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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