

Expression of 8-OHdG in *Zosterisessor ophiocephalus* from the Venetian lagoon, Italy

E. Negrato,¹ M. Vascellari,² F. Capolongo,¹ G. Binato,² L. Da Dalt,¹ M. Boscolo Papo,¹ G. Gioacchini,³ O. Carnevali,³ D. Bertotto,¹ G. Radaelli,¹ F. Pascoli¹

¹Dipartimento di Biomedicina Comparata e Alimentazione, Università di Padova, Legnaro (PD); ²Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro (PD); ³Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Ancona, Italy

Abstract

The aim of the present work was to evaluate the expression of 8-OHdG (8-hydroxy-2'-deoxyguanosine) in the benthic fish *Zosterisessor ophiocephalus* collected in two differently polluted sites of the Venetian lagoon (Porto Marghera and Caroman). We compared our data on 8-OHdG with those of CYP1A (Cytochrome P450, family 1, subfamily A, polypeptide 1), which is a well known biomarker for detoxification of contaminants. Immunohistochemistry with an antibody to 8-OHdG showed immunopositivity in nuclei of hepatocytes as well as in melanomacrophage centres of spleen and kidney, whereas an anti-CYP1A antibody exhibited positive immunostaining in the liver, kidney and ovary. The liver of males showed higher expression of both proteins than females. In animals from Porto Marghera site, the enzymatic assay for 8-OHdG exhibited higher levels in liver of males than in females. Western Blot analysis using the antibody anti-CYP1A recognized the presence of a band of about 60 kDa in the liver of males and females. Males exhibited a strong band, whereas in females the band showed a lower intensity. By using Real-Time PCR, the mRNA expression of CYP1A did not show any differences between males and females from each site, but it was at borderline significance level. Comparing the two sites, mRNA expression of CYP1A was significantly higher in the liver of both males and females from Porto Marghera than that of Caroman. The present data suggest that pollutants are bio-available as demonstrated by our biomarker analyses and may have a harmful effect on aquatic organisms such as *Z. ophiocephalus*. We report that the highest levels of hepatic 8-OHdG and CYP1A expression were detected in males, showing clear gender specificity.

Introduction

By definition, a biomarker is a biological response that can be related to an exposure to, or a toxic effect of an environmental chemical or chemicals.¹⁻³ Generally, biomarker responses provide qualitative and semi-quantitative information on the nature of the chemical insult and information on the relationship between the biological effects and levels of environmental contamination.¹

At cellular level, the metabolism of environmental stressors frequently results in the formation of reactive oxygen species (ROS).⁴ They are produced naturally during metabolism and their toxic effects are usually prevented by antioxidants, both molecular and enzymatic ones. During oxidative stress conditions, the production of ROS is greater than the ability of cells to remove them, leading to lipid peroxidation, protein carbonyl formation and DNA damage.⁵ DNA damage may include single and double strand breaks and the modification of bases, such as the oxidation of deoxyguanosine to form 8-hydroxy-2'-deoxyguanosine (8-OHdG).⁶ The high biological relevance of 8-OHdG is due to its ability to induce G to T transversions.⁷

The Cytochrome P4501A (CYP1A) subfamily is involved in the biotransformation of a variety of contaminants such as polychlorodibenzo dioxins (PCDDs), polycyclic aromatic hydrocarbons (PAHs), polyhalogenated aromatic hydrocarbons (PHAHs) and polychlorobiphenyls (PCBs).⁸ The induction of CYP1A mRNA and its protein is known to occur via high-affinity binding of contaminants to the cytosolic Ah (Aryl hydrocarbon) receptor.⁹ In fish, CYP1A is a widely accepted environmental biomarker, detecting biological effects of several xenobiotic groups (oil compounds, dioxins, PCBs, PAHs, etc.) present in aquatic environments, when evaluated in target tissues of a sentinel species.^{8,10} Although the liver represents the main site of CYP1A expression in fish,^{7,8} CYP1A appearance and induction is also detectable in numerous extrahepatic organs.¹¹ Interestingly, the epithelia of organs in direct contact with the environment (gills, intestine and kidney) and the vascular endothelia exhibit a CYP1A expression.¹¹

The Venice lagoon is a shallow transitional environment composed by a complex combination of intertidal marshes, mudflats, submerged mudflats and channels. Recent data¹² suggest that the areas located in front of Porto Marghera are the most polluted of the Venice lagoon, whereas the Caroman site exhibits a lower concentration of pollutants.

The present study aims to investigate the expression and the localization of 8-OHdG in relationship to the exposure to contaminants in the grass goby *Z. ophiocephalus*, that is an edible species of local commercial importance, col-

Correspondence: Giuseppe Radaelli, Dipartimento di Biomedicina Comparata e Alimentazione, Università degli Studi di Padova, Agripolis, viale dell'Università 16, 35020 Legnaro (PD), Italy. E-mail: giuseppe.radaelli@unipd.it

Key words: OHdG, CYP1A, fish, immunohistochemistry, molecular biology.

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lected in two differently polluted sites of the Venetian lagoon (Porto Marghera and Caroman). 8-OHdG is a fairly new biomarker of oxidative stress and very few data are available in literature. In order to support those results, the expression of 8-OHdG was compared to CYP1A used as a positive control of contaminant exposure (oil compounds, dioxins, PCBs, PAHs, etc.). Furthermore, PCB content in fish muscle was evaluated. PCBs belong to the most persistent, bioaccumulative and toxic pollutants. It is well established that the dioxin-like congeners can bind to and activate the aryl hydrocarbon receptor (AhR), a normally inactive transcription factor. The overactivation of the AhR can induce enzymes that produce cytotoxic metabolites or otherwise adversely affect cellular metabolism. Consequently, in order to assess the bio-availability of PCBs in the sediment, it has been performed analyses on the lateral muscle tissue of *Z. ophiocephalus* obtained from both sites.

Materials and Methods

Sampling site

One sampling site was selected near the industrial area of Porto Marghera (Central-Northern basin) and the other site was located at Caroman (southern basin) on the basis of data reported in the literature¹² (Figure 1). The

sampling was performed during March and April of 2008.

Animals and preparation

Two hundred and fifty-one adult fish (*Z. ophiocephalus*), 112 males and 139 females, of similar size (standard length 14.9 ± 0.3 cm; weight 63.5 ± 4.1 g) were caught with home made net traps at the Porto Marghera and Caroman sites, immediately transferred to the laboratory (by boat in aerated and insulated bags with sampling site water) and anaesthetized with MS222 (Sandoz, Origgio, Italy) before sacrifice. After the sacrifice, the celomic cavity was opened and different organs were sampled. The experiments comply with current Italian laws on the use of animals in scientific work.

For RNA expression, Western blot and 8-OHdG evaluation, samples of liver were immediately frozen in liquid nitrogen and stored at -80°C until analysis. Samples of liver from 20 animals/site (10 males and 10 females) were collected and aliquoted for each analysis. For immunohistochemistry, in addition to liver, several other organs were sampled (spleen, kidney, ovary and testis) from the 20 animals/site and fixed in 4% paraformaldehyde prepared in phosphate-buffered saline (PBS, 0.1 M, pH 7.4) at 4°C overnight, washed in PBS, dehydrated through a graded series of ethanol and embedded in paraffin. Consecutive sections were cut at a thickness of $4 \mu\text{m}$ using a microtome. For analysis of PCBs in muscle (Table 1), which represents the main source of edible tissue, three adult fish of the same sex were pooled on the basis of a similar Liver Somatic Index (LSI) the analytical requirements samples were organized in *macropools* (MP), each composed of three pooled samples (9 individuals in total). In total, 19 MP were organized: 11MP (99 individuals) for females (6MP for Porto Marghera; 5MP for Caroman); 8 MP (72 individuals) for males (5MP for Porto Marghera, 3MP for Caroman). Samples were stored at -80°C until the analysis.

Pollutants in sediments

Data concerning concentrations of heavy metals, acid volatile sulphides (AVS), PCBs, hexachlorobenzene (HCB) and PAHs in the upper sediment layer (0-15 cm) from the site of sampling (Porto Marghera and Caroman) were provided by *Ministero delle Infrastrutture, Magistrato alle Acque di Venezia, Consorzio Venezia Nuova* and are reported in Table 2.

RNA extraction and RT-PCR

Total RNA was extracted from 50 mg of liver using TRIzol[®] Reagent (Invitrogen, Milan, Italy).¹³ CYP1A primers were designed on the basis of the homology among the sequences available in GenBank. cDNA was amplified

with 5 units of Taq DNA polymerase (DynaZyme) in 20 μl of master mix containing 1 x PCR buffer, 1.5 mM MgCl_2 , 2.5 mM dNTPs, and CYP1A primers (forward: 5'-CCCTGCA-GACTTCATCCC-3', reverse: 5'-TTTGT-GCTTCATTGTGAGACC-3'). PCR amplification of CYP1A was carried out for 32 cycles with the following profile: denaturation at 94°C for 1 min, primer annealing at 58°C for 90 s, and primer extension at 72°C for 1 min. To verify the efficiency of the reverse transcription (RT) and to exclude genomic DNA contamination, a fragment (130bp) of 18S was amplified with primers designed to span an intron: (forward: 5'-GCCCTTCCGTCAAATCCTTT-3', reverse: 5'-AACGGTGCCAACTAGCGATC-3'). PCR products were electrophoresed on a 1.5% agarose gel and visualized under UV light.

Real-Time PCR

Real-Time PCRs were performed with SYBR green method in a iQ5 iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). Triplicate PCR reactions were carried out for each sample analyzed. The reactions were set on a 96-well plate by mixing, for each sample, 1 μL of dilut-

Table 1. Individual indicator PCB congeners evaluated in muscle of *Z. ophiocephalus*.

PCB congeners	
PCB 28	2,4,4' - trichlorobiphenyl
PCB 52	2,2',5,5' - tetrachlorobiphenyl
PCB 95	2,2',3,5',6 - pentachlorobiphenyl
PCB 99	2,2',4,4',5 - pentachlorobiphenyl
PCB 101	2,2',4,5,5' - pentachlorobiphenyl
PCB 105	2,3,3',4,4' - pentachlorobiphenyl
PCB 110	2,3,3',4,6 - pentachlorobiphenyl
PCB 118	2,3',4,4',5 - pentachlorobiphenyl
PCB 138	2,2',3,4,4',5' - hexachlorobiphenyl
PCB 146	2,2',3,4,4',5' - hexachlorobiphenyl
PCB 149	2,2',3,4',5',6 - hexachlorobiphenyl
PCB 151	2,2',3,5,5',6 - hexachlorobiphenyl
PCB 153	2,2',4,4',5,5' - hexachlorobiphenyl
PCB 170	2,2',3,3',4,4',5 - heptachlorobiphenyl
PCB 177	2,2',3,3',4,4',5,6 - heptachlorobiphenyl
PCB 180	2,2',3,4,4',5,5' - heptachlorobiphenyl
PCB 183	2,2',3,4,4',5',6 - heptachlorobiphenyl
PCB 187	2,2',3,4',5,5',6 - heptachlorobiphenyl



Figure 1. Map of the Venice Lagoon indicating the toxicity of sediments on the basis of pollutant concentrations (modified from *Ministero delle Infrastrutture, Magistrato alle Acque di Venezia, Consorzio Venezia Nuova*).

ed (1/20) cDNA, 5 μ L of 2X concentrated iQ TM SYBR Green Supermix (Bio-Rad), containing SYBR Green as a fluorescent intercalating agent, 0.3 μ M of forward primer and 0.3 μ M of reverse primer.¹⁴ The thermal profile for all reactions was 3 min at 95°C and then 45 cycles of 20 s at 95°C, 20 s at 58°C and 20 s at 72°C. Fluorescence monitoring occurred at the end of each cycle. Additional dissociation curve analysis was performed and showed in all cases one single peak. The 18S was used as an internal reference in each sample in order to standardize the results by eliminating variation in mRNA and cDNA quantity and quality. No amplification product was observed in negative controls and no primer dimer formation was observed in the control templates. The data obtained were analyzed using the iQ5 optical system software version 2.0 (Bio-Rad).

Immunohistochemistry

Immunohistochemical staining was done using the Envision system (goat anti-rabbit or goat anti mouse immunoglobulins conjugated to peroxidase-labeled complex, Dakocytomation, Milan, Italy). Before applying the primary antibodies overnight at 4°C, endogenous peroxidase activity was blocked by incubating the sections in 3% H₂O₂ in PBS (phosphate buffered saline). Non-specific binding sites were blocked by incubating the sections in normal goat serum (Dakocytomation). For CYP1A, a polyclonal antibody was raised against peptides 190-202 and 282-296 of rainbow trout CYP1A (Biosense laboratories, Bergen, Norway) and used at dilution of 1:200. A monoclonal antiserum against mouse 8-OHdG (ab48508; Abcam, Cambridge, MA, USA) was used at a dilution of 1 μ g/mL. The immunoreactive sites were visualized using a freshly prepared solution of 10 mg of 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, Milan, Italy) in 15 mL of a 0.5 M Tris buffer at pH 7.6, containing 1.5 mL of 0.03% H₂O₂. To ascertain structural details, sections were counterstained with Mayer's haematoxylin.

The specificity of the immunostaining was verified by incubating sections with: i) PBS instead of the specific primary antibody; ii) preimmune sera instead of the primary antiserum; iii) PBS instead of the secondary antibodies; iv) antiserum which was pre-absorbed with an excess of respective synthetic peptide (3 μ g/mL) before incubation with sections. The results of these controls were negative (*i.e.*, staining was abolished). Samples were evaluated for the presence and distribution of immunopositivity, and a grade from negative (-) to strong (+++) was assigned to the intensity of the reaction in liver samples.

Enzymatic immunoassay for 8-OHdG

Total DNA was purified from liver tissue

using the DNeasy® Blood and Tissue Kit (Qiagen, Milan, Italy) following the manufacturer's protocol. To assess the purity and the amount of DNA extracted, spectrophotometric A260/A280 readings were performed.

The 8-OHdG concentrations in DNA samples were determined using an enzyme immunoassay EIA kit (Cayman Chemical Co., Ann Arbor, MI, USA) according to the manufacturer's protocol. Sample preparation was performed by digesting DNA using nuclease P1 (Sigma-Aldrich, St. Louis, MO, USA) and adding 1 unit of alkaline phosphatase per 100 μ g of DNA. The product of enzymatic reaction was determined reading the plate at 405 nm. The 8-OHdG concentrations were expressed as pg of 8-OHdG per μ g of DNA.

Western blot

Protein concentration from liver was measured by Bradford assay (Sigma-Aldrich) using BSA as standard. 24 μ g of liver homogenate was separated using 10% SDS-polyacrylamide gel electrophoresis under reduction conditions and transferred to nitrocellulose filter (GE Healthcare, UK) at 250 mA for 2 h at 4°C. Filters were treated with blocking solution (3% non-fat milk, 0.5% Tween-20 in Tris-buffered saline, TBS, pH 7.6) overnight at 4°C to prevent nonspecific binding and then incubated with the primary polyclonal rabbit CYP1A antiserum (Biosense Laboratories) in 3% blocking solution for 60 min.¹⁵ Membranes were next incubated with HRP-labeled goat anti-rabbit IgG diluted 1:8000 (Bio-Rad) for 60 min. All membranes were visualized using Chemiluminescent HRP substrate (Millipore, Billerica, MA, USA).

PCBs in muscle

Sample preparation

Lateral muscle of *Z. ophiocephalus* was carefully homogenised before analysis. 70 g of each macropooled sample (see *Animals and preparation*) was then weighed and thoroughly mixed with an equivalent amount of sodium sulphate; the sample was then extracted with 100 mL of n-hexane/acetone 50:50 in ultrasonic bath for 10 minutes; this procedure was repeated three times. The combined organic phase was filtered through sodium sulphate, after which the solvent was evaporated. The lipid content was determined by weighing.

Clean-up

2.5 g of fat dissolved in 25 mL n-hexane was cleaned up by a double SPE extraction [Extrelut NT20 (VWR International, Lutterworth, UK) and Florisil FL-PR (Phenomenex, Torrance, CA, USA)]. The eluate was evaporated under a gentle stream of nitrogen, the residue was re-dissolved in 200 μ L of n-hexane.

GC - μ ECD analysis

The final sample was analysed by dual column GC- μ ECD using an Agilent 6890 Plus (Agilent Technologies, Palo Alto, CA, USA) gaschromatograph equipped with double μ ECD ⁶³Ni detectors, split/splitless injection port and an 7683B autosampler injector. Capillary column apparatus: Zebron ZB5 (30 m \times 0.25 mm i.d. \times 5 μ m f.t.) and Zebron ZB1701 (30 mt \times 0.25 mm i.d. \times 0.25 μ m f.t.). Both columns were connected to a splitter; the splitter to injector connection was made with a Zebron

Table 2. Concentrations of heavy metals, acid volatile sulphides (AVS), polychlorinated biphenyl (PCBs), hexachlorobenzenes (HCBs) and polycyclic aromatic hydrocarbons (PAHs) in the upper sediment layer (0-15 cm) from different sites of the Venice lagoon. Values are expressed as means of three years of samplings (Ministero delle Infrastrutture, Magistrato alle Acque di Venezia, Consorzio Venezia Nuova, modified from Pascoli *et al.*³³).

	Porto Marghera	Caroman
mg/Kg d.w.		
As	17.5	6.4
Cd	2.3	0.4
Cr	48.5	45.5
Cu	49.3	8.0
Hg	1.5	0.1
Ni	23.0	16.0
Pb	46.1	10.1
μ g/Kg d.w.		
Zn	353.7	48.5
AVS	224.8	42.3
Σ PCB*	4.6	0.3
Aroclor*	31.5	1.9
HCB	11.5	0.3
Σ PAH ^o	2473.7	324.7

d.w., dry weight. *PCB concentrations were determined as total Aroclor (calculated as the sum of a (1:1) 1254 and 1260 mixtures) and the sum (Σ PCB) of 7 congeners (77,105,118,156,167,170,180). ^oPAH concentrations were determined as the sum of 15 compounds.

Retention Gap 5 m × 0.25 mm i.d. uncoated guard column (Phenomenex).

Quantification of all target analytes according to their peak area was performed using a five point calibration curve (1, 5, 10, 20, 30 ng/mL). Recoveries of the target compound were found to be between 70 and 120%; the limit of detection (LOD) was 1 ng/mL for each PCB congener. Results were expressed as ng/g on fat.

Statistical analysis

Statistical analysis was carried out for Real-Time PCR and EIA data with STATISTICA 10 (StatSoft). All data are reported as mean ± SEM. Differences between samples were tested with Test U di Mann-Whitney. For PCBs analyses in muscle, data were reported as mean ± SD. Means were compared by two-tailed unpaired t-Test (GraphPad Prism 5.0). In all analyses a P<0.05 value was accepted as significant.

Results

8-OHdG levels determined by enzymatic immunoassay

In liver of males from Porto Marghera, 8-OHdG concentrations were significantly higher than those observed in females (P<0.01), whereas no differences were detected among males and females from Caroman (Table 3). No differences were detected comparing the two sites.

8-OHdG immunohistochemistry

The anti-8-OHdG antibody revealed an immunostaining in nuclei of hepatocytes as well as in melanomacrophage centres of spleen and kidney (Figure 2A-D). In both sites, liver of males showed higher expression of 8-OHdG than females, whereas no differences were detected among sites (Figure 2A,B; Table 4). No differences were detected comparing the two sites, although a tendency towards a significance was evidenced among males.

Analysis of PCBs in muscle

Results reported in Table 5 are expressed as the mean of the sum of the 18 different congeners detected in each macropooled sample analysed. In each sites, the mean level of total PCBs found in tissue from males was 1.7-2.9 time greater than that of females. These results were significantly different (P<0.01) between males and females from Porto Marghera.

Expression of CYP1A mRNA by Real-Time PCR

By using Real-Time PCR, mRNA expression

Table 3. Concentrations of 8-OHdG evaluated in liver of *Z. opiocephalus* expressed as pg/μg of DNA (N number of samples analysed). Data presented as mean±SEM. Significant differences between genders are indicated by different letters, as determined by test U Mann-Whitney.

	N	Porto Marghera	Caroman
Males	10	1.09±0.09 ^a	0.72±0.08
Females	10	0.56±0.05	0.54±0.04

^aMales vs females, P<0.01.

Table 4. Grade of immunopositivity to anti-8-OHdG antibody in *Z. opiocephalus* liver.

Samples	Porto Marghera		Caroman	
	M	F	M	F
1	+++	++	++	+
2	+++	+	+++	+
3	+++	+	++	+/-
4	++	+	+++	+
5	+++	+	+++	+
6	++	+	+++	+/-
7	+++	+	+++	+
8	+++	+	++	+/-
9	+++	+	+++	+
10	++	+	++	+

+/-, positivity mainly observed in the endothelial cells of arteries and veins; +, moderate positivity; ++, marked positivity; +++, strong positivity.

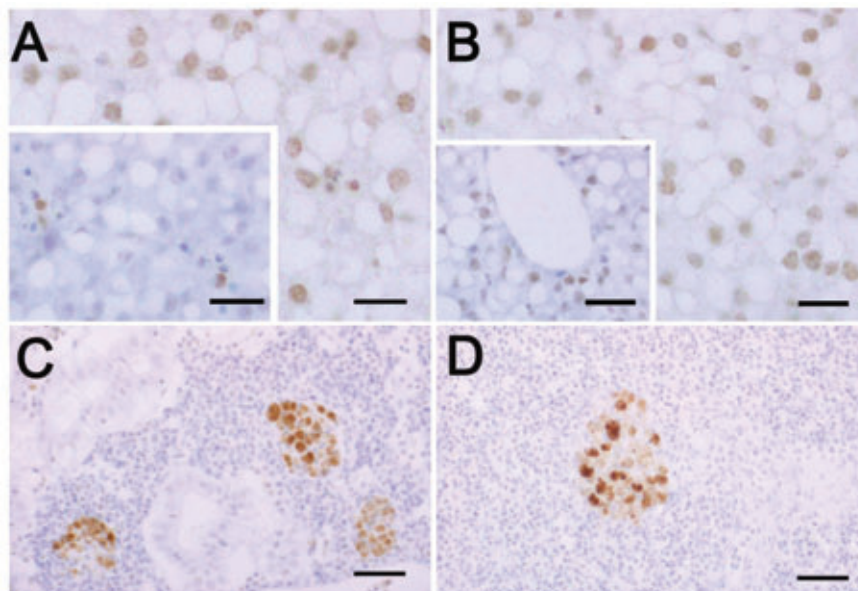


Figure 2. Immunohistochemical localization of 8-OHdG in *Z. opiocephalus*. All sections are counterstained with Mayer's haematoxylin. A) Liver of a male from Porto Marghera site, which exhibits a strong 8-OHdG immunostaining in nuclei of hepatocytes; insert in A shows the liver parenchyma of a female, which exhibits a faint immunostaining; scale bar: 10 μm, insert: 6 μm. B) Liver of a male from Caroman site, which exhibits a strong 8-OHdG immunostaining in nuclei of hepatocytes; insert in B shows the liver parenchyma of a female, which exhibits a faint immunostaining; scale bar: 10 μm, insert: 6 μm. C) Kidney of a male from Porto Marghera site, which exhibits a 8-OHdG immunostaining in melanomacrophage centers; scale bar: 20 μm. D) Spleen of a male from Porto Marghera site showing a 8-OHdG immunostaining in melanomacrophage centers; scale bar: 20 μm.

of CYP1A was significantly higher in the liver of both males and females from the Porto Marghera site if compared respectively to males and females from Caroman (M, $P < 0.01$; F, $P < 0.05$) (Figure 3). In each site, the liver mRNA expression of CYP1A did not show a difference between genders (Figure 3), but a tendency towards a significance was evidenced.

CYP1A immunohistochemistry

The anti-CYP1A antibody exhibited positive immunostaining in the liver, kidney and ovary, whereas spleen and testis were negative. In the liver, a granular immunostaining was diffusely detected in the cytoplasm of hepatocytes as well as in the endothelial cells of hepatic arteries and veins (Figure 4A,B). In both sites, male liver showed higher expression of CYP1A protein than females, whereas no differences were detected among sites (Figure 4A,B; Table 6). In the kidney of animals from both sites, CYP1A immunoreactivity was found in the epithelial cells of the proximal tubules (Figure 4C) and in the endothelial cells of vessels (Figure 4, insert in C), whereas the glomeruli were immunonegative. In the ovary of females from both sites, CYP1A immunostaining was found in the cortical cytoplasm under the plasma membrane (Figure 4D).

CYP1A Western blot

Western blot analysis using the antibody raised against peptides 190-202 and 282-296 of rainbow trout CYP1A confirms that the antibody cross-reacts specifically with CYP1A of *Z. ophiocephalus* and recognizes the presence of a band of about 60 kDa in the liver of males and females (Figure 5).

As shown in Figure 5, liver of males from Porto Marghera site exhibited a band of the strongest intensity at 60 kDa, whereas in females from both sites, the band showed the lowest intensity (Figure 5). The intensity of the band was higher in males from the Porto Marghera site compared to the males from Caroman.

Discussion

In recent years there has been an increasing interest in studies aimed at bio-monitoring environmental pollution in the Venice lagoon.^{11,12} According to Losso and Volpi Ghirardini,¹² the WATI (Weighted Average Toxicity Index) shows that very high toxicity sites are located in the Central-Northern Basin, closed to the industrial area of Porto Marghera. The Southern Basin, which includes the Caroman site, shows a general medium toxicity, probably due to urban contamination from the town of Chioggia.

In this study, the hepatic levels of 8-OHdG

and its expression have been evaluated in the grass goby *Z. ophiocephalus* sampled in the sites of Porto Marghera and Caroman, to test if there is DNA damage generated by nucleic base modification by pollutants, such as the oxidation of deoxyguanosine to form 8-OHdG. In fish, measurement of 8-OHdG has been used as a novel index of DNA damage induced by pollutants.^{6,16-20}

The 8-OHdG levels determined by an enzymatic immunoassay were significantly higher in the liver of males from the Porto Marghera than those of females, showing a clear gender specificity. No differences were detected comparing the two sites, although a tendency towards a significance was evidenced among males. In both sites, the immunohistochemical analysis in male liver (hepatocyte nuclei)

Table 5. Concentration of total PCBs in pooled samples of *Z. ophiocephalus* expressed as ng/g fat (n number of pooled samples analysed). Data are presented as means \pm SD.

	Porto Marghera Total PCBs (mean \pm SD)	Caroman Total PCBs (mean \pm SD)
Males	3603 \pm 632 (n=5) ^{a,b}	1397 \pm 1039 (n=3)
Females	2081 \pm 455 (n=6) ^b	481 \pm 179 (n=5)

^aMales vs females, $P < 0.01$; ^bPorto Marghera vs Caroman, $P < 0.001$.

Table 6. Grade of immunopositivity to anti-CYP1A antibody in *Z. ophiocephalus* liver.

Samples	Porto Marghera		Caroman	
	M	F	M	F
1	++	+/-	+++	+
2	+++	+/-	+++	+/-
3	+++	+/-	++	+/-
4	++	+/-	+++	+/-
5	++	+/-	++	+
6	+++	+/-	+++	+/-
7	+++	+/-	++	+/-
8	++	+/-	++	+/-
9	+++	+/-	+++	+
10	++	++	+++	+/-

+/-, positivity mainly observed in the endothelial cells of arteries and veins; +, moderate positivity; ++, marked positivity; +++, strong positivity.

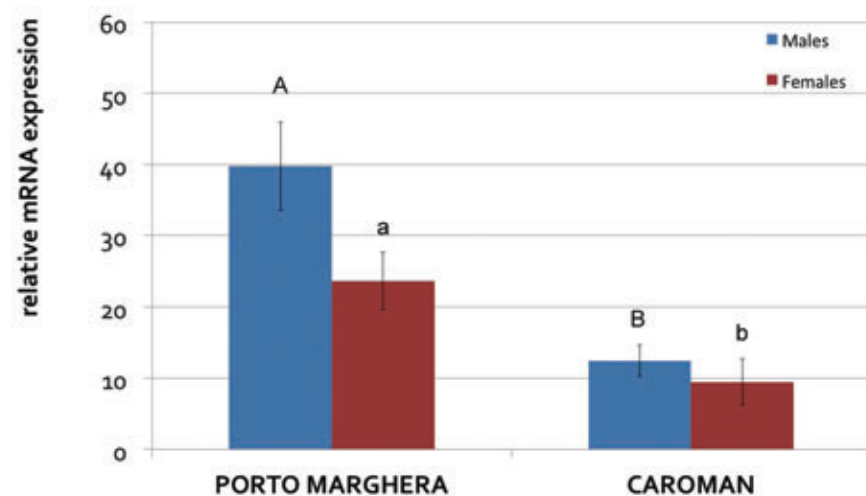


Figure 3. Quantitative relative CYP1A mRNA expression in *Zosterisessor ophiocephalus*. Data are expressed as mean \pm SEM. Different letters indicate significant differences ($P < 0.05$).

revealed an immunopositivity to 8-OHdG higher than that observed in females.

Since sediments exhibit higher contaminants concentrations respect to those of the water column (as observed from data provided by *Ministero delle Infrastrutture, Magistrato alle Acque di Venezia*), higher levels of hepatic 8-OHdG detected in males than in females, suggest a higher exposure of males to pollutants, probably due to their reproductive behaviour. *Z. ophiocephalus* is a benthic species, and during the breeding season, lasting from March to June, males dig and defend a nest under the sea grass rhizomes, where they court females, mate, and perform paternal embryo care.²¹ As a consequence of this behavior, males spend a lot of time in nests, in contact with sediments, whereas females stay in nests only for spawning their eggs.²¹

Since it is by now well established in vertebrates that interesting variations in oxidative balance often cannot be sufficiently represented by a single variable,^{22,23} we evaluated the expression of hepatic CYP1A and the PCBs content in muscle, in order to support the results obtained by 8-OHdG analyses. Although it is known that numerous contaminants are able to induce an oxidative stress, we decided to focus on PCB which are one of the most representative compounds useful to evaluate a CYP1A induction. The levels of PCBs in *Z. ophiocephalus*, performed by gas chromatography were significantly higher in muscle specimens of both males and females from the Porto Marghera site if compared respectively to those of males and females from Caroman. Moreover, in animals from Porto Marghera, males showed mean levels significantly higher than those observed in females, confirming a clear gender specificity. In animals from Caroman, no differences were detected comparing males and females, although a tendency towards a significance was detected, still confirming a gender specificity. Induction of CYP1A expression is a biomarker frequently used to indicate exposure to a variety of contaminants in fishes because of its high sensitivity, dose responsiveness, and wide window of response.^{24,25} In fish, CYP1A reveals biological effects of several xenobiotic groups (oil compounds, dioxins, PCBs, etc.) present in aquatic environments, when evaluated in target tissues of a sentinel species.⁸ In the present study, CYP1A was used as positive control of xenobiotic presence.

The expression of hepatic CYP1A mRNA evaluated by Real Time PCR was significantly higher in both males and females from the Porto Marghera site if compared respectively to that of males and females from Caroman. Owing to the high number of industries present all around the Porto Marghera site, it is probable that this is the most polluted area of the Venice lagoon, confirming recent data by Losso and Volpi Ghirardini.¹² Moreover, the

CYP1A mRNA expression reached only a tendency to significance for a difference between genders in each site, but Western blot analysis showed higher levels of protein in males, highlighting a marked sex differences and suggesting a greater exposure of males to pollutants

concentrated in the sediment during reproductive phase. Our results are also in accordance with those of other authors who recently demonstrated that CYP1A mRNA expression levels correlate with the concentration of contaminants in sediments from the sites inhabit-

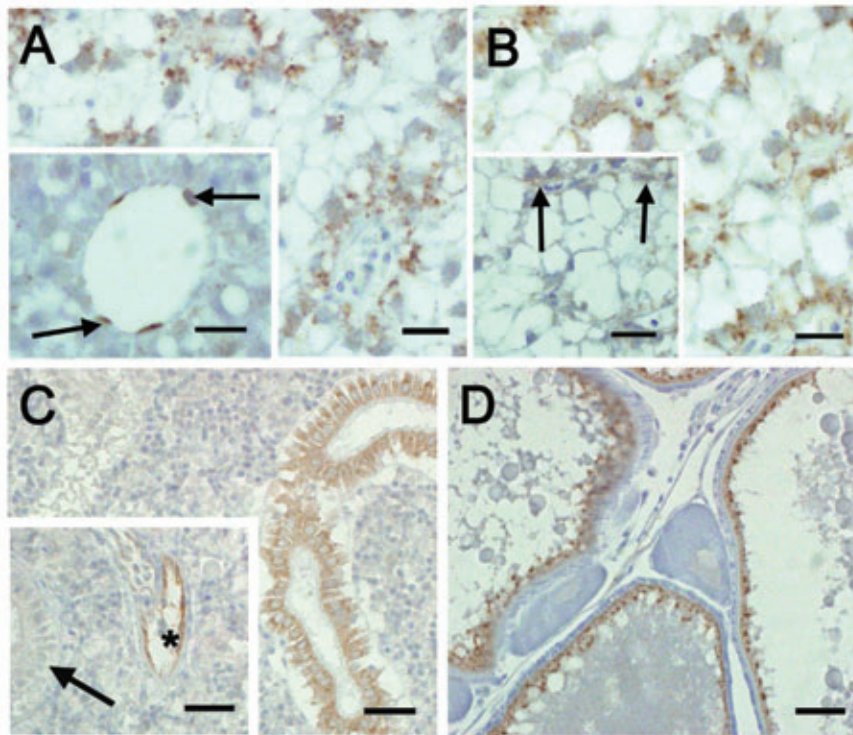


Figure 4. Immunohistochemical localization of CYP1A in *Z. ophiocephalus*. All sections are counterstained with Mayer's haematoxylin. A) Liver of a male from Porto Marghera site, which exhibits a strong CYP1A immunostaining; insert shows the liver parenchyma of a female, which exhibits a faint immunostaining in the cytoplasm of hepatocytes; arrow indicates immunostaining in endothelial cell; scale bar: 10 μ m, insert: 6 μ m. B) Liver of a male from Caroman, which exhibits a strong immunostaining in the cytoplasm of hepatocytes; insert shows the liver parenchyma of a female, which exhibits a faint immunostaining in the cytoplasm of hepatocytes; scale bar: 10 μ m, insert: 6 μ m. C) Kidney of a male from Porto Marghera site, which exhibits a CYP1A immunostaining in the cytoplasm of the epithelial cells of proximal tubules; insert shows positive endothelial cells in the kidney parenchyma (asterisk) and negative epithelial cells of distal tubules (arrow); scale bar: 20 μ m, insert: 20 μ m. D) Ovary of a female from Porto Marghera site showing CYP1A immunoreactivity in the cytoplasm just under the vitelline membrane of mature follicles; scale bar: 20 μ m.

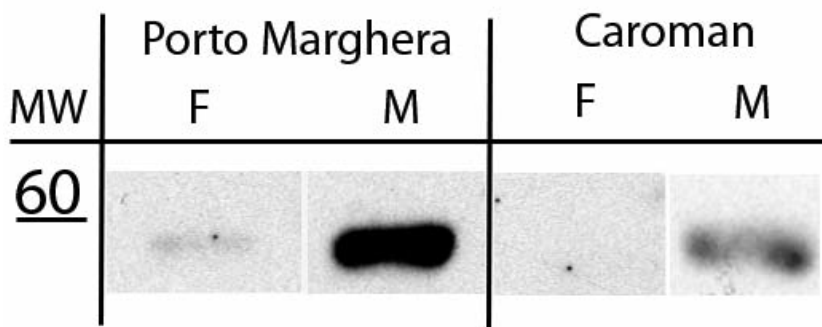


Figure 5. Representative Western blot analysis of liver homogenates in *Z. ophiocephalus*. Lanes represent CYP1A protein (about 60 kDa) (MW molecular weights in kDa, M males, F females).

ed by the fish *Aphanius fasciatus*,²⁶ *Barbus graellsii*,²⁷ *Cyprinus carpio*^{28,29} and *Salmo trutta*.³⁰ The immunolocalization of CYP1A protein demonstrated the presence of reactivity not only in the parenchyma of liver but also in the cytoplasm of renal tubules and in the cortical cytoplasm of mature follicles. Moreover, immunopositivity was observed in the endothelial cells of vessels. In both sites, the highest intensity of hepatic CYP1A immunostaining was detected in males. These results are in accordance with those of other authors who detected a CYP1A immunostaining in the liver but also in the brain, heart, gastrointestinal tract, gills, kidney and endothelial cells of *Sparus aurata*.^{11,31,32}

Benthic fish, which are in contact with sediment, represent a valid sentinel model since they reflect well the contamination state of the aquatic environment. Data reported in Table 2 suggest that the site located in front of the industrial area of Porto Marghera is the most polluted of the Venetian lagoon,¹² where the WATI (Weighted Average Toxicity Index) indicated a high toxicity in the same area. Investigating different oxidative stress biomarkers in *Z. ophiocephalus*, we previously detected the highest levels of malondialdehyde (MDA), 4-hydroxynonenal (HNE) and nitrotyrosine (NT) in animals from Porto Marghera.³³ Moreover, the highest concentration of PCB, HCB and PAH was observed in the upper sediment layer from Porto Marghera. Chemical analysis of that sediment, although providing important data, is not able to predict the biological effects on aquatic organisms living there.

In conclusion, this study provides new data on the expression and the localization of 8-OHdG in a benthic fish, suggesting it as a potential indicator for biomonitoring studies of the exposure of fish to contaminants that can induce oxidative damage to DNA. The results are supported by those of CYP1A (a well-known biomarker for exposure to contaminants) and of PCBs levels in muscle. Moreover, the gender differences found in this bioindicator highlight an intriguing possible relationship between behaviour and chemical exposure which merits more investigation.

References

- Henderson RF, Bechtold WE, Bond JA, Sun JD. The use of biological markers in toxicology. *Toxicology* 1989;20:65-82.
- Stevens DK, Bull RC, Nauman CH, Blancato JN. Decision models for biomarkers of exposure. *Regul Toxicol Pharmacol* 1991;14:286-296.
- Timbrell JA, Draper R, Waterfield CJ. Biomarkers in toxicology: new uses for some old molecules? *Toxicol Ecotoxicol* 1994;1:4-14.
- Winston GW, Di Giulio RT. Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquat Toxicol* 1991;19:137-161.
- Halliwell B, Gutteridge JMC. *Free radicals in biology and medicine*, 4th ed. Oxford, Oxford University Press, 2001.
- Ploch SA, Lee Y, MacLean E, Di Giulio RT. Oxidative stress in liver of brown bullhead and channel catfish following exposure to tert-butyl hydroperoxide. *Aquat Toxicol* 1999;46:231-240.
- Stegeman JJ, Hahn ME. Biochemistry and molecular biology of monooxygenases: current perspective on forms, functions, and regulation of cytochrome P450 in aquatic species. In: Malins DC, Ostrander GK, editors. *Aquatic toxicology - molecular, biochemical and cellular perspectives*. Boca Raton, Aquatic Publisher, 1994; pp. 87-203.
- Nebert DW, Gonzalez FJ. P450 genes. structure, evolution and regulation. *Annu Rev Biochem* 1987;56:945-93.
- Ribocco C, Hardiman G, Sasik R, Vittori S, Carnevali O. Teleost fish (*Solea solea*): A novel model for ecotoxicological assay of contaminated sediments. *Aquat Toxicol* 2012;109:133-42.
- Sarasquete C, Segner H. Cytochrome P4501A (CYP1A) in teleostean fishes. A review of immunohistological studies. *Sci Total Environ* 2000;247:313-32.
- Nesto N, Romano S, Moschino V, Mauri M, Da Ros L. Bioaccumulation and biomarker responses of trace metals and micro-organic pollutants in mussels and fish from the Lagoon of Venice, Italy. *Mar Pollut Bull* 2007;55:469-84.
- Losso C, Volpi Ghirardini A. Overview of ecotoxicological studies performed in the Venice Lagoon (Italy). *Environ Int* 2010; 36:92-121.
- Bertotto D, Poltronieri C, Negrato E, Richard J, Pascoli F, Simontacchi C, et al. Whole body cortisol and expression of HSP70, IGF-I and MSTN in early development of sea bass subjected to heat shock. *Gen Comp Endocrinol* 2011;174:44-50.
- Carnevali O, Tosti L, Speciale C, Peng C, Zhu Y, Maradonna F. DEHP impairs zebrafish reproduction by affecting critical factors in oogenesis. *PLoS One* 2010;5: e10201.
- Maradonna F, Polzonetti V, Bandiera SM, Migliarini B, Carnevali O. Modulation of hepatic CYP1A1 system in the marine fish *Gobius niger*, exposed to xenobiotic compounds. *Environ Sci Technol* 2004;38: 6277-82.
- Ferraris M, Radice S, Catalani P, Francolini M, Marabini L, Chiesara E. Early oxidative damage in primary cultured trout hepatocytes: a time course study. *Aquat Toxicol* 2002;59:283-96.
- Oliveira M, Ahmad I, Maria VL, Ferreira CS, Serafim A, Bebianno MJ, et al. Evaluation of oxidative DNA lesions in plasma and nuclear abnormalities in erythrocytes of wild fish (*Liza aurata*) as an integrated approach to genotoxicity assessment. *Mutat Res* 2010;703:83-9.
- Oliveira M, Maria VL, Ahmad I, Teles M, Serafim A, Bebianno MJ, et al. Golden grey mullet and sea bass oxidative DNA damage and clastogenic/aneugenic responses in a contaminated coastal lagoon. *Ecotoxicol Environ Saf* 2010;73: 1907-13.
- Celino FT, Yamaguchi S, Miura C, Ohta T, Tozawa Y, Iwai T, et al. Tolerance of spermatogonia to oxidative stress is due to high levels of Zn and Cu/Zn superoxide dismutase. *PLoS One* 2011;6:e16938.
- Sepici-Dincel A, Sahin D, Karasu Benli AC, Sarikaya R, Selvi M, Erkoc F, et al. Genotoxicity assessment of carp (*Cyprinus carpio* L.) fingerlings by tissue DNA damage and micronucleus test, after environmental exposure to fenitrothion. *Toxicol Mech Methods* 2011;21:388-92.
- Mazzoldi C, Scaggiante M, Ambrosin E, Rasotto MB. Mating system and alternative male mating tactics in the grass goby *Zosterisessor ophiocephalus* (Teleostei: Gobiidae). *Mar Biol* 2000;137:1041-8.
- Costantini D, Coluzza C, Fanfani A, Dell'omo G. Effects of carotenoid supplementation on colour expression, oxidative stress and body mass in rehabilitated captive adult kestrels (*Falco tinnunculus*). *J Comp Physiol B* 2007;177:723-31.
- Cohen AA, McGraw KJ, Robinson WD. Serum antioxidant levels in wild birds vary in relation to diet, season, life history strategy, and species. *Oecologia* 2009;161: 673-83.
- Wirgin I, Theodorakis CW. Molecular biomarkers in aquatic organisms: DNA damage and RNA expression. In: Adams SM, editor. *Biological indicators of aquatic ecosystem stress*. Bethesda, American Fish Societies, 2002; pp. 43-110
- Van der Hoost R, Beyer J, Vermeulen NPE. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ Toxicol Pharmacol* 2003;13:57-149.
- Kessabi K, Navarro A, Casado M, Said K, Messaoudi I, Piña B. Evaluation of environmental impact on natural population of the Mediterranean killifish *Aphanius fasciatus* by quantitative RNA biomarkers. *Mar Environ Res* 2010;70:327-33.
- Quiròs L, Piña B, Solé M, Blasco J, Lòpez MA, Riva MC, et al. Environmental monitoring by gene expression biomarkers in *Barbus graellsii*: laboratory and field studies. *Chemosphere* 2007;67:1144-54.

28. Fisher MA, Mehne C, Means JC, Ide CF. Induction of CYP1A mRNA in carp (*Cyprinus carpio*) from the Kalamazoo river polychlorinated biphenyl-contaminated superfund site and in a laboratory study. *Arch Environ Contam Toxicol* 2006;50:14-22.
29. Olivares A, Quirós L, Pelayo S, Navarro A, Bosch C, Grimalt JO, et al. Integrated biological and chemical analysis of organochlorine compound pollution and of its biological effects in a riverine system downstream the discharge point. *Sci Total Environ* 2010;408:5592-9.
30. Jarque S, Gallego E, Bartrons M, Catalan J, Grimalt JO, Piña B. Altitudinal and thermal gradients of hepatic Cyp1A gene expression in natural populations of *Salmo trutta* from high mountain lakes and their correlation with organohalogen loads. *Environ Pollut* 2010;158:1392-8.
31. Ortiz-Delgado JB, Segner H, Sarasquete C. Cellular distribution and induction of CYP1A following exposure of gilthead seabream, *Sparus aurata*, to waterborne and dietary benzo(a)pyrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin: an immunohistochemical approach. *Aquat Toxicol* 2005;75:144-61.
32. Ortiz-Delgado JB, Segner H, Sarasquete C. Brain CYP1A in seabream, *Sparus aurata* exposed to benzo(a)pyrene. *Histol Histopathol* 2009;24:1262-73.
33. Pascoli F, Negrato E, Di Giancamillo A, Bertotto D, Domeneghini C, Simontacchi C, et al. Evaluation of oxidative stress biomarkers in *Zosterisessor ophiocephalus* from the Venice Lagoon, Italy. *Aquat Toxicol* 2011;101:512-20.