp 70 in zebra mussels (Dreissena polymorpha) exposed to copper and tributyltin. Aquat. Toxicol. 47, 213–226.

Collins, A.R., Dusinska, M., Gedik, C.M., Stetina, R., 1996. Oxidative damage to DNA: do we have a reliable biomarker? Environ. Health Perspect. 104 (Suppl 3), 465–469.

Conti, G.O., Copat, C., Ledda, C., Fiore, M., Fallico, R., Sciacca, S., Ferrante, M., 2012. Evaluation of heavy metals and polycyclic aromatic hydrocarbons (PAHs) in *Mullus barbatus* from Sicily Channel and risk-based consumption limits. Bull. Environ. Contam. Toxicol. 88, 946–950.

Copat, C., Bella, F., Castaing, M., Fallico, R., Sciacca, S., Ferrante, M., 2012a. Heavy metals concentrations in fish from Sicily (Mediterranean Sea) and evaluation of possible health risks to consumers. Bull. Environ. Contam. Toxicol. 88, 78–83.

Copat, C., Maggiore, R., Arena, G., Lanzafame, S., Fallico, R., Sciacca, S., Ferrante, M., 2012b. Evaluation of a temporal trend heavy metals contamination in Posidonia oceanica (L.) Delile, (1813) along the western coastline of Sicily (Italy). J. Environ. Monit. 14, 187–192.

de Andrade, V.M., de Freitas, T.R., da Silva, J., 2004a. Comet assay using mullet (*Mugil* sp.) and sea catfish (*Netuma* sp.) erythrocytes for the detection of genotoxic pollutants in aquatic environment. Mutat. Res. 560, 57–67.

De Andrade, V.M., Silva, J., Silva, F.R., Heuser, V.D., Dias, J.F., Yoneama, M.L., Freitas, T.R., 2004b. Fish as bioindicators to assess the effects of pollution in two southern Brazilian rivers using th Comet assay and micronucleus test. Environ. Mol. Mutagen. 44, 459–468.

ESCODD, 2003. Measurement of DNA oxidation in human cells by chromatographic and enzymic methods. Free Radical Biol. Med. 34, 1089–1099.

Fasulo, S., Marino, S., Mauceri, A., Maisano, M., Giannetto, A., D'Agata, A., Parrino, V., Minutoli, R., De Domenico, E., 2010. A multibiomarker approach in *Coris julis* living in a natural environment. Ecotoxicol. Environ. Saf. 73, 1565–1573.

Flammarion, P., Devaux, A., Nehls, S., Migeon, B., Noury, P., Garric, J., 2002. Multibiomarker responses in fish from the Moselle River (France). Ecotoxicol. Environ. Saf. 51, 145–153.

Flora, S.J., Mittal, M., Mehta, A., 2008. Heavy metal induced oxidative stress & its possible reversal by chelation therapy. Indian J. Med. Res. 128, 501–523.

Frenzilli, G., Scarcelli, V., Del Barga, I., Nigro, M., Forlin, L., Bolognesi, C., Sturve, J., 2004. DNA damage in eelpout (Zoarces viviparus) from Goteborg harbour. Mutat. Res. 552, 187–195.

Griffiths, H.R., Moller, L., Bartosz, G., Bast, A., Bertoni-Freddari, C., Collins, A., Cooke, M., Coolen, S., Haenen, G., Hoberg, A.M., Loft, S., Lunec, J., Olinski, R., Parry, J., Pompella, S.B., Poulsen, H., Verhagen, H., Astley, S.B., 2002. Biomarkers. Mol. Aspects Med. 23, 101–208.

Hamza-Chaffai, A., Amiard, J.C., Pellerin, J., Joux, L., Berthet, B., 2000. The potential use of metallothionein in the clam Ruditapes decussatus as a biomarker of in situ metal exposure. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 127, 185–197.

Hartl, M.G., Kilemade, M., Sheehan, D., Mothersill, C., O'Halloran, J., O'Brien, N.M., van Pelt, F.N., 2007. Hepatic biomarkers of sediment-associated pollution in juvenile turbot. Scophthalmus maximus L. Marine environmental research 64, 191–208.

Hellou, J., 2011. Behavioural ecotoxicology, an "early warning" signal to assess environmental quality. Environ. Sci. Pollut. Res. Int. 18, 1–11.

Huang, X., Fang, C.W., Guo, Y.W., Huang, H.Q., 2011. Differential protein expression of kidney tissue in the scallop Patinopecten yessoensis under acute cadmium stress. Ecotoxicol. Environ. Saf. 74, 1232–1237.

Ingelman-Sundberg, M., Hagbjork, A.L., 1982. On the significance of the cytochrome P-450-dependent hydroxyl radical-mediated oxygenation mechanism. Xenobiotica; the fate of foreign compounds in biological systems 12, 673–686.

Ivanina, A.V., Taylor, C., Sokolova, I.M., 2009. Effects of elevated temperature and cadmium exposure on stress protein response in eastern oysters Crassostrea virginica (Gmelin). Aquat. Toxicol. (Amsterdam, Netherlands) 91, 245–254.

Ivankovic, D., Pavicic, J., Erk, M., Filipovic-Marijic, V., Raspor, B., 2005. Evaluation of the Mytilus galloprovincialis Lam. digestive gland metallothionein as a biomarker in a long-term field study: seasonal and spatial variability. Marine Poll. Bull. 50, 1303–1313.

Iwama, G.K., Thomas, P.T., Forsyth, R.B., Vijayan, M.M., 1998. Heat shock protein expression in fish. Rev. Fish Biol. Fisheries 8, 35–56.

Jebali, J., Ben-Khedher, S., Ghedira, J., Kamel, N., Boussetta, H., 2011. Integrated assessment of biochemical responses in Mediterranean crab (Carcinus maenas) collected from Monastir Bay, Tunisia. J. Environ. Sci. (China) 23, 1714–1720.

Kligermann, D., 1982. Fishes as biological detectors of the effects of genotoxic agents. In: Heddle, J. (Ed.), Mutagenicity: New Horizons in Genetic Toxicology. Academic Press, New York, pp. 435–456.

Knapen, D., Reynders, H., Bervoets, L., Verheyen, E., Blust, R., 2007. Metallothionein gene and protein expression as a biomarker for metal pollution in natural gudgeon populations. Aquat. Toxicol. (Amsterdam, Netherlands) 82, 163–172.

Kohler, H.R., Bartussek, C., Eckwert, H., Farian, K., Granzer, S., Knigge, T., Kunz, N., 2001. The epatic stress protein (hsp70) responce to interacting abiotic parameters in fish exposed to various levels of pollution. J. Aquat. Ecosyst. Stress Recov. 8, 261–279.

Kohler, H.R., Sandu, C., Scheil, V., Nagy-Petrica, E.M., Segner, H., Telcean, I., Stan, G., Triebskorn, R., 2007. Monitoring pollution in river Mures, Romania, Part III: Biochemical effect markers in fish and integrative reflection. Environ. Monit. Assess. 127, 47–54.

Lemaire, P., Matthews, A., Forlin, L., Livingstone, D.R., 1994. Stimulation of oxyradical production of hepatic microsomes of flounder (Platichthys flesus) and perch (Perca fluviatilis) by model and pollutant xenobiotics. Arch. Environ. Contam. Toxicol. 26, 191–200.

Matsumoto, S.T., Mantovani, M.S., Malagutti, M.I.A., Dias, A.L., Fonseca, I.C., Marin-Morales, M.A., 2006. Genotoxicity and mutagenicity of water contaminated with tannery effluents, as evaluated by the micronucleus test and comet assay using the fish Oreochromis niloticus and chromosomes aberrations in onion root-tips. Genet. Mol. Biol. 29, 148–158.

Nwani, C.D., Lakra, W.S., Nagpure, N.S., Kumar, R., Kushwaha, B., Srivastava, S.K., 2010. Mutagenic and genotoxic effects of carbosulfan in freshwater fish Channa punctatus (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. Food Chem. Toxicol. 48, 202–208.

Padmini, E., Usha Rani, M., 2008. Impact of seasonal variation on HSP70 expression quantitated in stressed fish hepatocytes. Comp. Biochem. Physiol. 151, 278–285.

Parolini, M., Binelli, A., Cogni, D., Provini, A., 2010. Multi-biomarker approach for the evaluation of the cyto-genotoxicity of paracetamol on the zebra mussel (Dreissena polymorpha). Chemosphere 79, 489–498.

Rocha, P.S., Luvizotto, G.L., Kosmehl, T., Bottcher, M., Storch, V., Braunbeck, T., Hollert, H., 2009. Sediment genotoxicity in the Tiete River (Sao Paulo, Brazil): in vitro comet assay versus in situ micronucleus assay studies. Ecotoxicol. Environ. Saf. 72, 1842–1848.

Rotchell, J.M., Clarke, K.R., Newton, L.C., Bird, D.J., 2001. Hepatic metallothionein as a biomaker for metal contamination: age effects and seasonal variation in European flounders (Pleuronectes flesus) from the Severn Estuary and Bristol Channel. Mar. Environ. Res. 52, 151–171.

Russo, C., Rocco, L., Morescalchi, M.A., Stingo, V., 2004. Assessment of environmental stress by the micronucleus test and the Comet assay on the genome of teleost populations from two natural environments. Ecotoxicol. Environ. Saf. 57, 168–174.

Sanchez, W., Ait-Aissa, S., Palluel, O., Ditche, J.M., Porcher, J.M., 2007. Preliminary investigation of multi-biomarker responses in three-spined stickleback (Gasterosteus aculeatus L.) sampled in contaminated streams. Ecotoxicology (London, England) 16, 279–287.

Sanders, B.M., Martin, L.S., 1993. Stress proteins as biomarkers of contaminant exposure in archived environmental samples. Sci. Total Environ. 139-140, 459–470.

Sasaki, Y.F., Izumiyama, F., Nishidate, E., Ishibashi, S., Tsuda, S., Matsusaka, N., Asano, N., Saotome, K., Sofuni, T., Hayashi, M., 1997. Detection of genotoxicity of polluted sea water using shellfish and the alkaline single-cell gel electrophoresis (SCE) assay: a preliminary study. Mutat. Res. 393, 133–139.

Shariati, F., Shariati, S., 2011. Review on methods for determination of metallothioneins in aquatic organisms. Biol. Trace Elem. Res. 141, 340–366.
Sinaie, M., Bastami, K.D., Ghorbanpour, M., Najafzadeh, H., Shekari, M., Haghparast, S., 2010. Metallothionein biosynthesis as a detoxification mechanism in mercury exposure in fish, spotted scat (Scatophagus argus). Fish Physiol. Biochemistry 36, 1235–1242.
Smolkova, B., Dusinska, M., Raslova, K., McNeill, G., Spustova, V., Blazicek, P., Horska, A., Collins, A., 2004. Seasonal changes in markers of oxidative damage to lipids and DNA; correlations with seasonal variation in diet. Mutat. Res. 551, 135–144.
Sokolova, I.M., Lanning, G., 2008. Interactive effects of metal pollution and temperature on metabolism in aquatic ectotherms: implications of global climate change. Clim. Res. 37, 181–201.
Steinert, S.A., Streib-Montee, R., Leather, J.M., Chadwick, D.B., 1998. DNA damage in mussels at sites in San Diego Bay. Mutat. Res. 399, 65–85.
Sureda, A., Box, A., Ensenat, M., Alou, E., Tauler, P., Deudero, S., Pons, A., 2006. Enzymatic antioxidant response of a labrid fish (*C. julis*) liver to environmental caulerpenyne. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 144, 191–196.
Tigano, C., Ferrito, V., Calandra, M.L., Scifo, C., Renis, M., 2004. Stima della genotossicità di ambienti marini inquinati tramite Comet assay in pesci della costa ionica siciliana, 65° Congresso Unione Zoologica Italiana. Giardini Naxos (ME), 136.
Tigano, C., Tomasello, B., Pulvirenti, V., Ferrito, V., Copat, C., Carpinteri, G., Mollica, E., Sciacca, S., Renis, M., 2009. Assessment of environmental stress in Parablennius sanguinolentus (Pallas, 1814) of the Sicilian Ionian coast. Ecotoxicol. Environ. Saf. 72, 1278–1286.
van der Oost, R., Beyer, J., Vermeulen, N.P., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57–149.
Vijayavel, K., Balasubramanian, M.P., 2008. DNA damage and cell necrosis induced by naphthalene due to the modulation of biotransformation enzymes in an estuarine crab Scylla serrata. Journal of Biochem. Mol. Toxicol. 22, 1–7.
Wang, Y., Xu, J., Sheng, L., Zheng, Y., 2007. Field and laboratory investigations of the thermal influence on tissue-specific Hsp70 levels in common carp (Cyprinus carpio). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 148, 821–827.
Webb, D., Gagnon, M.M., 2009. The value of stress protein 70as an environmental biomarker of fish health under field conditions. Environ. Toxicol. 24, 287–295.
Whitfield, A.K., Elliot, M., 2002. Fishes as indicators of environmental and ecological changes within estuaries: a review of progress and some suggestion for the future. J. Fish Biol. 61, 229–250.