BIOMARKERS IN ZEBRA MUSSEL FOR MONITORING AND QUALITY ASSESSMENT OF LAKE MAGGIORE (ITALY)

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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 as indicators of chemical exposure and early response has been used for the last two decades. Among biomarkers, the measure of ethoxy resorufin-O-deethylase (EROD) activity is one of the best documented responses to planar chemicals, while 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 (SCGE) assay, also known as the Comet assay, is a rapid and reliable method for evaluating DNA damage induced by chemicals in eukaryotic individual cells. Particular care is needed in biomarker data interpretation which is seldom applied in field studies. Although the final benchmark of EROD activity is generally the enzyme activation, some studies highlight a clear decrease in EROD activity in organisms that have been exposed to heavy metals [1-2]. Moreover, some chemicals that lead to an increase in EROD activity also decrease its activity at high concentrations [3-4], which is explained by competitive inhibition of the EROD-substrate reaction. Notwithstanding that AChE activity is inhibited by OPs and carbamates, the effect of trace metals on this enzyme is still controversial [5-6-7].

The main aim of this study was water quality assessment of the chemically polluted Lake Maggiore by analyzing a battery of biomarkers (EROD and AChE activities, DNA damages) using Zebra mussel (*Dreissena polymorpha*) specimens collected at 6 different sampling sites. A comparison between 2005 data of EROD and AChE activities and those found in a previous campaign carried out in the same period of 2003 is also shown to point out the contamination trend of some pollutants. We also analysed several chemical classes in the soft tissues of mussel by GC-ECD and GC-MSn to support biomarker data: pp' dichloro-diphenyl-trichloroethane and its 5 homologues (DDTs), 23 congeners of polychlorinated biphenyls (PCBs), 11 polycyclic aromatic hydrocarbons (PAHs), 14 polybrominated diphenyl ethers (PBDEs) and hexachlorobenzene (HCB).

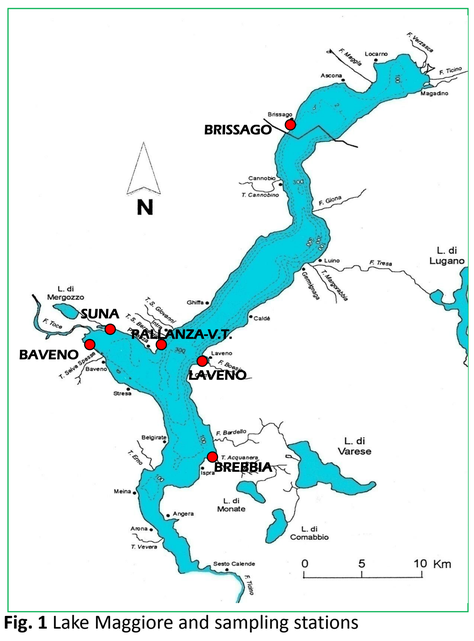

Fig. 1 Lake Maggiore and sampling stations

Materials and Methods:

Several hundred mussel specimens were collected at 4-5 m of depth by a scuba-diver at the end of April 2005 (Fig. 1), when Zebra mussels are in their pre-reproductive stage. Molluscs were divided in two different pools. The organisms for chemical analyses 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, for which only adult specimens of a shell length greater than 15 mm were used. Specimens for biomarker assays were tied on rocks, 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. AChE activity was determined on 5 replicate pools (10 mussels per pool) at 23 °C, pH=7 and with the acetylthiocholine (ASCh) as the substrate, as described by Ellman et al. [8]. EROD activity was determined on 5 replicate pools (10 mussels per pool) according to the protocol of Burke and Mayer [9]. Several chemical classes were also analysed in the soft tissues of mussel by GC-ECD and GC-MSn.

Tab. 1 Temperatures measured at each sampling sites, average of AChE (nmol/min/mg of proteins), EROD (pmol/min/mg of proteins) activities and LDRs (Length Diameter Ratios) measured in controls and in Zebra mussels from Lake Maggiore. Percentage differences between controls and experimental data were also shown. Standard deviations are in

	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

General results:

The Pearson's correlation coefficients 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.

Biomarker results did not exhibit high variability at every site, as shown by low standard deviations in Table 1. 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 % (Tab. 2) relative to an efficiency of 33.3% for EROD activity and only 19% for AChE activity, which is, however, a more specific biomarker. Levels of measured man-made chemicals are shown in table 3.

Tab. 2 Results of the Tukey's range test for each biomarkers. Groups with same letters are significantly different.

	AChE activity	EROD activity	Log LDR
Sites	**Group**	**Group**	**Group**
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 EROD and log LDR, while ANCOVA ($p<0.05$) was carried out for the measure of the AChE activity.

Tab. 3 Concentrations (ng/g lipids) of several chemical classes measured in zebra mussel soft tissues from 6 sampling sites of Lake Maggiore.

Sites	*ΣDDTs*	*ΣPCBs*	*ΣPAHs*	*ΣPBDEs*	*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

AChE activity:

Comparisons between mussels collected in Lake Maggiore and depurated controls (Tabs. 1-2) pointed out 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. Since a significant 20% inhibition of AChE activity relative to controls signifies a clear toxicological effect and that AChE inhibition higher than 50-60% can damage the most sensible populations, the AChE levels found at Brissago and Baveno indicate a potential danger both for the mussel population and for the entire aquatic community.

Data shown in Figure 2 confirmed the interference made by temperature since 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:

Field data results (Tabs. 1-2) 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 anthropized areas of the lake. The high pollution due to EROD inducers revealed at Pallanza-VT is also confirmed by the significant difference ($p<0.05$) with all of the other sites (Tab. 3). Field data interpretation is sometimes difficult because chemical mixtures in the environment can interfere with this biomarker in different ways. Even though measurement of this enzymatic activity is normally performed to evaluate chemical pollution with enzymatic inducers, several authors [10-11] (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. Comparisons between 2003-2005 data (Fig. 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, the ecosystem quality was not worsened 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.

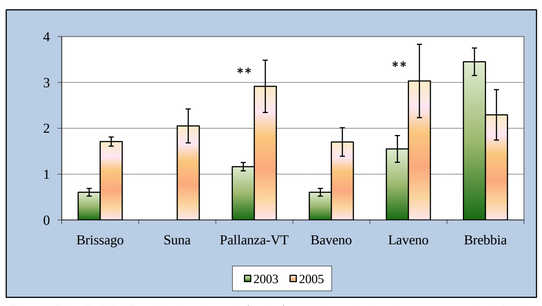

Fig. 2 Fig. 2 AChE activity (nmol/min/mg of proteins) measured at Lake Maggiore sampling stations in the two monitoring campaigns. ANCOVA, Tukey's *post-hoc* test (** < 0.01).

SCGE assay:

Baveno and Brebbia were the most contaminated sites, having 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 above all of the sampling stations (Tabs. 1-2). Brissago and Pallanza-VT were the only areas that were not contaminated by chemicals that were able to produce significant strand breakages in Zebra mussel cells (Tab. 2). 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. The LDR calculated at Brissago, as well as the sampling stations of Suna, Laveno and Brebbia, was in full accordance with low EROD activity (Tab. 1). On the contrary, DNA damage found at Pallanza-VT and Baveno had behaviour that was different from that of EROD activity. This difference suggests different pollution in these areas which are located outside and inside Baveno Bay: the DNA damages noticed at Baveno is likely created by chemicals that directly produce 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. Criteria reported by Mitchelmore et al. (1998) to categorize the grade of damage through the percentage of DNA in the tail (Fig. 4) was also used and compared to the LDR approach. These two metrics fit properly for Baveno, since it has the detected highest LDR in the whole lake basin (Tab. 2) and the majority of the haemocytes fall into mid (46.2%) and high (28.6) damage classes, the highest levels calculated for all of the sampling stations (Fig. 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 5 classes of damage relative to those measured in mussels maintained at laboratory conditions.

On the contrary, although the LDRs calculated for Suna and Laveno were very similar (Tabs. 2-3), the tail % 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 categorization of DNA damage, this last metric is more sensitive and provides much more information on the pollution effects among sampling sites.

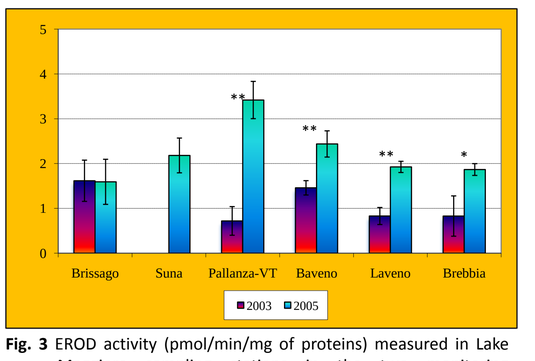

Fig. 3 EROD activity (pmol/min/mg of proteins) measured in Lake Maggiore sampling stations in the two monitoring campaigns. Student's t-test (* < 0.05; ** < 0.01).

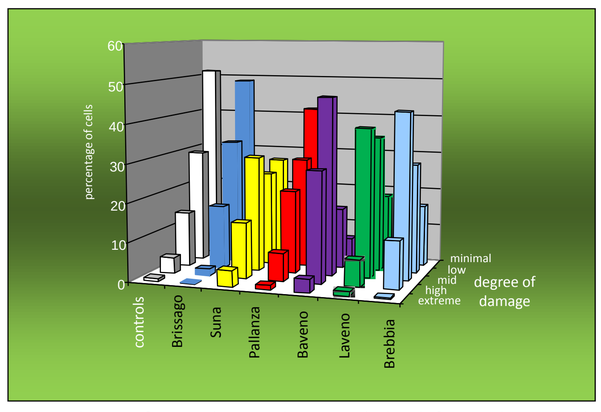

Fig. 4 Classes of DNA damage degrees calculated from Zebra mussel haemocytes from 6 sampling sites of Lake Maggiore and their comparison with controls. Grade of damage (using tail % DNA): minimal<10%; low 10-25%; mid 25-50%; high 50-75%; extreme >75%.

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. 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.

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