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Integrated use of biomarkers and bioaccumulation data in Zebra mussel (*Dreissena polymorpha*) for site-specific quality assessment

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Abstract
One of the useful biological tools for environmental management is the measurement of biomarkers whose changes are related to the exposure to chemicals or environmental stress. Since these responses might vary with different contaminants or depending on the pollutant concentration reached in the organism, the support of bioaccumulation data is needed to prevent false conclusions. In this study, several persistent organic pollutants — 23 polychlorinated biphenyl (PCB) congeners, 11 polycyclic aromatic hydrocarbons (PAHs), six dichlorodiphenyltricholroethane (DDT) relatives, hexachlorobenzene (HCB), chlorpyrifos and its oxidized metabolite — and some herbicides (lindane and the isomers α, β, δ; terbutilazine; alachlor; metolachlor) were measured in the soft tissues of the freshwater mollusc Zebra mussel (*Dreissena polymorpha*) from 25 sampling sites in the Italian portions of the sub-alpine great lakes along with the measure of ethoxyresorufin dealkylation (EROD) and acetylcholinesterase (AChE) activity. The linkage between bioaccumulation and biomarker data allowed us to create site-specific environmental quality indexes towards man-made chemicals. This classification highlighted three different degrees of xenobiotic contamination of the Italian sub-alpine great lakes: a high water quality in Lake Lugano with negligible pollutant levels and no effects on enzyme activities, an homogeneous poor quality for Lakes Garda, Iseo and Como, and the presence of some xenobiotic point-sources in Lake Maggiore, whose ecological status could be jeopardized, also due to the heavy DDT contamination revealed since 1996.

Keywords: *Water quality, ethoxyresorufin dealkylation (EROD), acetylcholinesterase (AChE), bioaccumulation, Italian lakes*

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Introduction

The recent European Union Directive 2000/60/EC, establishing a framework for European Community action in the field of water policy, points out the importance of a multidisciplinary approach for the assessment of water quality to establish some connections among external values of exposure to pollutants, their levels in organisms and possible early adverse effects (Van der Oost et al. 2003). Environmental risk assessment is generally based on information derived from research on the physical–chemical characteristics of pollutants (quantitative structure–activity relationship (QSAR)-based approach) and from laboratory-based toxicity tests

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(Moore et al. 2004). These procedures identify the possible effects only when real damage to the selected organism has occurred and they do not directly identify other adverse effects due to chemical mixtures (Howard 1997, Kortenkamp & Altenburger 1998). There is therefore a priority requirement to implement the use of a robust, simple, easy-to-learn, cost-effective test system that can identify early diagnostic changes in biota, which can be linked to ecologically relevant end-points to facilitate a predictive ranking of the conditions of the ecosystem (Moore et al. 2004).

The final goal indicated by international agencies for environmental protection is then to establish environmental quality indexes obtained by chemical and/or biological criteria in order to classify the monitored sites in a scale of pollution (Narbonne et al. 2005).

Useful approaches based on the biological responses (biomarkers) produced by an organism, population or community due to a chemical exposure have been proposed for the identification of the environmental quality by biological assays (Depledge & Fossi 1994). Biomarkers can be used to obtain early-warning signals of environmental risk (Payne et al. 1987) and they can detect either exposure to or the effects of pollutants. However, the translation of biomarker results into environmental information is limited due to a difficulty in explaining some of the biological responses, whose variations from the basal activity are influenced by several biotic and abiotic variables. Therefore, many international programmes (BEEP, CITY FISH, BEQUALM) are being carried out in order to standardize the measurement of some biomarkers (Van der Oost et al. 2003).

Biomarker assays are well known and standardized for aquatic vertebrates and several invertebrates, such as in the marine mussels of the genus *Mytilus* (Livingstone et al. 1997, Regoli 1998, Mora et al. 1999, Sheehan & Power 1999, Banni et al. 2005, Narbonne et al. 2005). On the other hand, the resistance to pesticides in invertebrates by detoxification is the only population-level response that has been directly correlated with enzyme biomarkers such as mixed-function oxidase (MFO), glutathione *S*-transferase (GST) and acetylcholinesterase (AChE) activities or other esterase activities (Lagadic et al. 1994, Gunning et al. 1997).

Recently, there has been considerable interest in the use of biochemical indexes in the freshwater Zebra mussel (*Dreissena polymorpha*) (Dauberschmidt et al. 1997, De Lafontaine et al. 2000, Bolognesi et al. 2004, Binelli et al. 2005, Ricciardi et al. 2006), which is a suitable sentinel organism due to its wide geographical distribution (a large part of the European and American water bodies and it is sporadically present in the Far East and Africa), a great sensitivity to environmental pollutants and a high accumulation rate. Moreover, Zebra mussel is stationary and is normally the dominant species in the freshwater ecosystems. It has been commonly used in the biomonitoring of persistent organic pollutants (POPs), trace metals and radionuclides, but very limited data are found on its possible use in early-warning systems. New information on these biomarkers is available for *D. polymorpha*, such as the opportunity of using mussels depurated in laboratory as controls (Binelli et al. 2005, 2006) and the strong influence of temperature on the AChE activity in the range 15–25°C (Ricciardi et al. 2006).

Two of the most used biomarkers are the variation of ethoxyresorufin dealkylation (EROD) and the inhibition of AChE. The EROD activity is induced by the presence of several planar compounds such as polycyclic aromatic hydrocarbons (PAHs), dioxin-like polychlorinated biphenyls (PCBs), polychlorinated dibenzo-dioxins

(PCDDs) and polychlorinated dibenzofurans (PCDFs) (Snyder 2000, Whyte et al. 2000), and therefore it is a measure of the detoxifying ability of the organism by enzymes related to the cytochrome P450 family. AChE is a serine hydrolase that catalyses the hydrolysis of choline esters and it is mainly involved in cholinergic neurotransmission (Mora et al. 1999). Two chemical classes have a reversible (carbamates) and irreversible (organophosphates) inhibition action on this enzyme, blocking the natural substrate binding.

The aim of the present paper was to assess the site-specific quality assessment with regard to man-made chemicals at 25 sampling stations on the portions in the sub-alpine great lakes (Maggiore, Lugano, Como, Iseo, Garda) obtained through the combination between bioaccumulation and biomarker data found in *D. polymorpha* specimens. Concentrations were measured of several POPs (23 PCB congeners, 11 PAHs, six dichlorodiphenyltricholroethane (DDT) relatives, hexachlorobenzene (HCB), chlorpyrifos and its oxidized metabolite) and some herbicides (lindane and the isomers α, β, δ; terbutilazine; alachlor; metolachlor) in the mussel soft tissues along with the measure of EROD and AChE activities as biomarkers to evaluate the contamination effects.

Separated site-specific quality assessments both for bioaccumulation and biomarker measurements were first created. Afterwards, the combination between these two different sets of data allowed the creation of a complementary approach for the environmental management through the use of site-specific quality classification.

Materials and methods

Study area and sample treatment

The Italian sub-alpine Great Lakes represent more than 70% of overall Italian freshwaters and are located in the Po River plain, one of the most populated and industrialized European areas (Figure 1). They are included in the geological district of the Southern Alps and have similar characteristics of climate and of pH (7.3–8.5), conductivity (146–258 $\mu S\ cm^{-1}$ at 20°C), dissolved oxygen (8.8–12.6 $mg\ l^{-1}$), alkalinity (0.8–2.2 $meq\ l^{-1}$) (Osservatorio dei Laghi Lombardi (OLL) 2005). The selection of 25 sampling sites was planned to account for different environmental conditions of these water bodies that have different morphometric characteristics and anthropic impact levels. Lake Maggiore was sampled at 14 sites to monitor the heavy DDT contamination found in it since the early 1990s (Binelli et al. 2004a).

About 500 mussels were collected by a scuba diver at 4–5 m of depth at each sampling site during the pre-reproductive period of *D. polymorpha* (Bacchetta et al. 2001) to minimize every possible interference due to abiotic and biotic factors.

Mussels used for biochemical assays were transported still tied on rocks to the laboratory in bags filled with lake water within a few hours from sampling. Only live adult specimens (visual inspection) were then separated from the substrate by bissus excision and immediately stored at -80°C pending biomarker assays. Mussels to be used as controls underwent a different handling regimen (Binelli et al. 2006): rocks were rinsed, introduced in several glass aquaria filled with about 100 litres of dechlorinated tap water, all the while maintaining a natural photoperiod, constant temperature (20°C) and oxygenation ($> 90\%$ of saturation). Animals were fed daily by an algal suspension of *Pseudokirchneriella subcapitata*, and water was constantly

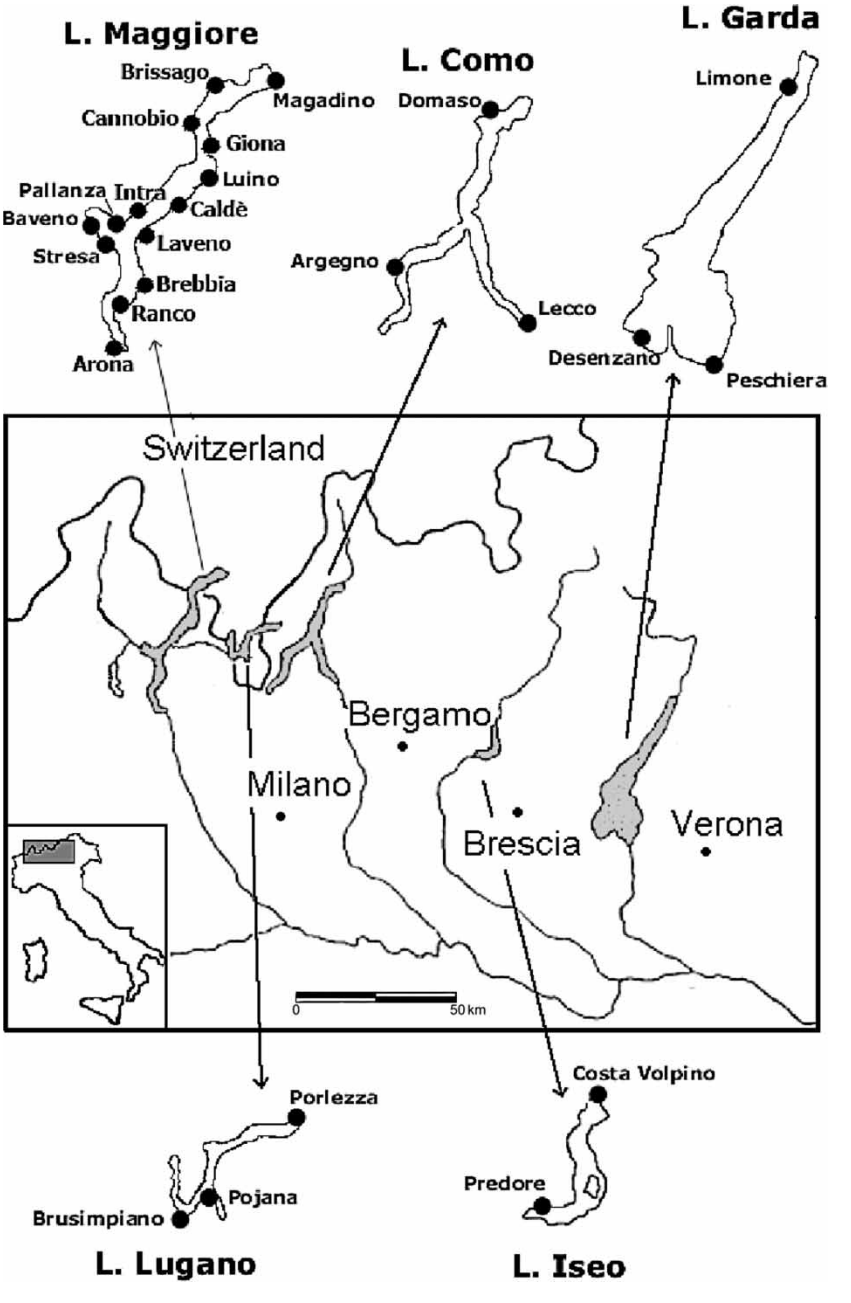

Figure 1. Detail of the study area with the Italian sub-alpine great lakes and sampling stations of Zebra mussel specimens.

changed every day for 45 days to depurate molluscs by any accumulated xenobiotic, whose elimination was checked by gas chromatographic analysis. After this control, mussels were separated from the substrate and stored at -80°C.

Specimens used for chemical analyses were washed with lake water, transported to the laboratory into aluminium sheets in refrigerated bags and frozen at -18°C

pending chemical analysis, for which only adult specimens of a shell length greater than 15 mm (older than 1 year) were used (Binelli et al. 2001).

Chemical analyses

Several classes of POPs were analysed (23 PCB congeners, six DDT relatives, HCB), the insecticide organophosphate chlorpyrifos and its oxidized metabolite (oxon), and some herbicides (lindane and the isomers α, β, δ; terbutilazine; alachlor; metolachlor) by gas chromatography-electron capture detection (GC-ECD), while 11 PAHs (phenanthrene, naphthalene, fluorene, anthracene, fluoranthene, pyrene, benzo(α)anthracene, chrysene, benzo(β)fluoranthene, benzo(k)fluoranthene, benzo(α)pyrene) were measured by high-performance liquid chromatography (HPLC) only at some sampling stations due to their low levels. Some of these xenobiotics are in the European Union list of priority substances for the monitoring of water bodies (European Union 2455/2001/EC).

Gas chromatographic analyses. About 1.5 g of dried tissue were extracted for 12 h with 100 ml of acetone/*n*-hexane (50/50) mixture using a Soxhlet and dried with a rotating evaporator (RV 06-LR, IKA, Staufen, Germany) and 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 with 6 ml of H_2SO_4 (96%) for the organic phase digestion. After 24–36 h the supernatant was recovered, concentrated to 1 ml and put in a glass column (length = 35 cm; i.d. = 1 cm) filled with 18 g of Florisil (Supelco PR, 60-100 Mesh, Sigma Aldrich, Milan, Italy) for the following clean-up.

The column was eluted first with *n*-exane and then with a dichloro-methane/*n*-exane (85/15) mixture (Lazar et al. 1992, Binelli et al. 2004a). The two different fractions were injected separately into a GC chromatograph equipped with ECD.

DDTs, HCB, chlorpyrifos and hexachlorocyclohexanes (HCHs) were identified with a Varian CP-sil 19 CB column (length = 50 m; i.d. = 0.25 mm; film thickness = 0.20 μm), while PCBs were analysed with an HP CP/sil 8 CB column (length = 100 m; i.d. = 0.25 mm; film thickness = 0.25 μm). Helium and nitrogen were used as carrier and make-up gases, respectively. Xenobiotics were quantified by comparing the peak areas of individual compounds with external standards obtained adding the single certified compounds (Dr Ehrenstorfer GmbH, Augsburg, Germany) to reach a similar pattern of the environmental mixture. The hexa-CB 209 not found in the environmental samples was used as internal standard (recovery rate up to 80%) and the detection limit was 0.5 ng l^{-1}; analyses of samples were carried out in duplicate. This method was tested in two different International ring-tests. One blank was carried out every six complete analytical procedures.

Several samples were confirmed by a GC-MSn Polaris Q 'Ion-Trap' (Thermo-Ultron, Austin, TX, USA) equipped with a Programmed Temperature Vaporizer (PTV) injector (split flux = 20 ml min^{-1}) and a Restek column RTX-5 MS (length = 30 m; i.d. = 0.25 mm). GC peaks were revealed in tandem mass spectrometry (MS-MS) mode and checked by the Excalibur software (Thermo-Ultron).

High-performance liquid chromatography (HPLC) analyses. About 2 g of freeze-dried sample were extracted with 100 ml of dichloromethane (DCM) in a Soxhlet for 6–7 h using dark glassware. The extract was gently concentrated to about 5 ml under a

nitrogen gas stream, recovered with 20 ml of cyclohexane and digested in darkness for about 24 h with 100 ml of 0.5 M KOH in a water/methanol mixture (1:9). The hexanic phase was recovered and the polar mixture washed twice with small aliquots of cyclohexane and then discharged. The sample was gently evaporated in a nitrogen gas stream to about 1.5 ml and run on a column filled with 1 g of Florisil, then eluted with 15 ml of DCM and evaporated to about 2 ml. Finally, 5 ml of acetonitrile were added and the sample concentrated to 1 ml under a nitrogen gas stream. HPLC analyses were carried out with a Jasco PU-980 Liquid Chromatograph equipped with a 20 µl loop, a 20 cm long C 18 column (Lichrospher®, Merck, Haar, Germany) maintained at 20°C with a Peltier-column thermostat, and a Jasco FP-920 fluorimeter. The flow rate was 0.5 ml min^{-1} and the mobile phase gradient was: 50+50 acetonitrile/water for 5 min, a constant increase of acetonitrile phase during 15 min and an isocratic phase from 15 to 35 min. Two HPLC runs with different wavelengths were used to avoid peak overlaps. One blank was carried out every six complete analytical procedures. The 11 PAHs (Dr Ehrenstorfer GmbH) most found in the aquatic ecosystems were quantified by comparing peak areas with an external standard obtained by single certified compounds to reach a similar pattern of the environmental mixture. The detection limit was 1 ng l^{-1}; analyses of samples were carried out in duplicate and the benzo(*g*,*h*,*i*)perylene was used as an internal standard (recovery rate up to 75%).

This method was tested with a certified PAH standard (100 ng g^{-1} of dry weight for each compound) spiked in an unpolluted rainbow trout fillet, obtaining a recovery rate up to 80% for each PAH.

Biomarker assays

Thirty mussels of similar size (20±0.2 mm) divided into three replicate pools were used for each assays and stored at −80°C before biochemical analyses. Protein concentration was measured by the Bradford (1976) method using bovine serum albumin (BSA) as standard.

AChE activity measurement. AChE activity was determined at 23°C, pH 7, and with acetylthiocholine (ASCh) as substrate, as described by Ellman et al. (1961). Soft tissues were excised and washed in 0.15 M of KCl at 4°C. About 1 g of tissue maintained in an ice bath was homogenized in a Tris-HCl buffer-saline solution (100 mM, pH 7.6), containing 0.1% Triton X-100 (Bocquené et al. 1997). S10 supernatant was obtained by centrifugation at 10 000*g* for 15 min at 4°C, repeated twice and stored at 4°C overnight.

A total of 50 µl of S10 extract, pre-warmed at 23°C for 15 min, was reacted with 0.43 mM of 5,5-dithiobis(-2-nitrobenzoic acid) (DTNB), phosphate buffer (pH 7) in the presence of ASCh. The absorption of the 2-nitro-5-thiobenzoate anion formed from the reaction was recorded at 412 nm every 60 s for 7 min at room temperature using a double-beam spectrophotometer Perkin Elmer Lambda 2 to read each sample against blank.

The kinetics were calculated in the linear range after the subtraction of blank activities due to substrate autohydrolysis. Each pool was analysed in triplicate and results were expressed as nmol min^{-1} mg^{-1} protein.

Ethoxyresorufin-O-deethylase (EROD) activity measurement. The principle of the assay is based on the hydrolysis of the substrate ethoxyresorufin to the fluorescent compound resorufin, according to Burke & Mayer (1974). Zebra mussel soft tissues, maintained in an ice bath, was rinsed in 150 mM of KCl and homogenized (50–60 s) by an Ultra-Turrax grinder in a buffer solution containing 100 mM of Tris-HCl (pH 7.6) and sucrose (250 mM). ethylenediamine tetra-acetic 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 the enzyme activity was measured using 50 µl of S9 extract and a reaction buffer containing Tris-HCl (50 mM, pH 7.4), $MgCl_2$ (5 mM) and β-nicotinamide adenine dinucleotide phosphate (NADPH) (10 µM) with ethoxyresorufin (10 µM) as substrate. Dicumarol (10 µM) was added as a specific inhibitor of DT diaphorase (Risso-de Faverney et al. 2000).

The fluorescence ($\lambda_{ex} = 520$ nm, $\lambda_{em} = 590$ nm) was recorded for 20–30 min by a Jasco FP-920 fluorescence detector at 20 ± 1°C using only data measured in the linear range of the kinetic curve. Each pool was analysed in duplicate and EROD activity was measured as pmol min^{-1} mg^{-1} protein.

Statistical approach

A significant correlation ($r = 0.68$, $p < 0.05$) between temperatures measured at each sampling site and AChE activity was observed. In laboratory conditions, a strong relationship between the two variables has also been found (Ricciardi et al. 2006). Hence, the analysis of covariance (ANCOVA) was used to test statistical significance for all variables. A Fischer LSD post-hoc test was used when necessary.

EROD data were not normally distributed (Kolmogorov–Smirnov test, $p > 0.05$), thus a logarithmic ($\log_{10}$) transformation was applied before one-way analysis of variance (ANOVA), followed by a Fisher LSD post-hoc test.

Correlations between biomarkers and chemical data were checked by Pearson correlation coefficients ($p < 0.05$). Due to insufficient data obtained for PAHs, the average value calculated for each lakes was used.

All statistical tests were carried out by the Statistica software, version 6.0.

Results and discussion

Bioaccumulation data

Table I shows some mussel characteristics and temperatures measured at each sampling site. Biomonitoring results in Lake Maggiore (Table II) showed a homogeneous DDT contamination, which is well-known since 1996 and due to a chemical plant located on one of the main lake inlets (River Toce). The concentrations found are higher than those measured in bivalves from other Western countries and comparable with those found in several developing countries (Tanabe et al. 2000).

Previous research (Binelli et al. 2004b) showed the clear effect of *p,p′*-DDT and their metabolites: a delay in oocyte maturation and a high incidence rate of pathological pictures mainly referable to oocyte degeneration and haemocyte infiltration was noticed in Zebra mussel specimens from the most polluted area of Lake Maggiore (Pallanza Bay).

Table I. Temperature measured at each sampling site and some characteristics of mussel specimens from the Italian sub-alpine great lakes.

Lakes	Sampling stations	Identification	Temperature (°C)	Average shell length (cm) ± S.D.	Whole body lipid (%) (d.w.)	Protein content (mg ml^{-1} ± S.D.)
Maggiore	Magadino	Mag	15	2.1 (± 0.2)	18.7	25.6 (± 1.6)
	Brissago	Bri	14.5	2.1 (± 0.2)	18.4	22.8 (± 1.5)
	Cannobio	Can	14	2.0 (± 0.2)	18.8	12.2 (± 0.2)
	Giona	Gio	11.5	2.0 (± 0.2)	19.4	25.2 (± 1.0)
	Luino	Lui	15	2.1 (± 0.2)	17.7	27.1 (± 2.0)
	Caldè	Cal	14.5	2.0 (± 0.2)	19.2	25.5 (± 1.3)
	Intra	Int	17	2.0 (± 0.3)	17.3	25.1 (± 0.6)
	Pallanza	Pal	18	2.1 (± 0.2)	15.9	14.3 (± 0.8)
	Laveno	Lav	14	1.7 (± 0.2)	15.4	21.3 (± 2.3)
	Baveno	Bav	17	2.3 (± 0.2)	17.2	19.4 (± 11.2)
	Stresa	Str	16	2.0 (± 0.2)	17.9	26.9 (± 2.4)
	Brebbia	Bre	20	2.4 (± 0.3)	13.7	11.4
	Ranco	Ran	18	2.1 (± 0.2)	15.5	24.5 (± 3.4)
	Arona	Aro	18	2.1 (± 0.3)	14.4	14.4 (± 5.2)
Lugano	Brusimpiano	Bru	20	2.0 (± 0.1)	12.5	27.1
	Porlezza	Por	21.5	1.9 (± 0.2)	11.6	25.2
	Pojana	Poj	21.5	1.9 (± 0.1)	14.0	23.9
Como	Domaso	Dom	12	1.7 (± 0.2)	16.3	25.7 (± 0.6)
	Argegno	Arg	15.5	1.7 (± 0.3)	15.0	25.3
	Lecco	Lec	14	1.7 (± 0.2)	15.1	23.6 (± 1.3)
Iseo	Costa Volpino	C.V.	16.5	1.7 (± 0.2)	14.8	26.8 (± 3.8)
	Predore	Pre	16	1.7 (± 0.2)	13.6	22.8 (± 3.7)
Garda	Desenzano	Des	18.5	1.7 (± 0.2)	12.6	24.1 (± 3.6)
	Peschiera	Pes	18	1.6 (± 0.2)	14.3	18.9
	Limone	Lim	15	1.5 (± 0.1)	10.3	23.4 (± 2.4)

S.D., standard deviations; d.w., dry weight (the percentage of lipids was calculated by the ratio between lipid content and dry weight).

In almost all sampling sites of Lake Maggiore the analogue with the highest concentration was the *p,p′*-DDD dichlorodiphenyldichloroethylane that increased during the floods that occurred in 2001–02, while the most abundant homologue found in the other lake basins was the *p,p′*-DDE dichlorodiphenyldichloroethylene that is more toxic, volatile and able to interfere the endocrine system than the parent compound (Olea et al. 1998). The high values of this metabolite (mean value of 67% of total DDTs) indicated an old contamination, also confirmed by the low levels of the *p,p′*-DDT:*p,p′*-DDE ratio. Moreover, the homogenous DDT values measured in all sampling sites indicated that no point-sources are still present in Lakes Garda, Iseo, Como and Lugano.

On the contrary, several point-sources are probably still discharging these chemicals in the Italian sub-alpine great lakes due to the variability of PCB concentrations among the selected sampling stations (Table II). The PCB composition monitored is equivalent to a mixture of Aroclor 1260 (65%) and 1254 (35%), the two commercial mixtures used in Italy until the end of the 1980s (Provini et al. 1995). Hexachlorobiphenyls were the main chlorine-based class, followed by the epta- and pentachlorobiphenyls, while very low concentrations of the other classes were measured.

Table II. Concentrations (ng g^{-1} lipids) of several chemicals measured in Zebra mussel soft tissues.

Lakes	ID	α-HCH	β-HCH	γ-HCH	δ-HCH	HCB	CP	CPO	TBA	Ala	Met	ΣDDTs	ΣPAHs	ΣPCBs
Maggiore (14 sampling sites)	Mag	5.6	0.8	3.0	8.1	0.3	5.9	1.3	44.0	< d.l.	31.2	804.5		428.5
	Bri	2.8	2.6	5.5	5.4	8.0	21.5	17.4	20.0	79.0	41.2	457.9	31.5	833.8
	Can	0.8	2.9	4.4	2.0	8.3	11.8	16.9	36.0	27.0	20.6	442.1		870.9
	Gio	< d.l.	< d.l.	10.6	< d.l.	15.2	7.1	< d.l.	< d.l.	19.0	< d.l.	1116.8		747.4
	Lui	0.9	1.2	7.1	< d.l.	2.6	< d.l.	< d.l.	< d.l.	< d.l.	18.0	945.3		486.3
	Cal	< d.l.	2.7	3.0	1.3	3.6	< d.l.	5.0	10.0	< d.l.	18.0	829.4		538.0
	Int	< d.l.	1.0	2.6	56.1	7.3	8.5	< d.l.	< d.l.	8.0	20.9	948.7		527.2
	Pal	0.8	1.4	2.5	35.8	8.0	8.2	< d.l.	< d.l.	< d.l.	10.2	729.9		365.9
	Lav	< d.l.	< d.l.	10.3	< d.l.	7.6	19.5	< d.l.	42.0	27.0	13.4	992.5		1288.1
	Bav	< d.l.	1.5	2.7	72.5	13.3	72.7	< d.l.	< d.l.	< d.l.	27.6	1386.2	58.8	400.2
	Str	< d.l.	68.3	< d.l.	< d.l.	17.4	< d.l.	< d.l.	61.0	24.0	18.6	1417.3		893.7
	Bre	1.8	< d.l.	11.5	< d.l.	14.6	6.3	12.5	60.0	3.0	26.6	972.8		1846.0
	Ran	1.4	11.6	9.9	1.7	9.5	15.7	0.0	34.0	39.0	56.7	479.1		921.3
	Aro	1.6	2.6	7.5	< d.l.	5.1	18.1	26.2	55.0	< d.l.	15.1	686.3	199.5	1521.1
Lugano (3)	Des	< d.l.	6.6	2.0	16.9	5.0	6.6	20.2	< d.l.	< d.l.	39.7	147.2		1029.8
	Pes	< d.l.	5.7	1.6	55.2	4.0	6.4	16.2	< d.l.	< d.l.	33.4	134.3	119.2	793.3
	Lim	< d.l.	5.6	2.4	7.9	4.1	3.4	6.4	< d.l.	< d.l.	36.0	100.4		691.1
Como (3)	Pre	0.8	< d.l.	4.8	< d.l.	< d.l.	12.3	24.1	< d.l.	< d.l.	9.7	161.2	150.4	1121.5
	C.V.	0.9	0.9	7.0	< d.l.	3.9	31.9	43.3	55.0	< d.l.	29.7	147.6		2508.5
	Dom	< d.l.	< d.l.	< d.l.	< d.l.	4.1	10.4	36.8	42.0	5.0	14.2	224.1		1115.9
Iseo (2)	Arg	0.8	< d.l.	8.2	< d.l.	5.8	16.5	53.8	56.0	3.0	11.5	148.3	149.1	1189.4
	Lec	0.9	< d.l.	8.1	< d.l.	7.2	11.3	43.0	49.0	< d.l.	19.3	193.7		1234.1
Garda (3)	Poj	0.2	2.4	1.9	0.8	3.0	7.5	4.0	< d.l.	150.0	33.6	116.2		530.4
	Bru	< d.l.	1.2	2.7	0.7	7.1	12.7	10.4	< d.l.	8.0	27.4	116.2	109.9	915.9
	Por	< d.l.	< d.l.	< d.l.	< d.l.	5.6	11.8	10.1	< d.l.	13.0	15.8	165.1		429.2

<d.l., lower than the detection limit; Ala, alachlor; CP, chlorpyrifos; CPO, chlorpyrifos oxon; DDTs, *p,p′*-dichlorodiphenyltrichloroethane relatives; HCB, hexachlorobenzene; HCH, hexachlorocyclohexane; Met, metalachlor; PAHs, polycyclic aromatic hydrocarbons; PCBs, polychlorinated biphenyls; TBA, terbutilazine.

On the other hand, CBs 153, 138 and 118 (hexachlorosubstituted) are the prevailing congeners usually present in biological samples, followed by the epta-CB 180 (Bayarri et al. 2001).

Low levels of chlorpyrifos and HCB were found, while the contamination of lindane and its isomers, terbutilazine, alachlor and metolachlor was absolutely negligible (Table II) because they either have been banned or their use has been heavily limited by law since the 1990s after the discovery of their presence in the ground waters of the Po River basin.

We carried out the analyses of PAHs only in few sampling stations, given the low levels of several polycyclic aromatic hydrocarbons found, including the most toxic benzo(α)pyrene.

The main contamination source might be due to random discharges of hydrocarbons rather than a pyrogenic or combustion origin, since the percentage of less condensed PAHs (2- and 3-rings) ranged from 58.3 to 74.7%, with the exception of Baveno and Brissago (Lake Maggiore).

The scarce literature data did not show any other xenobiotic contamination in the examined basins.

Acetylcholinesterase (AChE) activity

Between- and within-lakes differences and the comparison between enzyme activity measured at each sampling station and depurated controls were tested by the analysis of covariance (ANCOVA). Unfortunately, it was not possible to analyse three pools for the AChE determination at three sampling sites (Brusimpiano, Brebbia and Peschiera) because of the insufficient quantity of soft tissues.

Results obtained in the Italian sub-alpine great lakes (Figure 2) indicated an homogeneous pollution by the AChE inhibitors in these aquatic ecosystems. We noticed a significant decrease of the enzyme activity compared with controls for all the sampling stations ($p < 0.05$), except for Ranco (Lake Maggiore).

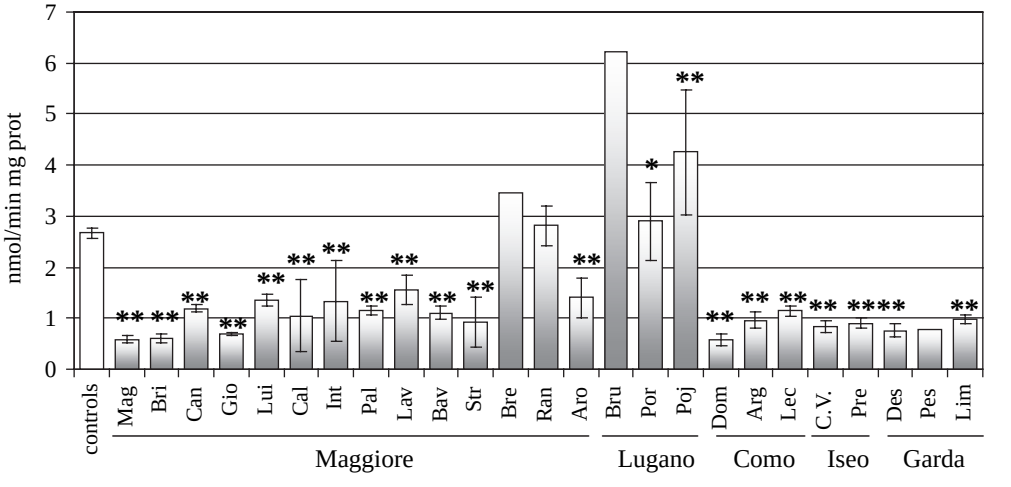

Figure 2. AChE activity measured in the 25 sampling sites of the Italian sub-alpine great lakes by Zebra mussel and control levels (white bar). Levels of significance (*$p < 0.05$, **$p < 0.01$) of ANCOVA test are indicated.

The between-lakes comparison showed a significant difference between Lake Lugano and the other ones ($p < 0.05$) probably because its watershed does not allow for an intensive agriculture and a subsequent drainage of the AChE inhibitors. Another possible reason could be the high copper levels found in *D. polymorpha* from this basin (Camusso et al. 2001), which are perhaps able to increase the AChE activity (Dethloff et al. 1999, Romani et al. 2003).

Within-lakes comparisons showed a significant difference among sampling stations only in Lake Maggiore, when the southernmost sampling site (Arona) highlighted a significant difference of AChE activity with Magadino and Brissago, located in the northern part of the lake. Moreover, the AChE activity measured at Ranco was significantly different from that of all the other sampling sites ($p < 0.05$).

Ethoxyresorufin dealkylation (EROD) activity

EROD activity results seemed to show a similar contamination of compounds able to bind the Aryl hydrocarbons receptor (AhR) in Lakes Como, Iseo and Garda with no significant between-lakes differences, while Lake Maggiore is significantly different from the other environments ($p < 0.05$). Lake Lugano showed significant differences only with Lake Garda and Maggiore ($p < 0.05$).

We did not find within-lakes differences for all the ecosystems, except for Lake Maggiore, where a much more complex picture for pollution was observed. This is confirmed by the comparisons between each sampling station and depurated mussels used as controls (Figure 3): EROD activity measured at all sites of Lakes Como, Iseo and Garda was significantly different from controls and the same holds true for several sites of Lake Maggiore, while in Lake Lugano only the Brusimpiano site is significantly different from controls ($p < 0.05$).

Surprisingly, very low levels of EROD activity were found in some sampling stations of Lake Maggiore with a mean inhibition rate of 41% compared with controls. These data seem to highlight the lack of pollution in this basin; however, as discussed in

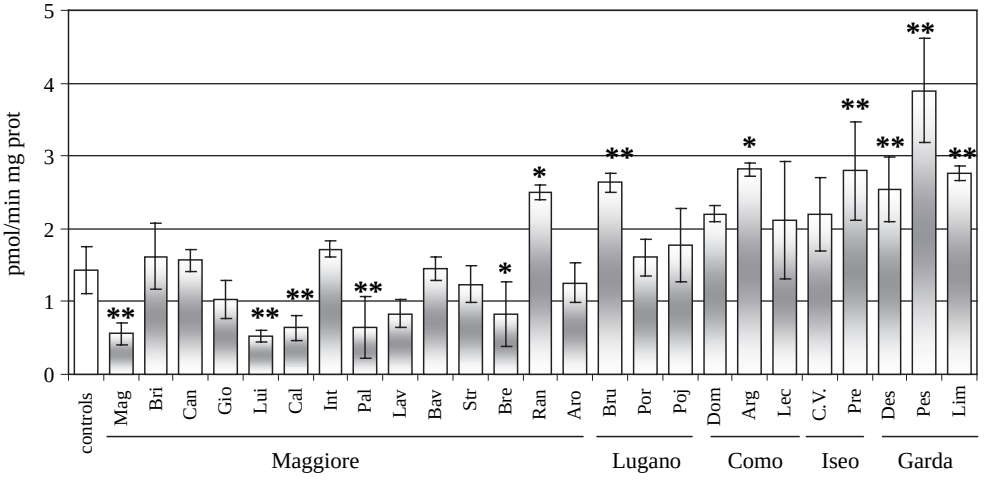

Figure 3. EROD activity of Zebra mussel specimens from 25 sampling sites of the Italian sub-alpine great lakes and related control levels (white bar). Levels of significance (*$p < 0.05$, **$p < 0.01$) of ANOVA test are indicated.

detail by Binelli et al. (2005), this lack of contamination could be only apparent due to the presence of EROD-inhibiting compounds, which means Lake Maggiore is contaminated by both DDTs and trace metals (Camusso et al. 2001) that can interfere with the CYP1A induction cycle (George 1989, Fent & Stegeman 1993, Fent et al. 1996, Viarengo et al. 1997).

Site-specific quality assessment

The use of depurated mussels as controls, instead of the values of the less contaminated station, allowed us to identify an EROD activity inhibition that is not usually considered as a common biological end-point (Whyte et al. 2000). This particular behaviour demonstrates the need to support the biomarker results with bioaccumulation data to carry out a correct biomonitoring and the subsequent environmental management.

EROD activity exhibited a significant correlation with chlorpyrifos oxon (Table III) that is the first oxidized metabolite of the parent compound after a metabolic transformation by cytochrome P450-dependent mono-oxygenases (Belden & Lydy 2000). Moreover, a strong inverse correlation between DDTs and EROD activity was found, pointing out a probable inhibiting action of these insecticide relatives, whose pollution has been particularly troublesome in Lake Maggiore.

Arukve et al. (2000) showed similar results, even though the EROD activity decrease found in the Atlantic Salmon (*Salmo salar*) treated with *o*,*p*′-DDT was not significant. Moreover, a previous study (Binelli et al. 2006) exhibited a significant decrease of EROD activity in Zebra mussel specimens exposed to 100 ng l^{-1} of *p*,*p*′-DDT in laboratory conditions. Since DDT levels measured in treated mussels (about 1550 ng g^{-1} lipids) in the latter study were similar to those found in *D. polymorpha* at some sampling sites of Lake Maggiore, the possible counteracting action of heavy metals and DDTs compared with planar chemicals could be the reason of EROD inhibition observed in this basin. Moreover, this behaviour was not noticed in the other basins that are not affected by heavy DDT and metal pollution (Camusso et al. 2001, Binelli et al. 2006, table 2).

Notwithstanding that the CYP450 induction by PCBs had been widely demonstrated in several fish species (Whyte et al. 2000), in the Blue mussel (Livingstone et al. 1997) and Zebra mussel (Binelli et al. 2006), the Pearson's coefficient matrix did not show a significant correlation with EROD activity. However, several authors had shown that this enzyme activity does not follow a convention saturation curve as the inducer concentration increases, but reaches a maximum and then declines (Hahn et al. 1993, Schmitz et al. 1995, Kennedy et al. 1996).

Besselink et al. (1998) had previously reported convincing evidence for inhibition of the EROD reaction in the Flounder (*Platichthys flesus*) by the CBs 77, 126, 169 (coplanar congeners), the CB 153 and the commercial mixture Clophen A50, while Gooch et al. (1989) showed an EROD competitive inhibition in scup (*Stenotomus chryops*) due to the exposure of CB 77. This behaviour was also shown in previous laboratory tests carried out with Zebra mussel specimens exposed to the dioxin-like CB 126 (100 ng l^{-1}): a significant increase of EROD activity compared with controls was noticed only in the first 48 h (Student's *t*-test, $p < 0.01$), followed by a fast decrease towards values equal to controls when the pollutant concentration in mussels was higher than 800 ng g^{-1} lipids (Binelli et al. 2006).

Table III. Pearson correlation coefficients between chemicals and biomarkers.

	EROD	AChE	HCB	HCHs	DDTs	PAHs	PCBs	CP	CPO	TBA	Ala	Met
EROD	–											
AChE	0.04	–										
HCB	−0.29	0.09	–									
HCHs	0.02	−0.21	0.58**	–								
DDTs	−0.72***	−0.20	0.63***	0.57**	–							
PAHs	0.22	0.04	−0.30	−0.25	−0.22	–						
PCBs	0.19	−0.01	0.05	−0.26	−0.21	0.30	–					
CP	0.04	−0.06	0.21	0.33	0.18	−0.22	0.13	–				
CPO	0.50**	−0.21	−0.29	−0.39	−0.59**	0.36	0.63***	0.10	–			
TBA	−0.17	−0.15	0.22	−0.09	0.13	0.23	0.66***	0.02	0.47*	–		
Ala	−0.08	0.35	0.03	−0.12	−0.13	−0.30	−0.18	−0.04	−0.21	−0.11	–	
Met	0.30	0.22	−0.09	0.14	−0.27	−0.37	0.01	0.12	−0.13	−0.04	0.32	–

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

The action mechanism has recently been discovered and it is due to the capability of the dioxin-like compounds to increase the aryl hydrocarbon receptor repressor (AhRR) protein that can create an inactive complex with the Ah receptor nuclear translocator (Arnt) (Hahn 2002, Karchner et al. 2002).

A graphical representation of EROD activity trend is shown in Figure 4, where the increase of the planar compound concentration produces a decrease of enzyme level due to competitive inhibition. That no misunderstanding in environmental data interpretation is possible when the enzyme levels fall into the squared zone because of the activating effect due to chemicals is directly shown by the EROD activity measured in the biological model. On the contrary, the main problem is the capability of discriminating between the two striped zones that show a similar EROD activity, but very different contamination conditions that can be a confusing factor for the interpretation of results without chemical data support. Moreover, Petrulis et al. (2001) showed that EROD activity in mixtures can be lower than that of one of the components alone and it is frequently much less than would be expected on the basis of additivity.

Bioaccumulation indexes. Since the creation of site-specific quality indexes does not appear to be easy because of the above-mentioned problems, we proceeded systematically. First, we separated quality indexes for both biomarkers and bioaccumulation data were created, then they were combined to reach a complementary quality assessment.

Only PCB and DDT results were considered for the chemical approach because other xenobiotics showed negligible pollution with levels not dangerous for the ecosystem and the chlorpyrifos oxon (CPO), the other chemical correlated with EROD, was not found in several sampling sites (Table II). Ranges defining some different chemical quality classes (Table IV) were selected starting from the background levels of PCBs and DDTs measured in these lakes. The average value for the ΣDDT was 150 ng g^{-1} lipids, excluding data from Lake Maggiore, which is comparable with residue levels of low contaminated sites (Monirith Ueno et al. 2003). We used the mean concentration of the less polluted sampling stations (400 ng g^{-1} lipids) as a PCB background level, which is a typical reference value measured in locations merely receiving diffuse atmospheric loading (Brevik et al. 1996) or with a

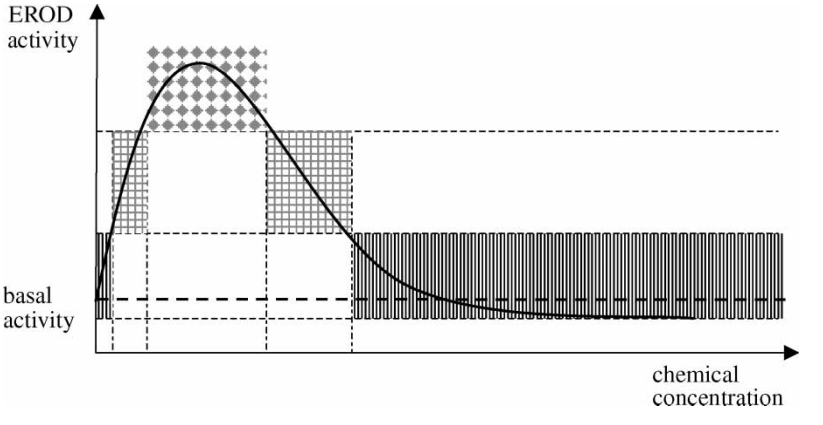

Figure 4. Representation of EROD activity variation in comparison with pollutant level increase.

Table IV. Quality classes based on DDT and PCB levels (ng g^{-1} lipids) measured in Zebra mussel specimens.

ΣDDTs	ΣPCBs	Quality class
0–300	0–600	0
> 300–600	> 600–1200	1
> 600–900	> 1200–1800	2
> 900–1200	> 1800–2300	3
> 1200	>2300	4

low level of industrialization (Nhan et al. 2001). The five quality classes (bad, scarce, moderate, good and high) are the same proposed by European Union Directive 2000/60/EC for the ecological status assessment of freshwaters.

The two classes obtained from the experimental data for each sampling site (Table II) were crossed in the matrix shown in Table V to obtain the site-specific quality index for the chemical approach only (Table VII).

The selection of bioaccumulation indexes followed specific criteria: the worst quality class (E) was assigned even when only one of the two parameters fell in the highest class (4) of Table V, assuring the greatest environmental protection. Moreover, the same weight was assigned to the sum of PCBs and DDTs because of their very similar environmental and toxicological dangers (ATSDR 1998, 2000).

Obviously, the selection of different ranges and classes is arbitrary because no literature data are still available. On the other hand, any environmental quality assessment assumes similar criteria, such as Directive 2000/60/EC that defines the ecological status of water bodies through arbitrary classifications. Moreover, when data on persistence and bioaccumulation are available, this Directive stated that these shall be taken into account in deriving the final value of the environmental quality standard, but without indications about the way to behave.

Biomarker indexes. The creation of site-specific quality indexes for biomarkers is much more difficult, above all for EROD activity. The main problem is the choice of the arbitrary biomarker ranking for the creation of the final classification scale (Narbonne et al. 1999, Banni et al. 2005).

We used the AChE activities lower than 20% from controls as background values (Table VI), in accordance with the US Environmental Protection Agency (1998) which considered a significant activity inhibition of 20% as a clear toxicological effect.

Table V. Quality classes based on the combination between bioaccumulation data only.

		PCBs				
		0	1	2	3	4
DDTs	0	A	B	C	D	E
	1	B	B	C	D	E
	2	C	C	C	D	E
	3	D	D	D	D	E
	4	E	E	E	E	E

A, high; B, good; C, moderate; D, poor; E, bad.

Table VI. Quality classes based on biomarker activities measured in mussel specimens. Percentage differences are referred to controls depurated at laboratory conditions.

		AChE activity (%)		
		> 50	> 20–50	0–20
EROD activity (%)	> 100	E	D	C
	> 40–100	D	C	B
	0±40	C	B	A
	> 40–100	D	C	B
	> 100	E	D	C

Since several studies (Osterloh & Pond 1983, Fleming et al. 1995, Sibley et al. 2000) have pointed out that an AChE inhibition higher than 50–60% can kill some organisms or damage the most sensible populations, an inhibition rate higher than 50% was chosen as a worst case. The five sampling sites (Figure 2) with enzyme activities not significantly higher than controls were considered to be in the range 0–20% of inhibition (waiting for future researches on the possible AChE activation by heavy metals), as found in Lake Lugano.

No literature data are available about specific EROD benchmarks because its inhibition is not a common end-point and its activation does not directly represent a potential danger for organism survival, but only an early-warning system for planar chemical exposure. Since the statistically significant EROD value (Brebbia) more similar to basal activity was 42% higher than controls (Table VII), we considered every site with activity differences lower than 40% as having a low pollution level (Table VI). The second limit was arbitrarily chosen, supposing that the doubling or halving of basal activity can point out a worsening of environmental quality.

Undoubtedly, the inhibitory potential of some halogenated aromatic compounds (HACs) plays a basic role in the connection between EROD activity and pollution conditions because a decrease in the enzyme activity to basal levels sometimes is diagnostic of an environmental quality worse than that obtained with high EROD values. Since it is very difficult to categorize this effect, also bearing in mind the interfering action of the real EROD inhibitors such as DDTs and heavy metals, we used the same quality classes for the activation and inhibition effects and delegated the chemical approach to distinguish between them.

Final site-specific classification. Table VII summarizes the site-specific indexes obtained by chemical and biomarker data after and before their combination. We assigned the same weight to the two approaches, so that the quality class for each sampling site was automatically obtained by the worst single index.

The complementary use of the two sets of data allowed a better identification of the contamination in nine sites, whose EROD activities fall in the range 0±40% of the difference from controls (highlighted with grey boxes in Table VII). In these sites, the activity similar to controls can be due to the two opposite effects mentioned above. Bioaccumulation data revealed that Baveno, Giona and Stresa are subjected to heavy pollution due to EROD inhibitors, as indicated by DDT levels higher than those measured in all the other sites (Table II). Moreover, PCB concentrations higher than 800 ng g^{-1} lipids were measured at Stresa, showing a possible inhibition effect on

Table VII. Quality classes obtained by bioaccumulation data at each sampling site; biomarker differences (%) with controls and their final quality indexes; site-specific quality ranking obtained by the link between bioaccumulation and biomarker data.

Lake	Site	DDT classes	PCB classes	Chemical index	Differences with controls (%) AChE	Differences with controls (%) EROD	Biomarker index	Site-specific quality classification
Maggiore	Mag	2	0	C	−8.1	−61.8	D	D
	Bri	1	1	B	−77.4	13.1	C	C
	Can	1	1	B	−55.3	9.3	C	C
	Gio	3	1	D	−74.1	−28.2	C	D
	Lui	3	0	D	−49.1	−63.2	C	D
	Cal	2	0	C	−60.8	−55.2	D	D
	Int	3	0	D	−50.0	21.5	B	D
	Pal	2	0	C	−56.5	−55.0	D	D
	Lav	3	2	D	−42.0	−42.7	C	D
	Bav	4	0	E	−58.7	2.1	C	E
	Str	4	1	E	−65.5	−14.5	C	E
	Bre	3	3	D	29.2	−42.0	C	D
	Ran	1	1	B	5.6	75.8	B	B
	Aro	2	2	C	−47.6	−12.9	B	C
Lugano	Bru	0	1	B	133.5	84.4	B	B
	Por	0	0	A	8.5	12.4	A	A
	Poj	0	0	A	59.3	24.3	A	A
Como	Dom	0	1	B	−78.1	54.1	D	D
	Arg	0	1	B	−64.0	97.7	D	D
	Lec	0	2	C	−57.4	48.7	D	D
Iseo	C.V.	0	4	E	−68.8	54.9	D	E
	Pre	0	1	B	−66.2	96.6	D	D
Garda	Des	0	1	B	−71.7	78.5	D	D
	Pes	0	1	B	−70.9	173.6	E	E
	Lim	0	1	B	−63.0	94	D	D

enzyme activity. Arona is subjected to some DDT pollution, but above all to heavy PCB contamination with levels (1.4 μg g^{-1} lipids) lower than those of Brebbia only.

Since DDT concentrations measured at Brissago and Cannobio are the lowest found in this lake and the PCB level is not much higher than the threshold of 800 ng g^{-1} lipids, the assigned quality class should be correct, as well as for Intra, affected by moderate DDT and AChE inhibitor contamination.

Porlezza and Pojana are not contaminated sites, as shown by the agreement between chemical and biological data. The bad environmental quality found at Peschiera was not justified by the low levels of xenobiotics found, but the very high EROD activity was probably due to other pollutants (dioxins, furans and hydrocarbons) produced and released by the recreation area located near the sampling site.

The site-specific classification showed three different categories of the xenobiotic contamination of the Italian sub-alpine great lakes. The quality of Lake Lugano is very good because of the lack of industrial plants and the peculiar characteristics of its watershed that do not allow for intensive agricultural activity. However, the reason for the high AChE activity of Brusimpiano and Pojana should be investigated.

Lakes Como, Iseo and Garda are subjected to a diffuse xenobiotic pollution since their quality ranges from poor for some sites to bad for Peschiera (Lake Garda) and

Costa Volpino (Lake Iseo). A not homogeneous contamination was observed for Lake Maggiore, where the worst environmental quality was obtained for two sampling sites and the ecological status could be compromised in several other areas. The pollution found at Baveno and Stresa is particularly troublesome, bearing in mind that these places are near one of the most important wetlands of Northern Italy (Fondotoce), as well as the Ramsar site of Magadino, a reproduction area of several aquatic bird species.

The interaction between bioaccumulation and biomarker data allowed a better comprehension of several EROD activity results and a more correct definition of the quality indexes for four sampling stations (Intra, Baveno, Stresa and Arona). Moreover, this combination might allow a wider range of environmental monitoring because it can identify pollution due to some different chemical classes.

Notwithstanding chemical data discriminated among sampling sites with similar EROD activity, the use of biomarkers demonstrated the presence of a different kind of pollution not shown by the former approach. All the sampling stations of Lakes Como, Iseo and Garda, with the exception of Costa Volpino, indicated an environmental quality worse than that pointed out by the biomonitoring alone (Table VII), as well as for several sites of Lake Maggiore. The presence of environment xenobiotic mixtures and the introduction of new industrial and agricultural contaminants make the monitoring based only on chemical analyses less effective for the environmental quality assessment of man-made pollutants. The support of suitable and well-known biomarkers might supply a powerful tool to highlight the areas exposed to environmental risk.

Conclusions

The use of biomarkers in the Zebra mussel is a promising approach for the assessment of freshwater quality, even if much work has to be done in order to test and interpret biological responses and to develop acceptable quality assessment procedures. The present results showed that the use of biomarkers needs particular care in the interpretation of data, mostly for EROD activity, whose inhibition must be considered as a normal end-point for a correct environmental management.

The present study is an example of how bioaccumulation and biomarker data could be treated to obtain a simple-quality site-specific classification that can address more exhaustive research aimed at the discovery of pollutant point-sources. Future laboratory studies on biochemical responses in Zebra mussel should set out the environmental quality classes more accurately, as well as the interference effects of competitive inhibitors.

The use of a biomarker battery should be even more suitable for this purpose, but at this moment only a few assays are well standardized and none is accepted by international legislation. Only when scientific and legal credibility is established will this approach perhaps be applied in routine monitoring programmes.

References

Arukve A, Celius T, Walther BT, Goksøyr A. 2000. Effects of xenoestrogen treatment on zona radiata protein and vitellogenin expression in Atlantic Salmon (Salmo salar). Aquatic Toxicology 49:159–170.

ATSDR. 1998. Toxicological profile for selected PCBs. Atlanta, GA: US Department of Health and Human Services, Public Health Service. p. 393.

ATSDR. 2000. Toxicological profile for DDT, DDE and DDD. Syracuse, NY: US Department of Health and Human Services, Public Health Service. p. 765.

Bacchetta R, Mantecca P, Vailati G. 2001. Reproductive behaviour of the freshwater mussel Dreissena polymorpha in Italy: a comparison between two populations. Archives of Hydrobiology 151:247–262.

Banni M, Jebali J, Daubeze M, Clerandeau C, Guerbej H, Narbonne JF, Boussetta H. 2005. Monitoring pollution in Tunisian coasts: application of a classification scale based on biochemical markers. Biomarkers 10(2–3):105–116.

Bayarri S, Turrio Baldassarri L, Iacovella N, Ferrara F, di Domenico A. 2001. PCDDs, PCDFs, PCBs and DDE in edible marine species from the Adriatic Sea. Chemosphere 43:601–610.

Belden JB, Lydy MJ. 2000. Impact of atrazine on organophosphate insecticides toxicity. Environmental Toxicology and Chemistry 19:2266–2274.

Besselink HT, Denison MS, Hahn ME. 1998. Low inducibility of CYP1A activity by polychlorinated biphenyls (PCBs) in flounder (Platichthys flesus): characterization of the Ah receptor and the role of CYP1A inhibition. Fundamentals of Applied Toxicology 43:161–171.

Binelli A, Bacchetta R, Mantecca P, Ricciardi F, Provini A, Vailati G. 2004b. DDT in Zebra mussels from Lake Maggiore: level of contamination and endocrine disruptions. Aquatic Toxicology 69(2):175–188.

Binelli A, Galassi S, Provini A. 2001. Factors affecting the use of Dreissena polymorpha as a bioindicator: the PCB pollution in Lake Como (N. Italy). Water, Air and Soil Pollution 125(1–4):19–35.

Binelli A, Ricciardi F, Provini A. 2004a. Present status of POP contamination in Lake Maggiore (Italy). Chemosphere 57(1):27–34.

Binelli A, Ricciardi F, Riva C, Provini A. 2005. Screening of POP pollution by AChE and EROD activities in Zebra mussels from the Italian great lakes. Chemosphere 61(8):1074–1082.

Binelli A, Ricciardi F, Riva C, Provini A. 2006. New evidences for old biomarkers: effects of several xenobiotics on EROD and AChE activities in Zebra mussel (D. polymorpha). Chemosphere 62:510–519.

Bocquené G, Roig A, Fournier D. 1997. Evidence for the presence of a soluble acetylcholinesterase insensitive to organophosphate and carbamate inhibitors. FEBS Letters 407:261–266.

Bolognesi C, Buschini A, Branchi E, Carboni P, Furbini M, Martino A, Monteverde M, Poli P, Rossi C. 2004. Comet and micronucleus assays in zebra mussel cells for genotoxicity assessment of surface water treated with three different disinfectants. Science of the Total Environment 333:127–136.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein–dye binding. Analytical Biochemistry 72:248–254.

Brevik EM, Grande M, Knutzer J, Polder A, Skaare JU. 1996. DDT contamination of fish and sediments from Lake Ørsjøen, Southern Norway: comparison of data from 1975 and 1994. Chemosphere 33:2189–2200.

Burke MD, Mayer RT. 1974. Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. Drug Metabolism and Disposition 2:583–588.

Camusso M, Balestrini R, Binelli A. 2001. Use of zebra mussel (Dreissena polymorpha) to assess trace metal contamination in the largest Italian sub-alpine lakes. Chemosphere 44:263–270.

Dauberschmidt C, Dietrich DR, Schlatter C. 1997. Investigations on the biotransformation capacity of organophosphates in the mollusc Dreissena polymorpha. Aquatic Toxicology 37:283–294.

De Lafontaine Y, Gagné F, Blaise C, Costan G, Gagnon P, Chan HM. 2000. Biomarkers in zebra mussel (Dreissena polymorpha) for the assessment and monitoring of water quality of the St Lawrence River (Canada). Aquatic Toxicology 50:51–71.

Depledge M, Fossi MC. 1994. The role of biomarker in environmental assessment: invertebrates. Ecotoxicology 3:173–179.

Dethloff GM, Schlenk D, Hamm JT, Bailey HC. 1999. Alteration in physiological parameters of rainbow trout (Oncorhyncus mikyss) with exposure to copper and copper/zinc mixtures). Ecotoxicology and Environmental Safety 42:253–264.

Ellman GL, Courtney KD, Andres V Jr, Featherstone RM. 1961. A new and rapid colorimetric determination of acetilcholynesterase activity. Biochemical Pharmacology 7:88–95.

European Union Directive 2000/60/EC establishing a framework for Community action in the field of water policy: OJ L327, 22.12.2000; Bull-9–2000:66.

Europena Union Decision No. 2455/2001/EC establishing the list of priority substances in the field of water policy and amending Directive 2000/60/EC, L331:5.

Fent K, Stegeman JJ. 1993. Effects of tributyltin in vivo on hepatic cytochrome P450 forms in marine fish. Aquatic Toxicology 24:219–240.

Fent K, Woodin B, Stegeman JJ. 1996. Effects of triphenyltin on the hepatic monooxigenase system in marine fish. Marine Environmental Research 42:51–52.

Fleming WJ, Augspurger TP, Alderman JA. 1995. Freshwater mussel die-off attributed to anticholinesterase poisoning. Environmental Toxicology and Chemistry 14:877–879.

George SG. 1989. Cadmium effects on plaice liver xenobiotic and metal detoxification systems: dose–response. Aquatic Toxicology 15:303–310.

Gooch JW, Elskus AA, Kloepper-Sams PJ, Hahn ME, Stegeman JJ. 1989. Effects of ortho and non-ortho substituted polychlorinated biphenyl congeners on the hepatic monooxygenases system in scup (Stenotomus chryops). Toxicology and Applied Pharmacology 98:422–433.

Gunning RV, Moores GD, Devonshire AL. 1997. Biochemical resistence detection in Helicoverpa armigera in Australia. Recent Research in Developmental Entomology 1:203–213.

Hahn ME. 2002. Aryl hydrocarbon receptors: diversity and evolution. Chemico-Biological Interactions 141(1–2):131–160.

Hahn ME, Lamb TM, Schultz ME, Smolowitz RM, Stageman JJ. 1993. CYP4501A induction and inhibition by 3,3′,4,4′-tetrachlorobiphenyl in an Ah receptor-containing fish hepatoma cell line (PLHC-1). Aquatic Toxicology 26:185–208.

Howard CV. 1997. Synergistic effects of chemical mixtures: can we rely on traditional toxicology? Ecologist 27:192–195.

Karchner SI, Franks DG, Powell WH, Hahn ME. 2002. Regulatory interactions among three members of the vertebrate aryl hydrocarbon receptor family: AhR repressor, AhR1 and AhR2. Journal of Biological Chemistry 277(9):6949–6959.

Kennedy SW, Lorenzen A, Norstrom RJ. 1996. Chicken embryo hepatocyte bioassay for measuring cytochrome P4501A-based 2,3,7,8-tetrachlorodibenzo-p-dioxin equivalent concentrations in environmental samples. Environmental Science and Technology 30:706–715.

Kortenkamp A, Altenburger R. 1998. Synergisms with mixtures of xenoestrogens: a reevaluation using the method of isoboles. Science of the Total Environment 221:59–73.

Lagadic L, Caquet T, Ramade F. 1994. The role of biomarkers in environmental assessment (5). Invertebrate populations and communities. Ecotoxicology 3:193–208.

Lazar R, Edwards RC, Metcalfe CD, Metcalfe T, Gobas FAPC, Haffner GD. 1992. A simple, novel method for the quantitative analysis of coplanar (non-ortho substituted) polychlorinated biphenyls in environmental samples. Chemosphere 25:493–504.

Livingstone DR, Nasci C, Montserrat S, Da Ros C, O'Hara S, Peters L, Fossato V, Wootton A, Goldfarb P. 1997. Apparent induction of a cytochrome P450 with immunochemical similarities to CYP1A in digestive gland of the common mussel (Mytilus galloprovincialis L.) with exposure to 2,2′,3,4,4′,5′-hexachlorobiphenyl and Arochlor 1254. Aquatic toxicology 38:205–224.

Monirith Ueno D, Takahashi S, Nakata H, Sudaryanto A, Subramanian A, Karuppiah S, Ismail A, Muchtar M, Zheng J, Richardson BJ, Prudente M, Hue ND, Tana TS, Tkalin AV, Tanabe S. 2003. Asia-Pacific mussel watch: monitoring contamination of persistent organochlorine compounds in coastal waters of Asian countries. Marine Pollution Bulletin 46:281–300.

Moore MN, Depledge MH, Readman JW, Leonard DR. P. 2004. An integrated biomarker-based strategy for ecotoxicological evaluation of risk in environmental management. Mutation Research 552:247–268.

Mora P, Michel X, Narbonne J. F. 1999. Cholinesterase activity as potential biomarker in two bivalves. Environmental Toxicology and Pharmacology 7:253–260.

Narbonne JF, Aarab N, Clérandeau C, Daubèze M, Narbonne J, Champeau O, Garrigues P. 2005. Scale of classification based on biochemical markers in mussels: application to pollution monitoring in Mediterranean coasts and temporal trends. Biomarkers 10(1):58–71.

Narbonne JF, Daubèze M, Clérandeau C, Garrigues P. 1999. Scale of classification based on biochemical markers in mussels: application to pollution monitoring in European coasts. Biomarkers 4(6):415–424.

Nhan DD, Carvalho FP, Am NM, Tuan NQ, Yen NTH, Villeneuve JP, Cattini C. 2001. Chlorinated pesticides and PCBs in sediments and molluscs from freshwater canals in the Hanoi region. Environmental Pollution 112:311–320.

Olea N, Pazos P, Exposito J. 1998. Inadvertent exposure to xenoestrogens. European Journal of Cancer Prevention 7:17–23.

Osservatorio dei Laghi Lombardi (OLL). 2005. Qualità delle acque lacustri in Lombardia. 1st OLL Report 2004. Milan: Fondazione Lombardia per l'Ambiente. p. 351.

Osterloh JLM, Pond SM. 1983. Toxicologic studies in a fatal overdose of 2,4-D, MCCP and chlorpyrifos. Journal of Analytical Toxicology 7:125–129.

Payne JF, Fancey LL, Rahimtula AD, Porter EL. 1987. Review and perspective on the use of mixed-function oxygenase enzymes in biological monitoring. Comparitive Biochemistry and Physiology 86C:233–245.

Petrulis JR, Bunce NJ. 1999. Competitive inhibition by inducer as a confounding factor in the use of the ethoxyresorufin-O-deethylase (EROD) assay to estimate exposure to dioxin-like compounds. Toxicology Letters 105:251–260.

Petrulis JR, Chen G, Benn S, LaMarre J, Bunce NJ. 2001. Application of the ethoxyresorufin-O-deethylase (EROD) assay to mixtures of halogenated aromatic compounds. Environmental Toxicology 16(2):177–184.

Provini A, Galassi S, Guzzella L, Valli G. 1995. PCB profiles in sediments of Lake Maggiore, Como and Garda (Italy). Marine and Freshwater Research 46:129–136.

Regoli F. 1998. Trace metals and antioxidant enzymes in gills and digestive gland of the Mediterranean mussel Mytilus galloprovincialis. Archives in Environmental Contaminants and Toxicology 34:48–63.

Ricciardi F, Binelli A, Provini A. 2006. Use of two biomarkers (CYP450 and AChE) in zebra mussel for the monitoring of Lake Maggiore (N. Italy). Ecotoxicology and Environmental Safety 63:406–412.

Risso-de Faverney C, Lafaurie M, Girard JP., Rahmani R. 2000. The nitroxide stable radical tempo prevents metal-induced inhibition of CYP1A1 expression and induction. Toxicology Letters 111:219–227.

Romani R, Antognelli C, Baldracchini F, De Santis A, Isani G, Giovannini E, Rosi G. 2003. Increased AChE activities in specimens of Sparus auratus exposed to sublethal copper concentrations. Chemico-Biological Interactions 145:321–329.

Schmitz HJ, Hagenmaier A, Hagenmaier HP, Bock KW, Schrenk D. 1995. Potency of mixtures of polychorinated biphenyls as inducers of dioxin receptor-regulated CYP1A activity in rat hepatocytes and H4IIE cells. Toxicology 99:47–54.

Sheehan D, Power A. 1999. Effects of seasonality on xenobiotic and antioxidant defence mechanisms of bivalve molluscs. Comparative Biochemistry and Physiology 123:193–199.

Sibley PK, Chappel MJ, George TK, Solomon KR, Liber K. 2000. Integrating effect of stressors across levels of biological organisation: examples using organophosphorus insecticides mixtures in field-level exposures. Journal of Aquatic Ecology and Stress Research 7:117–130.

Snyder MJ. 2000. Cytochrome P450 enzymes in aquatic invertebrates: recent advances and future directions. Aquatic Toxicology 48:529–547.

Tanabe S, Prudente MS, Kan-Atireklap S, Subramanian A. 2000. Mussel watch: marine pollution monitoring of butyltins and organochlorines in coastal waters of Thailand, Philippines and India. Ocean and Coast Management 43:819–839.

US Environmental Protection Agency (EPA). 1998. SCE policy issues related to the food quality protection act. Office of pesticide program's science policy on the use of cholinesterase inhibition for risk assessment of organophosphate and carbamate pesticides. OOP Docket # 00560. Federal Register 63. p. 214.

Van der Oost R, Beyer J, Vermeulen NPE. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environmental Toxicology and Pharmacology 13:57–149.

Viarengo A, Bettella E, Fabbri R, Burlando B, Lafaurie M. 1997. Heavy metal inhibition of EROD activity in liver microsomes from the bass Dicentrarchus labrax exposed to organic xenobiotics: role of GSH in the reduction of heavy metal effects. Marine Environmental Research 44:1–11.

Whyte JJ, Jung RE, Schmitt CJ, Tillitt DE. 2000. Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure. Critical Reviews in Toxicology 30:347–570.