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Biomarkers in Zebra mussel for monitoring and quality assessment of Lake Maggiore (Italy)

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Abstract
Three different biomarkers (acetylcholinesterase (AChE), ethoxy resorufin-*O*-deethylase (EROD) and DNA strand breaks) were measured in Zebra mussel (*Dreissena polymorpha*) specimens collected in April 2005 at six different sampling sites on Lake Maggiore, the second largest Italian lake in terms of depth and volume, in order to assess the spatial variation of exposure to man-made contaminants. Mussels maintained at fixed laboratory conditions were used as controls to eliminate potential interference due to environmental factors. Biomarker data were also supported by the analysis of several chemicals (six dichlorodiphenyltrichloroethane (DDTs), 23 polychlorinated biphenyls (PCBs), 14 polybrominated diphenyl ethers (PBDEs), 11 polycyclic aromatic hydrocarbons (PAHs) and hexachlorobenzene (HCB)) measured in the mussel soft tissues by gas chromatographic analyses. We found a negative correlation between temperature and AChE activity, while any measured environmental or physiological factor seemed to influence EROD activity and DNA strand breaks. A positive relationship was found between EROD activity and all of the measured chemicals, except for PAHs, which correlated with the amount of DNA strand breaks. Significant differences were noted for all biomarkers, both among sampling stations and between control and experimental data, even if the general level of variability was low. The biomarkers showed a distinct pattern of spatial variation, but the evaluation of DNA strand breaks was the strongest discriminating power between sites. In addition, the comparison between AChE and EROD activity measured in 2005 was compared with results obtained in a previous study carried out over the same sampling period in 2003. Results indicated a strong influence of temperature on AChE activity and probable interference of substrate inhibition of EROD activity, pointing out the need to take care in the interpretation of data comparisons. The results obtained with two different metrics used for the measure of DNA strand breaks is also discussed, as well as the relationship between EROD activity data and potential genotoxicity.

Keywords: *Biomarkers, Zebra mussel, acetylcholinesterase (AChE), ethoxy resorufin-O-deethylase (EROD), DNA strand breaks, Lake Maggiore*

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Introduction

Water quality assessment is generally a complex problem because of the diversity of anthropogenic pollutants that have been released in the environment and the possible additive, antagonistic or synergistic effects of contaminant mixtures on aquatic organisms. A complementary approach based on biochemical and genetic markers

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as indicators of chemical exposure and early response has been used for the last two decades (Depledge & Fossi 1994, Flammarion et al. 2002, Lee & Steinert 2003). These approaches can be used to monitor several pollutant levels or to assess the health status of organisms by obtaining early warning signals of environmental risk since the primary effect of xenobiotics is initially revealed at the subcellular level. Fast evaluation can enable the activation of measures for pollutant reduction before the damage has reached the entire community.

The development of biomarkers involves an in-depth knowledge of their biological function and requires the identification of any interference that can influence the biological responses in order to standardise the analytical procedure (Stegeman et al. 1992).

Among biomarkers, the level of ethoxyresorufin-*O*-deethylase (EROD) activity is one of the best documented responses to dioxins, furans, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Snyder 2000, Whyte et al. 2000), but the real mechanism involved in its activation is currently under discussion since the presence of CYP1A, from the well-known subfamily of the cytochrome P450 monooxygenase system found in vertebrates, is still controversial in invertebrates. Several results indicate the existence of a CYP1A isoform (CYP1A-*like*) in the digestive gland of *Mytilus* spp. and other molluscs (Porte et al. 1995, Wootton et al. 1996), but no *CYP1* genes have been sequenced (Livingstone et al. 1997). On the contrary, Chaty et al. (2004) argued that CYP1A is not present in invertebrates and they suggested that P450 isozymes other than CYP1A can be induced after exposure to chemicals like PCBs in the freshwater mussel *Unio tumida*. Recently, Grøsvik et al. (2006) assumed that changes in levels of CYP1A-immunopositive proteins in mussels could be due to a combination of cross-reactivities to other CYP proteins in mussels, combined with cross-reactivities to cytoskeletal proteins and major vault proteins (MVPs).

On the other hand, there is evidence of an increase of EROD activity in invertebrates exposed to planar chemicals both in field and laboratory studies (Heffernan & Winston 2000, Fisher et al. 2003, Pan et al. 2005, Ricciardi et al. 2006, Binelli et al. 2006a), even if total P-450 proteins is typically 10-fold lower than in mammals (Snyder 2000).

Acetylcholinesterase (AChE) is mainly involved in cholinergic neurotransmission and its activity can be decreased by organophosphates (OPs) and carbamates by blockage of natural substrate binding. The single cell gel electrophoresis assay, also known as the Comet assay, is a rapid and reliable method for evaluating DNA damage induced by chemicals in eukaryotic individual cells (Kim et al. 2002). The Comet assay is considered to be one of the most sensitive methodologies available for DNA strand break detection (Collins et al. 1997); its versatility allows for the detection of single-strand breaks (ssb) and alkali-labile sites, double-strand breaks (dsb), base damage and cross-links (Olive 1999). It was first used in radiobiology, clinical work and toxicology, and has now been extended to environmental biomonitoring (Rojas et al. 1999).

Particular care is needed in biomarker data interpretation which is seldom applied in field studies. Although the final benchmark of EROD activity is generally enzyme activation, some studies highlight a clear decrease in EROD activity in organisms that have been exposed to heavy metals (George 1989, Fent et al. 1996, Viarengo et al. 1997). Moreover, some chemicals that lead to an increase in EROD activity also

decrease its activity at high concentrations, which is explained by competitive inhibition of the EROD-substrate reaction (Petrulis & Bunce 1999). Recently, Kuiper et al. (2004) found a significant decrease of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced EROD activity in primary cultured carp hepatocytes exposed to some congeners of polybrominated diphenyl ethers (PBDEs).

Notwithstanding that AChE activity is inhibited by OPs and carbamates, the effect of trace metals on this enzyme is still controversial. Several studies (Dethloff et al. 1999, Romani et al. 2003) have demonstrated the capacity of these compounds to increase AChE activity, while Frasco et al. (2005) clearly demonstrated the potential of Cu, Zn, Cd and Hg ions to inhibit the enzyme's activity *in vitro*. Thus, the use of organisms depurated in laboratory conditions as controls instead of those from the less-contaminated sampling site may be more suitable for the measure of real basal enzyme activity and for the identification of possible double opposite effects as mentioned above (Binelli et al. 2005, 2006a).

Recently, there has been considerable interest in the use of biochemical indices of the freshwater mollusc Zebra mussel (*Dreissena polymorpha*) (Dauberschmidt et al. 1997, de Lafontaine et al. 2000, Binelli et al. 2005, Ricciardi et al. 2006) due to its useful ecological and physiological characteristics. The main aim of this study was assessment of the water quality of the chemically polluted Lake Maggiore by analysing a battery of biomarkers (EROD and AChE activities, DNA damages) from Zebra mussel specimens collected at different sampling sites. A comparison between the 2005 data on the activities of EROD and AChE with those found in a previous study carried out in the same period during 2003 is also given to point out the contamination trend of some pollutants. We also analysed several chemical classes in the soft tissues of mussels by gas chromatography with electron-capture detection (GC-ECD) and solid-phase microextraction GC (GC-MS) to support biomarker data: pp'dichlorodiphenyltrichloroethane (DDT) and its five homologues, 23 congeners of PCBs, 11 PAHs, 14 PBDEs and hexachlorobenzene (HCB).

Materials and methods

Study area and sampling

Lake Maggiore (Figure 1), the second largest Italian lake in terms of depth and volume, has been subject to heavy DDT contamination since the early 1990s and to other pollutants (heavy metals, PCBs) in the last decade (Camusso et al. 2001, Binelli et al. 2004). We selected six different sampling stations along the shores of the lake with regard to different anthropic levels and the source of DDT pollution, located on a tributary of River Toce that flows into Baveno Bay. Several hundred mussel specimens were collected at 4–5 m depth by a scuba-diver at the end of April 2005, when Zebra mussels are in their pre-reproductive stage. Molluscs were divided in two different pools. The organisms for chemical analysis were separated from rocks by byssus excision, washed with lake water, transported to the laboratory in refrigerated bags and frozen at -18°C pending chemical analysis. Only adult specimens of a shell length greater than 15 mm were used for analysis. Specimens for biomarker assays were tied on to rocks and transported alive to the laboratory, where adult specimens of shell length greater than 1.5 cm were separated from the substrate by byssus excision. Pools for EROD and AChE assays were stored at -80°C until analysis, while mussels for the Comet assay were immediately sacrificed and processed.

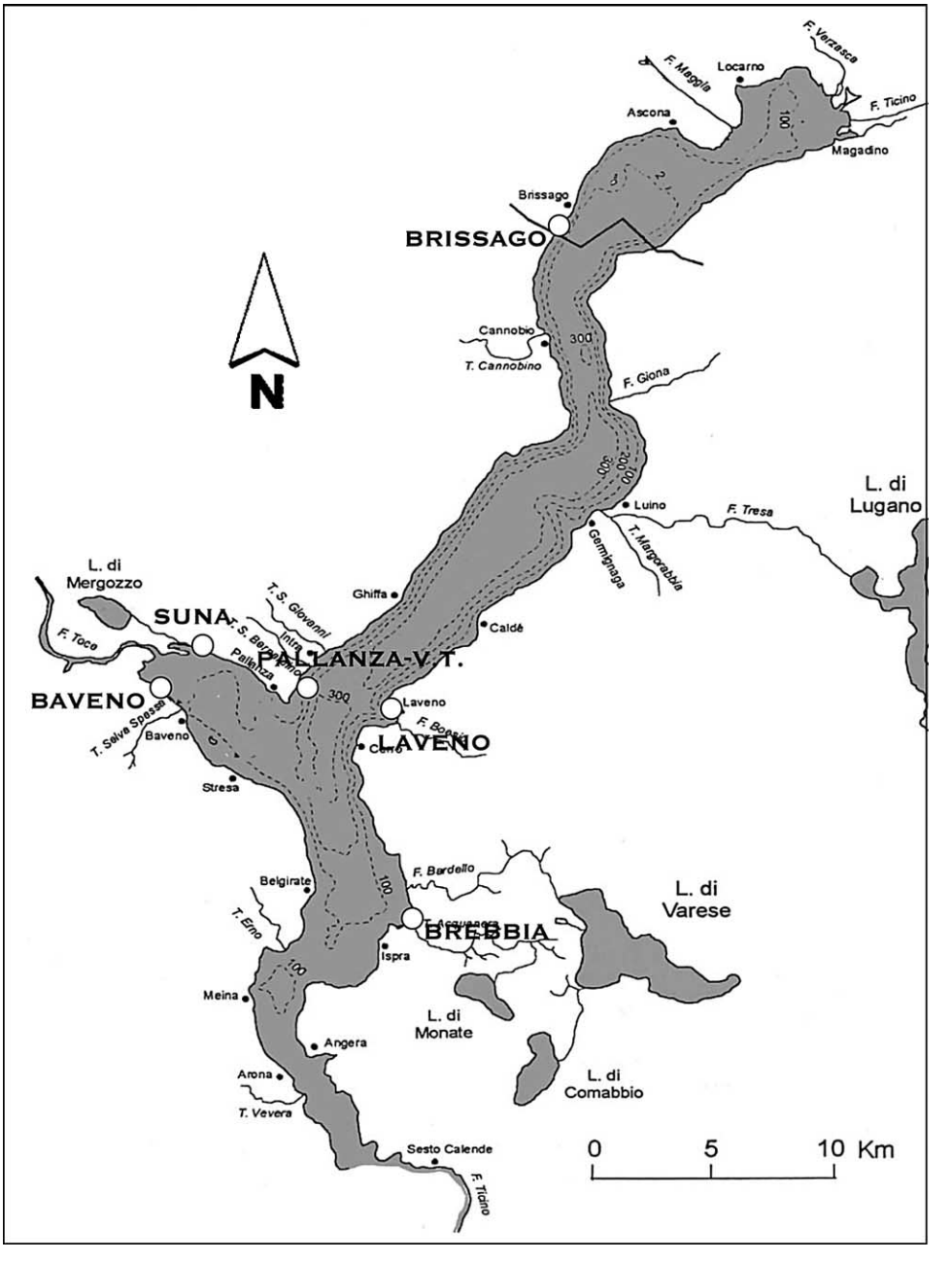

Figure 1. Lake Maggiore with the six sampling sites (white circles).

Chemical analyses

Mussel soft tissues were lyophilised for about 24 h, weighed, crumbled with a tissue-grinder and stored in dark jars. About 1.5 g of dried tissue from each sampling site was extracted for 12 h with 100 ml of an acetone/*n*-hexane (50:50) mixture using a Soxhlet and was dried with a rotating evaporator (RV 06-LR, IKA-Werke GmbH &

Co, KG, Staufen, Germany) and then under nitrogen flow for the gravimetric determination of lipids. Samples were recovered with small aliquots of *n*-hexane and the final volume (3 ml) was added to 6 ml of H_2SO_4 (96%) for organic phase digestion. This step was not carried out for PBDE analyses since acid digestion dramatically decreases the recovery of several BDE congeners.

After about 24 h, the supernatant was recovered, concentrated to 1 ml and put in a glass column filled with a double layer of 10 g of Florisil PR 60-100 Mesh (Supelco, Bellefonte, Pennsylvania, USA) and 10 g of silica gel 70-230 Mesh (Sigma-Aldrich, Staufen,Germany). The column was washed with *n*-hexane/acetone/dichloromethane (80:10:10) and eluted first with 50 ml of *n*-hexane and then with 50 ml of *n*-hexane/dichloromethane (50:50) mixture. Iso-octane (1 ml) was added to the two different fractions that were concentrated to 1 ml and injected separately into a GC chromatograph (TRACE GC, Thermo-Ultron, Texas, USA) equipped with ECD and a Hewlett Packard HP-5 column (length 60 m, i.d. 0.25 mm, film thickness 0.25 μm) for the analytical determination of DDTs (pp′DDT, DDD, DDE and op′DDT, DDD, DDE), PCBs (23 congeners) and HCB. Helium (1 ml min^{-1}) and nitrogen (30 ml min^{-1}) were used as carrier and make-up gases, respectively.

Eleven PAHs (phenanthrene, naphthalene, fluorene, anthracene, fluoranthene, pyrene, benzo α anthracene, chrysene, benzo β fluoranthene, benzo(k)fluoranthene and benzo α pyrene) and PBDEs (14 congeners) were quantified in selective ionization monitoring (SIM) and MS-MS modes, respectively, by a GC-MS (Polaris Q, Thermo-Electron, Texas, USA) equipped with a PTV injector and a Restek Rtx-5MS column (length 30 m, i.d. 0.25 mm, film thickness 0.25 μm). Helium was used as a carrier (1.2 ml min^{-1}) and damping (2 ml min^{-1}) gas.

BDE-209 was analysed by an on-column injection in a short piece (50 cm) of J&W DB-1 megabore column (0.53 mm i.d., 0.15 μm film thickness), which was used as a retention gap, connected to a short Restek Rtx-5MS column (7 m length, 0.32 mm i.d., 0.25 μm film thickness). The measurements were performed by an ECD-40 detector coupled with a TRACE GC Ultra (Thermo-Electron, Austin, Texas, USA).

PCBs, DDTs, HCB and PAHs were quantified by comparing the peak areas of individual compounds with external standards obtained by adding the single certified compounds (Dr Ehrenstorfer GmbH, Germany) to reach a similar environmental pattern, while a mixture of the commonly occurring 14 congeners (AccuStandard, New Haven, CT, USA) was used for the quantification of PBDEs. The method limits of quantification (LOQ) for all of the chemicals was 0.5 μg l^{-1}.

Biomarker assays

Preliminary assays performed on Zebra mussels (Ricciardi et al. 2006) showed that (acetylthiocholinesterase (AChE) was the only substrate that bound the cholinesterasic enzyme, while EROD activity had a higher response in comparison with other CYP-450 substrates (PROD and MROD activity). Consequently, only these two substrates were selected for biomarker assays.

Fifty mussels of similar size (20 ± 0.2 mm) per site were used for EROD and AChE tests, while ten specimens were used for the Comet assay, which analysed the DNA tail length, the Comet head diameter (LDR) ratio and the DNA percentage in the tail. Since seasonal variability with a minimum of DNA integrity in winter was found in

Mytilus galloprovincialis (Frenzilli et al. 2001), the selection of early spring as the sampling period minimised the possible role of temperature on DNA damage.

We preferred the use of mussels depurated at the laboratory as controls since all sampling sites were polluted by several chemicals. The controlled conditions (time, food, temperature, water characteristics) for depuration were explained by Binelli et al. (2006b).

AChE activity determination

AChE activity was determined on five replicate pools (ten mussels per pool) at 23°C, pH 7 and with the acetylthiocholine (ACh) as the substrate, as described by Ellman et al. (1961). Soft tissues were excised and washed in 0.15 M KCl at 4°C. About 1 g of tissue maintained in an ice bath was homogenised in a Tris–HCl buffer-saline solution (1 ml), pH 7.6, containing 0.1% of Triton X-100. S10 supernatant was obtained by centrifugation at 10 000*g* for 15 min at 4°C, repeated twice, and was stored at 4°C overnight.

Fifty microlitres of S10 extract, pre-warmed at 23°C for 15 min, was combined with a molar excess of 5,5-dithiobis(-2-nitrobenzoic acid) (DTNB) in the presence of ACh. Absorption of the 2-nitro-5-thiobenzoate anion, formed from reaction, was recorded at 412 nm every 60 s for 7 min at room temperature using the double-beam spectrophotometer Perkin Elmer Lambda 2 to read each sample against a blank.

Kinetic data were calculated in the linear range after subtraction of blank activities due to substrate autohydrolysis. Protein concentration was measured by the Bradford method (Bradford 1976) using bovine serum albumin (BSA) as a standard; each pool was analysed in triplicate and results were expressed as nmol min^{-1} mg^{-1} of proteins.

EROD activity determination

The principle of the assay is based on the oxidation of a substrate, ethoxyresorufin, to a fluorescent compound, resorufin, according to the protocol of Burke & Mayer (1974). EROD activity was determined on five replicate pools (ten mussels per pool). Zebra mussel soft tissues, maintained in an ice bath, were rinsed in 150 mM KCl and homogenised (50–60 s) with an Ultra-Turrax grinder in a buffer solution containing 25 mM of Tris-acetate (pH 7.6) and sucrose (250 mM). Ethylenediaminetetraacetic acid (EDTA) (1 mM) and phenylmethylsulphonyl fluoride (1 mM) were added as protease inhibitors.

The homogenate was centrifuged at 9000*g* for 15 min at 4°C, repeated twice, and enzyme activity was measured using 50 μl of S9 extract and a reaction buffer containing Tris, NaCl and β-NADPH with EROD as the substrate. The fluorescence (λ_{ex} 520 nm; λ_{em} 590 nm) was recorded for 20–30 min by the Jasco FP-920 detector at 20 ± 1°C; only data measured in the linear range of the kinetic curve were acquired. Protein concentration was measured by the Bradford method (Bradford 1976) using BSA as standard and each pool was analysed in duplicate (pmol min^{-1} mg^{-1} of proteins).

Comet assay

The Comet assay was basically performed according to the method of Singh et al. (1988) and the successive modifications for Zebra mussel (Buschini et al. 2003).

The mussel haemolymph was gently aspirated from the posterior adductor muscle sinus with a hypodermic syringe containing 20 µl of phosphate-buffered saline (PBS). The volume of recovered haemolymph was 10 µl per mussel with a final density of about 10^7 cells ml^{-1}. Cell viability was checked by the Trypan blue exclusion method immediately after withdrawal and the Comet assay was performed only with cells that were more than 90% viable.

Eighty-five microlitres of 0.7% low melting agarose (LMA) in PBS (37°C), mixed with 10 µl of cell suspension, were added to the coated slides (previously dipped in 1% normal melting agarose). The slides were covered by a cover glass and placed at 4°C for 5 min until the agarose layer hardened. A third agarose layer was added to the slides in the same way.

After agarose solidification, the slides were placed in a lysing solution (2.5 M NaCl, 100 mM Na_2 EDTA, 8 mM Tris–HCl, 1% Triton X-100 and 10% DMSO, pH 10) in a Coplin jar at 4°C in the dark for at least 1 h.

Alkaline DNA unwinding was carried out for 5 min in a gel electrophoresis chamber containing freshly prepared buffer (1mM Na_2 EDTA, 300 mM NaOH, pH 13) and placed in an ice-water bath (4°C); electrophoresis was then performed at 0.78 V cm^{-1} and 300 mA for 10 min.

Slides were washed after electrophoresis in a neutralisation buffer (0.4 M Tris–HCl, pH 7.5) and fixed in absolute methanol. After staining with FLUO*plus*® DNA dye (IKZUS, Genoa, Italy), the slides were covered with a cover glass and observed under a fluorescence microscope (Leitz DMR, Germany) equipped with a FTC filter.

All steps were performed in the dark to minimise additional UV-induced DNA damage and positive controls were carried out with H_2O_2 to check the effectiveness of electrophoresis conditions. Moreover, each slide was coded and randomly scanned. Fifty cells per slide were analysed using an image analysis system (Comet Score®), obtaining a total of 500 analysed cells per sample.

The ratio between the migration length and the LDR was chosen to represent DNA damage data (Bolognesi et al. 2004) coupled with the percentage of DNA in the tail, following criteria proposed by Mitchelmore et al. (1998): grade of damage zero or minimal $<10\%$, low damage 10–25%, mid-damage 25–50%, high damage 50–75% and extreme damage $>75\%$.

Statistical approach

The Statistica 6 software package was used for statistical calculation. Correlations between biomarkers and abiotic parameters or chemical data were checked by Pearson's correlation coefficients. One-way analysis of variance (ANOVA) was performed to measure EROD activity and LDRs (log_{10} transformed), while analysis of covariance (ANCOVA), using temperature as a covariate, was performed to compare AChE activity data between sites and years. A *post-hoc* test was carried out for all of the biomarkers using Tukey's correction when equal variance was assumed (Levene's statistic). A simple Student's *t*-test was carried out for the between-years comparison of EROD activity. Comparisons among the different classes of DNA percentage in the tail determined in the Comet assay were performed by the non-parametric χ^2 test.

Results and discussion

The Pearson's correlation coefficients (Table I) did not show any influence of environmental (temperature) and physiological characteristics (mussel size, protein content, lipid percentage) on EROD activity and LDRs, while a significant positive relationship ($r = 0.51$, $p < 0.01$) was found between temperature and AChE activity measured for each site (Table I). Other environmental parameters, such as pH, conductivity and hardness, were very similar in the whole basin of Lake Maggiore and they did not affect the biomarker measurements.

No correlations between biomarkers were found (Table I), in accordance with their very different biological targets and chemical behaviours. Biomarker results did not exhibit high variability at every site, as shown by low standard deviations in Table II. LDR was the most efficient biological response to discriminate among sampling stations, where 32 out of the 42 paired-sites comparisons resulted in significant differences, corresponding to a discriminating efficiency of 68.8% (Table III) relative to an efficiency of 33.3% for EROD activity and only 19% for AChE activity, which is, however, a more specific biomarker.

Chemical data

DDT pollution seems rather homogeneous in the whole basin, with an average level of about 1000 ng g^{-1} lipids (Table IV). Surprisingly, the highest concentration of ΣDDTs (about 1900 ng g^{-1} lipids) in Lake Maggiore was found at Pallanza-VT, located outside Baveno Bay, which experienced a dramatic increase (+155%) of ΣDDTs in the last 2 years, while Baveno, the nearest site to the River Toce, had a lower concentration of these chemicals (about 1000 ng g^{-1} lipids). This heavy contamination has never been found in the central area of the basin (CIPAIS 2004). It may be due to maintenance and heavy ground-stripping executed in the River Boesio which flows in front of this sampling station (Figure 1), which could have re-suspended old sediments with accumulated DDT relatives. This hypothesis is supported by the sediment analyses carried out in several lake inlets that indicated ΣDDT values at the River Boesio similar to those measured in the River Toce (CIPAIS 2005) with the same pattern of mussel contamination and quite high levels of the parent compound (pp′DDT).

The 2005 biomonitoring campaign established clear ageing of this insecticide pollution because the main homologues found in the mussel soft tissues at all of the sampling sites were the two metabolites pp′DDD and pp′DDE. This was confirmed by the level of the pp′DDT/pp′DDE ratio less than 1 calculated for all sites (0.08–0.47). Notwithstanding contamination ageing, the insecticide levels measured in the lake are still about one order of magnitude higher than those considered to be background concentrations (100–150 ng g^{-1} lipids) for the Italian subalpine great lakes (Binelli et al. 2005).

A clear decrease of PCB levels was noticed in the last 2 years, apart from the concentrations measured at Pallanza-VT which were more than double the 2003 levels. This is in accordance with the hypothesis that the contamination effect is due to chemical release from the sediments of the River Boesio. PCB levels measured in some sampling sites were about twice the background concentrations of the other Italian subalpine great lakes (about 400 ng g^{-1} lipids), but were much lower that the PCB

Table I. Pearson's coefficient matrix between chemical data, biomarkers and abiotic factors.

Variable	AChE	EROD	LDRs	Temp. (°C)	Lipid%	Proteins (EROD)	Proteins (AChE)	Shell length (EROD)	Shell length (AChE)	PCBs	PAHs	HCB	PBDEs	DDTs
AChE	–													
EROD	0.07	–												
LDRs	−0.31	0.07	–											
Temp. (°C)	0.51**	−0.12	0.36	–										
Lipid%	−0.39	−0.31	−0.20	−0.85***	–									
Proteins (EROD)	−0.37	0.06	0.04	−0.34	0.21	–								
Proteins (AChE)	−0.19	0.16	−0.03	−0.22	0.08	0.37	–							
Shell length (EROD)	−0.66***	−0.42	−0.07	−0.09	0.13	0.24	0.10	–						
Shell length (AChE)	0.02	0.50***	0.48	0.46	−0.67**	−0.15	−0.44	−0.09	–					
PCBs	0.48	0.59**	−0.35	0.06	−0.53*	−0.09	0.04	−0.43	0.45	–				
PAHs	0.26	−0.31	0.50*	0.92***	−0.61**	−0.31	−0.16	−0.14	0.23	−0.25	–			
HCB	0.22	0.73***	−0.44	−0.29	−0.24	0.21	0.38	−0.16	0.17	0.86***	−0.56*	–		
PBDEs	0.39	0.63**	−0.31	0.54*	−0.65**	−0.40	−0.31	0.01	−0.32	−0.28	0.60**	0.82***	–	
DDTs	0.34	0.82***	0.09	−0.09	−0.48*	0.33	0.50*	0.03	0.43	0.64**	−0.31	0.85***	0.84***	–

Temp., temperature measured at each sampling site; lipid%, percentage of lipid measured in mussel pools used for chemical analyses; proteins, total proteins measured for EROD (ethoxy resorufin-O-deethylase) and AChE (acetylcholinesterase) activity determination; shell length, length of mussel shell used for EROD and AChE measures; PCBs, sum of 23 polychlorinated biphenyls; PAHs, sum of 11 polycyclic aromatic hydrocarbons; HCB, hexachlorobenzene; PBDEs, sum of 14 polybrominated diphenylethers; DDTs, sum of pp'dichloro-diphenyl-trichloroethane and its five homologues. Significance levels: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table II. Temperatures measured at each sampling sites, average of acetylcholinesterase (AChE) (nmol min^{-1} mg^{-1} of proteins), ethoxy resorufin-O-deethylase (EROD) (pmol min^{-1} mg^{-1} of proteins) activities and length diameter ratios (LDRs) measured in controls and in Zebra mussels from Lake Maggiore.

Site	Temp. (°C)	AChE activity	Difference from controls (%)	EROD activity	Difference from controls (%)	LDR	Difference from controls (%)
Controls	20	2.98 (±0.26)		1.85 (±0.22)		1.28 (±0.04)	
Brissago	12	1.71 (±0.10)	−42.6	1.59 (±0.50)	−14.1	1.19 (±0.05)	−7.0
Suna	14	2.05 (±0.37)	−31.2	2.18 (±0.39)	15.1	1.62 (±0.17)	20.9
Pallanza-VT	14	2.91 (±0.57)	−2.3	3.42 (±0.42)	45.9	1.42 (±0.08)	9.8
Baveno	14	1.70 (±0.31)	−42.9	2.44 (±0.29)	24.2	2.72 (±0.54)	52.9
Laveno	15	3.03 (±0.79)	1.6	1.93 (±0.12)	4.1	1.53 (±0.17)	16.3
Brebbia	17	2.29 (±0.55)	−23.1	1.87 (±0.13)	1.0	1.97 (±0.15)	35.0

Results shown are means with standard deviations in brackets. Percentage differences between controls and experimental data are also shown.

levels found worldwide in highly polluted aquatic ecosystems (Minier et al. 2006). In contrast, HCB and PAH levels in Lake Maggiore were negligible (Table IV).

Hendriks et al. (1998) found that PBDE concentrations in freshwater biota were higher than in similar marine species, probably due to their local point-sources that are almost exclusively located inland (Covaci et al. 2005). Thus, attention must be paid to the monitoring of these chemicals, whose levels in the Italian freshwaters are still unknown.

The highest PBDE concentration was found at Pallanza-VT, in accordance with DDTs and PCBs (Table IV), while the contamination seems to be quite similar at the other sampling stations. Congeners 47, 99 and 100 were identified in all the sites, in accordance with similar observations found worldwide (Chen & Bunce 2003). The fully substituted deca-BDE 209 reached levels ranging from 6.1 ng g^{-1} lipids (Suna) to 84.6 ng g^{-1} lipids (Brebbia), indicating that it can accumulate in Zebra mussel in spite of its steric size.

Table III. Results of the Tukey's range test for each biomarker. Groups with same letters are significantly different.

	Group		
Sites	AChE activity	EROD activity	Log LDR
Controls	a	a	a
Brissago	a b	b	b c d e
Suna		c	a b c
Pallanza-VT		a b c d e	b d
Baveno	a c	b	a b c d e
Laveno	b c	d	a b e
Brebbia		e	a b c d e

ANOVA ($p < 0.05$) was performed for ethoxy resorufin-O-deethylase (EROD) and log length diameter ratio (LDR), while ANCOVA ($p < 0.05$) was carried out for the measure of the acetylcholinesterase (AChE) activity.

Table IV. Concentrations (ng g^{-1} lipids) of several chemical classes measured in Zebra mussel soft tissues from six sampling sites on Lake Maggiore.

Sites	□DDTs	□PCBs	□PAHs	□PBDEs	HCB
Brissago	884.0	310.2	11.5	206.6	13.4
Suna	746.8	645.8	19.2	216.7	9.8
Pallanza-VT	1858.0	975.0	17.0	446.5	47.1
Baveno	1096.0	333.0	28.8	217.0	2.0
Laveno	900.7	788.1	34.8	141.3	0.9
Brebbia	944.3	429.7	44.6	320.1	3.1

ΣDDTs, sum of pp'dichlorodiphenyltrichloroethane and its five homologues; ΣPCBs, sum of 23 polychlorinated biphenyls; ΣPAHs, sum of 11 polycyclic aromatic hydrocarbons; ΣPBDEs, sum of 14 polybrominated diphenylethers; HCB, hexachlorobenzene.

The average PBDE concentration (258 ng g^{-1} lipids) measured in Lake Maggiore is much higher than that found in eels (*Anguilla anguilla*) from several European aquatic ecosystems (Santillo et al. 2005) and in whitefish (*Coregonus* spp.) from some Swiss lakes (Zennegg et al. 2003), raising concerns about PBDE contamination in this lake and suggesting widespread contamination in other Italian freshwater environments.

AChE activity results

Notwithstanding that organophosphorus insecticides and carbamates are generally considered to be the most powerful cholinesterase inhibitors, lindane, organochlorine pesticides and pyrethroids may also play a minor role in AChE inhibition mechanisms (Galindo-Reyes et al. 2000, den Besten et al. 2001).

Comparisons between mussels collected in Lake Maggiore and depurated controls (Tables II and III) highlighted a clear effect of AChE inhibitors at Brissago and Baveno ($p < 0.05$), while no significant decrease of the enzyme activity was found at the other sites. The northern part of the lake is probably contaminated by pesticides that drain from several rivers (Maggia, Ticino, Tresa, Verzasca) that flow through agricultural areas. Baveno is near the mouth of the River Toce, one of the most significant inlets, through which DDT discharge from the chemical plant of Pieve Vergonte flows into the lake (Binelli et al. 2004).

Since a significant 20% inhibition of AChE activity relative to controls signifies a clear toxicological effect (US EPA 1998), and that AChE inhibition higher than 50–60% can kill some organisms or damage the most sensitive populations (Osterloh & Pond 1983, Fleming et al. 1995, Sibley et al. 2000), the AChE levels found at Brissago and Baveno indicate a potential danger both for the mussel population and for the entire aquatic community, bearing in mind the levels of inhibition found at Baveno are particularly troublesome since this area (Fondotoce) represents one of the most important wetlands of northern Italy.

The comparison between 2005 data and those found in a previous study carried out over the same period of 2003 (Binelli et al. 2006b) allows for the identification of the pollution trend of AChE inhibitors in Lake Maggiore. For the between-years comparison, it is absolutely necessary to take the water temperature into account. An active role of this environmental parameter on AChE activity was recently investigated in the freshwater mussel *Anodonta cygnea* by Robillard et al. (2003), in

the Pacific tree frog (*Hyla regilla*) by Johnson et al. (2005) and in *Mytilus* spp. from the south-western Baltic Sea by Pfeifer et al. (2005). A parabolic relationship between temperature and AChE activity was also found in Zebra mussel specimens maintained at laboratory conditions, with maximum enzymatic activity at 20°C (Ricciardi et al. 2006). Data shown in Figure 2 confirm the interference due to this environmental parameter because AChE activity increased significantly (ANCOVA, Tukey *post-hoc* test, $p < 0.05$) from 2003 to 2005 only at Pallanza and Laveno, while the variance between years noticed in the other sites was only due to temperature.

EROD activity results

Field data interpretation of EROD activity is sometimes difficult because chemical mixtures in the environment can interfere with biomarkers in different ways, as shown in Table I. This enzymatic activity is positively correlated with PCBs, HCB, PBDEs and, surprisingly, DDTs. Even though measurement of this enzymatic activity is normally performed to evaluate chemical pollution with enzymatic inducers, several authors (Hahn et al. 1993, Schmitz et al. 1995, Kennedy et al. 1996, Besselink et al. 1998) have reported convincing evidence suggesting that EROD activity is competitively inhibited by the inducer. This behaviour was also shown in laboratory tests carried out with Zebra mussels exposed to the *dioxin-like* CB 126 (100 ng l^{-1}), where a fast decrease of EROD activity towards controls was observed before a previous significant increase at concentrations higher than 800 ng g^{-1} lipids (Binelli et al. 2006a). The possible effects of the heavy DDT pollution measured in Lake Maggiore since 1996 should not be ignored. pp′DDT and its relatives were classified as phenobarbital-type cytochrome P450 inducers in the rat because they markedly induced CYP2B, and led to a lower induction of CYP3A and minimal or no induction of CYP1A (Nims et al. 1998). In contrast, a significant decrease (Student's *t*-test, $p < 0.05$) of EROD activity was recorded in Zebra mussel specimens (-43% compared with controls) exposed to pp′DDT (100 ng l^{-1}) in laboratory conditions (Binelli et al. 2006a) and a significant negative correlation was observed between DDT concentrations in Zebra mussel specimens from 14 sampling sites of Lake Maggiore and the EROD activity in the previous study carried out in 2003 (Binelli et al. 2006b).

Another possible interference affecting this enzyme is due to heavy metals that are able to decrease EROD activity through different effects in the CYP1A-induction cycle. Organotin compounds, such as tributyltin and triphenyltin, have been shown to reduce EROD activity in scup (Fent & Stegeman 1993, Fent et al. 1996) through interference with the reductase components of the microsomal mono-oxygenase system (Fent & Bucheli 1994). Cadmium injection in European plaice reduced EROD activity through a decrease in CYP1A protein rather than catalytic inhibition (George 1989), whereas copper and mercury inhibit EROD activity in fish by directly binding to exposed -SH groups (Viarengo et al. 1997). Lewis et al. (2006) showed a clear decrease of metal response element (MRE) induction in flounder treated with a mixture of PAH 3-methylcholanthrene (3-MC) and cadmium compared to 3-MC treatment alone.

Historically, Lake Maggiore has been a site of trace-metal contamination from industries located in the catchment area, with a peak in the 1970s (Baudo 1989). The entire basin is currently considered to be polluted by Cd and Cr (Zambon 2005), in

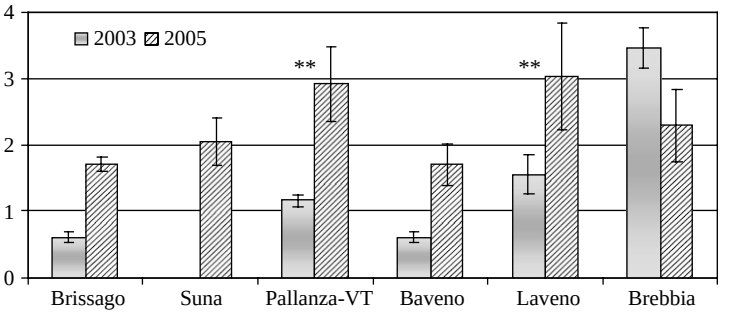

Figure 2. Acetylcholinesterase activity (nmol min^{-1} mg^{-1} of proteins) measured at Lake Maggiore sampling stations in the two monitoring campaigns. ANCOVA, Tukey's *post-hoc* test (**$p < 0.01$).

accordance with the classification proposed by Mersch (1993) based on the heavy metals measured in Zebra mussel.

Field data results (Tables II and III) highlight that Pallanza-VT was the only sampling site that was statistically different ($p < 0.05$) from controls, probably because it is located in one of the most anthropised areas of the lake. Since the Pearson's coefficients showed a significant positive correlation between EROD activity and the sum of PCBs, PBDEs, DDTs, and HCB (Table I), the enzyme activity seems to reflect pollution due to these chemicals. Moreover, this station was the site with the highest levels of these pollutants (Table IV).

EROD activity seems to be able to discriminate between sampling sites (Table III) better than AChE. The high pollution due to EROD inducers revealed at Pallanza-VT is also confirmed by the significant difference ($p < 0.05$) with all the other sites.

The role played by a single chemical class is very hard to assess because EROD activity in mixtures can be lower than that measured with a single component and it is frequently much less than would be expected on the basis of additivity (Petrulis et al. 2001). For instance, technical mixtures of PCBs (Aroclor 1254 and 1260) used in Italy until the end of the 1980s contained negligible levels of coplanar congeners (CBs 77, 81, 126, 169), but the percentage of the other *dioxin-like* PCBs (12.2 and 4.3%, respectively) may be enough to determine a low enzymatic response, as demonstrated by Newsted et al. (1995).

Notwithstanding the structural similarities of PBDEs with other classes of persistent organic pollutants (POPs), particularly the PCBs, that suggests a possible activation of the aryl hydrocarbon receptor (AhR) signal transduction pathway and the consequent induction of EROD activity, some pure BDE congeners and commercial mixtures had AhR-binding affinities 10^{-2}–10^{-5} times than that of TCDD in human, rat, chick and rainbow trout cells (Chen et al. 2001). Kuiper et al. (2004) found a significant reduction of TCDD-induced EROD activity in primary cultured carp (*Cyprinus carpio*) hepatocytes in the presence of BDE-47, 99, 153 and the commercial penta-BDE (DE-71) mixture. Relative induction potencies of the most active BDEs towards CYP1A1 are about 10^{-4} compared with TCDD in primary rat hepatocytes, similar to some *mono-ortho* PCBs and two orders of magnitude lower than those of co-planar PCBs (Chen & Bunce 2003). Thus, substrate competition due to PBDEs can hide the presence of another AhR agonist when organisms are exposed to chemical mixtures in the aquatic ecosystems.

Finally, it is not easy to identify the role played by DDT relatives because both the sum of DDTs and levels of each single homologue (not shown) were significantly and positively correlated with EROD activity (Table I). These data are diametrically opposed to those found in the 2003 study.

These observations about the different effects of polyhalogenated aromatic hydrocarbons (PHAHs) highlight the need for caution when using EROD activity as biomarker for environmental risk assessment, especially without the support of chemical data. Comparisons between 2003 and 2005 data (Figure 3) showed a possible misinterpretation of the environmental quality assessment: although the highest EROD activity was measured in the 2005 campaign at almost all of the sampling stations (Figure 3), the ecosystem quality was not worse in comparison with 2003 because chemical data showed a dramatic decrease of PCB pollution at Brissago (−63%), Brebbia (−77%) and Laveno (−39%) in 2005, while levels of the other monitored PHAHs did not change very much (Binelli et al. 2006b).

Unfortunately, PBDE concentrations were not measured in the 2003 study. Moreover, PCB levels found in 2003 at these three sites were higher than 800 ng g^{-1} lipids, the threshold up to which we observed competitive inhibition by these chemicals (Binelli et al. 2006a). Thus, the lower enzyme activity that was measured at some sampling sites in 2003 can only be due to this effect which masks the real contamination by PHAHs. On the other hand, EROD activity seems to indicate an increase in pollution due to man-made chemicals at Pallanza-VT and Baveno, which was confirmed by a good agreement with chemical data.

Comet assay results

The Comet assay has been shown to have broad range of applicability for aquatic organisms and DNA damage measured by this assay has been linked to exposure to a wide spectrum of genotoxic compounds, as well as to physical agents (Lee & Steinert 2003). The Comet assay seems to be the best biomarker for discrimination between the different sites (discriminating efficiency of 68.8%), as shown in Table III: Baveno and Brebbia were the most contaminated sites, showing significant differences ($p < 0.05$) with all of the other stations, while LDR values showed a significant increase in DNA damage ($p < 0.05$) in comparison with controls for all of the sampling stations (Tables II and III). Brissago and Pallanza-VT were the only areas that were not contaminated by chemicals that were able to produce significant strand

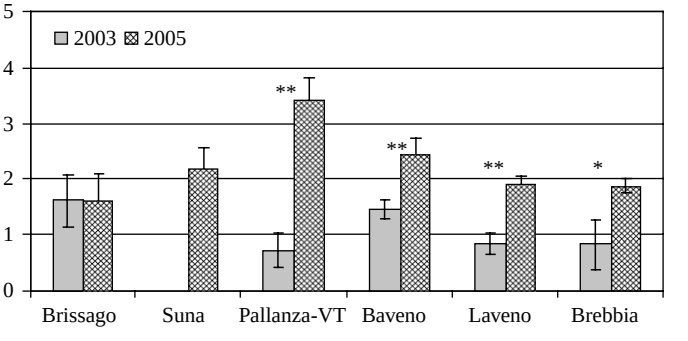

Figure 3. Ethoxy resorufin-O-deethylase activity (pmol min^{-1} mg^{-1} of proteins) measured in Lake Maggiore sampling stations in the two monitoring campaigns. Student's *t*-test (*$p < 0.05$; **$p < 0.01$).

breakages in Zebra mussel cells (Table III). The highest LDR found at Baveno, located near the mouth of the River Toce, can be explained by the heavy DDT pollution that has occurred since 1996. This insecticide and its metabolites are able to induce DNA damage both in animals (Sekihashi et al. 2002) and humans (Masoka et al. 2001) through different mechanisms (oxidative liver damage, DNA single-strand breaks and lipid peroxidation) that have been reported in DDT-treated rats (Yanez et al. 2004). Since chemical results highlighted the highest DDT level at Pallanza-VT (Table I), where little or no significant LDR difference from controls was noticed (Tables II and III), the high DNA damage found at Baveno is probably not solely due to insecticide pollution, but mainly to other xenobiotics transported through the River Toce or released from an unidentified point-source of this heavily urbanised and industrialised area. Measured chemical data (Table IV) did not explain the high LDR found at Brebbia, located near the inlet of the River Bardello which arises from Lake Varese and flows into a highly industrialised area.

Even though the Comet assay is a non-specific assay, these results can be used to better explain the EROD data, bearing in mind that some chemicals require metabolic activation through the CYP-450 system before DNA damage development occurs and that the cytochrome often involves production of electrophilic metabolites that can bind to the nucleophilic DNA, producing a variety of DNA lesions (Lee & Steinert 2003). The LDR calculated at Brissago, as well as the sampling stations of Suna, Laveno and Brebbia, was in full accordance with low EROD activity (Table II). In contrast, DNA damage found at Pallanza-VT and Baveno showed behaviour that was different from that of EROD activity. Pollutants that are able to activate the CYP-450 system at the former site probably do not produce many metabolites or agents, such as the reactive oxygen species (ROS), that create large DNA damage, whilst the contamination found at Baveno increases the LDR up to twice that measured for controls despite the lower EROD activity (Table II). This difference suggests different pollution in these areas which are located outside and inside Baveno Bay: the DNA damages noticed at Baveno is probably created by chemicals that directly produce

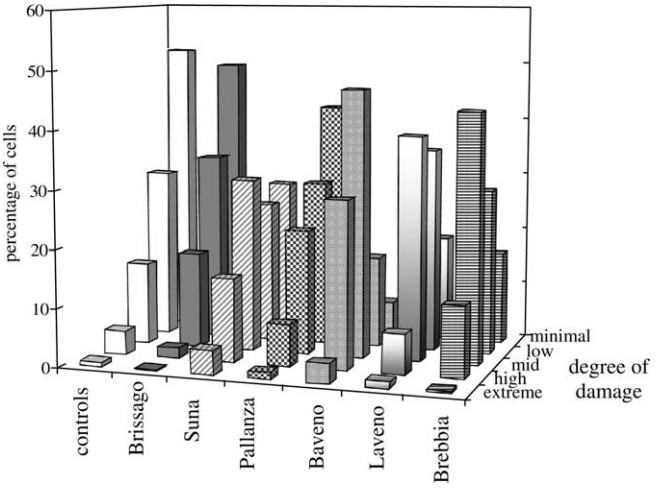

Figure 4. Classes of DNA damage degrees calculated from Zebra mussel haemocytes from six sampling sites of Lake Maggiore and their comparison with controls. Grade of damage (using tail DNA percentage): minimal <10%; low 10–25%; mid 25–50%; high 50–75%; extreme >75%.

bulky DNA adducts or that are unable to induce CYP-450 isoforms. The closeness of these two sampling stations and the consequent similar environmental conditions should rule out interference of oxidative stress depending on natural factors, such as temperature, dissolved oxygen, ultraviolet radiation, diet and reproductive status (Frenzilli et al. 2001).

Criteria reported by Mitchelmore et al. (1998) to categorise the grade of damage through the percentage of DNA in the tail (Figure 4) was also used and compared to the LDR approach. These two metrics fit properly for Baveno, since it has the highest detected LDR in the whole lake basin (Table II) and the majority of the haemocytes fall into mid- (46.2%) and high-damage (28.6%) classes, the highest levels calculated for all of the sampling stations (Figure 4).

The low genotoxic effects of contaminants noticed at Pallanza-VT were confirmed by the high percentages of cells falling into minimal (41.4%) and low (28.6%) degree of damage classes. The same results were also obtained for Brissago including a log LDR which was not different from controls and a very similar percentage of haemocytes in the five classes of damage relative to those measured in mussels maintained at laboratory conditions.

In contrast, although the LDRs calculated for Suna and Laveno were very similar (Tables II and III), the tail percentage DNA indicated a higher pollution level at the former site because several cells fell into high (14.4%) and extreme (4.2%) DNA damage classes, percentages that were more than double those recorded at Laveno.

It seems that although the approach based on the LDR measurement fits very properly with the categorisation of DNA damage, this last metric is more sensitive and provides much more information on the pollution effects among sampling sites.

Conclusions

Biomarker results obtained in Lake Maggiore indicate that pollution was due to a mixture of several man-made chemicals derived from industrial and agricultural activities rather than a single chemical class, as shown by the non-homogeneous responses of the three measured biomarkers at every site. The integrated use of different biomarkers also showed the probable presence of contamination due to xenobiotics which was not revealed by chemical data. The main drawback of this analysis is the inability to measure all of the pollutants present in an aquatic ecosystem. Thus, biomarkers in Zebra mussel seem to be a useful approach to monitor the bioavailability of contaminants and to identify potentially perturbed or contaminated sites. Undoubtedly, extreme attention has to be paid to the interpretation of several biomarker results and recourse to chemical data is often crucial to avoid mistakes that might wrongly direct environmental management. On the other hand, several biotic and abiotic interferences could modify the biochemical responses, as shown by the heavy influence of temperature on AChE activity and by substrate inhibition of EROD activity. For instance, we have reported that the between-years comparisons for these two biomarkers should be treated with great care due to the additive or opposite effects of the environmental and chemical variables.

Nowadays, the use of biomarkers is submitted to severe checks before their possible integration into legislation and they have been severely criticised for these specific reasons. On the other hand, any biological method implies a certain variability that can be further decreased only with in-depth knowledge of the real behaviour of the

selected biomarker, as well as knowledge of all interference, especially with a non-superficial approach to this potentially powerful tool for future environmental management.

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