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Biochemical Biomarker Responses and Chlorinated Compounds in the Fish *Leusciscus cephalus* Along a Contaminant Gradient in a Polluted River

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There is an urgent need to develop methods for assessing toxic risk in aquatic wildlife populations in order to evaluate the environmental quality of freshwater ecosystems. Field studies are essential condition for validating biomarkers in fish. These biomarkers can be useful tools for evaluating pollution exposure in aquatic organisms.

An ideal bioindicator organism must fulfil a series of requirements, including: a wide geographical distribution and understanding of the biology (life cycle, reproduction, feeding habitat, etc.) and it must be sensitive to environmental changes. Using biomarkers as indicators of health in bioindicator organisms we sampled one or more areas suspected of pollution, and compared health responses in these affected areas, with those of organisms collected from a reference area, this approach allow us to evaluate the potential danger to communities resulting from envirnmental stressors (Adams 1990; Fossi and Leonzio 1994).

The Lambro River (Lombardia region, northern Italy) has been described as one of the most polluted rivers in northern Italy, draining one of the most industrialized and urbanized areas within the river Po basin (Pettine et al. 1996). Heavy metals and organochlorine compounds have been described as typical pollutants in this riverine system (Vigano et al, 1996a; Galassi et al. 1996). Lambro river waters have been shown to be genotoxic in laboratory experiments with larval individuals of *Onchorinchus mykiss* (De Flora et al. 1993). Previous studies in this same experimental area has been conducted using caged fish upstream and downstream of the Lambro confluence with the River Po. Induction of EROD activity in fish exposed to Lambro river water has been observed together with increases of liver CYP P450 content in fish injected with sediment extracts from the Lambro River (Vigano et al. 1996b). However, there are no previous studies examining for biomarker responses in fish within the River Lambro, basically because for several years fish were practically absent from the river.

This present study was conducted to determine if *Leusciscus cephalus*, a fish species inhabiting the Lambro River, can be used as biomonitor to assess the water quality recovery of the river ecosystem. Two biochemical biomarkers widely used

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in field ecotoxicological studies, such as induction of the CYPP450 1A1 isoenzyme and inhibition of brain AchE activity, were measured in individuals collected along a transect established from upstream to dowstream in the Lambro River. In addition, organochlorine residues were measured in the muscle tissue of the collected fish in order to assess any relationship between biochemical responses and chlorinated compound levels. Sites along this gradient have been previously described as a pollution gradient.

MATERIALS AND METHODS

L.cephalus (chub) is one of the most widely distributed species in Europe, both in lotic and lentic ecosystems. This fish species is highly adapted to different environmental conditions and has high tolerance to pollution. *L.cephalus* is an omnivorous and opportunistic feeder, depending on the availability of aquatic invertebrates, sediments, organic matter (also sewage discharges), vegetal biomass and other small fish (Gandolfi et al. 1991). Reproductive period of this species in our study area occurs from May to June and male and females reach sexual maturity at 2-4 years of age (Gandolfi et al. 1991).

Three sampling sites (Pusiano 45°47' N 9°18'E, Monza 45°35' N 9°17'E and San Colombano al Lambro 45°11'N 9°32' E) were selected along the Lambro River (East Milan, Italy) based on a pollution gradient (Figure 1). Male specimens (lengh ranging from 26,6 to 28 cm) of *L. Cephalus* were caught by electrofishing (n=13 Pusiano, n=13 Monza and n=4 San Colombano) during March 1996. The average age of fish was found to be 2 years determined by scale analysis. At San Colombano station were not possible to find out more than four male specimens for the analysis and the capture per effort unit was much higher than the other two sampling sites.

Fish were sacrificed in the field, liver and brain were immediately removed and frozen in liquid nitrogen until enzymes could be assayed. Blood samples were taken with heparinized tubes from caudal vein and centrifuged at 3000 g for 5 min to remove the cellular debris and nuclei. The serum was decanted and immediately frozen at –80°C until esterase assays could be conducted

All analyses were conducted at the Biomarkers laboratory of the Department of Environmental Sciences at Siena University, Italy. For the microsomal fraction frozen livers was weighed and all subsequent procedures were carried out at 4° C. Liver tissue was homogenized within the ratio 1:5 W/V (tissue weight/buffer volume) in 0.25 M sucrose buffer at pH 7.5 with a potter teflon homogenizer. The homogenate was centrifuged at 9,000 x g for 20 min. The supernatant was centrifuged in an ultracentrifuge at 100,000 x g for 60 min and the resulting microsomal pellet was resuspended in 1.15% KCl solution at pH 7.5; these fractions were immediately used for enzyme activity determinations.

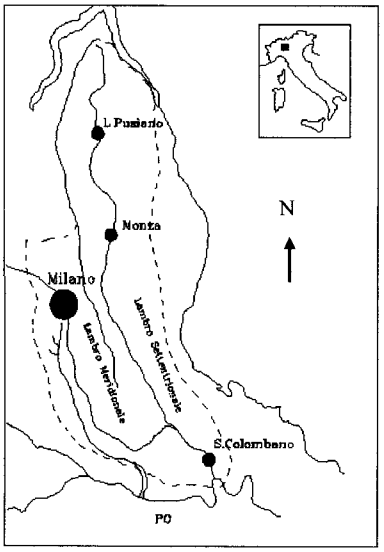

Figure 1. Sampling sites in the study area.

All enzyme assays were conducted at 20°C. CYP1A1 activity was measured in the liver microsomal fraction by ethoxyresorufin dealkylation (EROD)(Lubet et al. 1985). EROD activity was expressed in pmol/min/mg microsomal protein. Benzo(a)pyrene monooxigenase (BPMO) was determined by the method of Kurelec et al. (1977). The activity was expressed in Fluorescence Units UF/mg microsomal protein/min. Microsomal proteins were quantified by the Bio-Rad protein assay.

Esterase activity was evaluated through spectrophotometric determination of brain acetylcholinesterase (AChE), by the method of Ellman et al. (1961). Acetylthiocholine was used as substrate and the subsequent detection of released thiocoline was followed by reaction with 5,5-dithiobis (2-nitrobenzoic acid). DTNB was monitored over a 5 min period with recording spectrophotometer set at 410 nm. These activities were expressed in μmol/min/mg protein.

Composite frozen dried muscle samples (c.a. 2 g) were extracted for 8 hours in a soxhlet apparatus with hexane, 20 ml of concentrated sulphuric acid was added and allowed to stand overnight. The sample was concentrated to 1 ml, a clean-up procedures used a Florisil column of 1 g eluted with 25 ml of hexane. The extract was concentrated again down under Nitrogen to 100 μl adding a volume of concentrated sulphuric acid and left for 2 hours in a water bath at 70°C. After this treatment, hexane was added and the total sample (c.a. 1 ml) was subjected to a

clean -up in an activated silica 60 column (3 hrs at 130°C) (2.5 g silica plus anhydrous sodium sulphate). The first fraction containing the PCBs fraction was eluted with 32 ml of hexane and then concentrated to 1 ml; the second fraction containing the OC compounds was washed with 50 ml of an hexane:ethylacetate 85:15 mixture. 1 µl was injected to a Perkin Elmer gas chromatograph with an ECD detector. The chromatographic conditions were: injector temperature 280 °C; detector temperature 300°C , flowrate 1 ml/min argon:methane (95:5 v/v); chromatographic column SPB-5 60 m lenght i.d. 0.20 mm. The quantification was made using a standard of 34 PCBs congeners and a standard of OC pesticides including lindane and its metabolites, hexachlorobenzene and DDT an its metabolites (α-HCH, γ-HCH, HCB, DDT, DDE, and DDD). Tetrabromobenzene was used as an internal standard. Reference samples and blanks were analized for each batch of samples. Mean recovery rates were over 78% for PCBs and 86% for organochlorine pesticides. Data are expressed without correction by recovery rates.

Statistical differences among biomarker responses between different sites were tested using the non parametric Mann-Whitney U test and considering a probability level ≤ 0.05 as statistically significant.

RESULTS AND DISCUSSION

Induction of the P4501A1 isoform in fish liver is often used in biomonitoring to indicate pollution with 3-Methylcolanthrene-type inducers, such as polychlorodibenzo-p-dioxins.(PCDDs), polychlorodibenzofurans (PCDF's), certain polycyclic aromatic hydrocarbons (PAHs), and polychlorobyphenils (PCB's) (Schoor et al. 1991; Anderson and Forlin, 1992). The induction of CYP1A1 can be evaluated by means of the ethoxyresorufin O-deethylase activity (EROD) and Benzo (a) pyrene monooxigenase (BPMO) assays. Figure 2 shows the results of BPMO and EROD activities in liver where EROD activity is clearly induced in the San Colombano site (about eight times) compared to Pusiano and Monza stations($p<0.001$). Compared to the cleaner Pusiano area, induction of BPMO was significantly higher at the San Colombano site. Based on these results, the EROD assay appears to be more sensitive for detecting induction of CYP1A1 than the BPMO assay. The EROD activity in Pusiano area (reference) showed values consistent with similar studies performed using the same species in France (Vindimian et al. 1991)

Recent data, published by Viganó et al. (1998) confirm the results obtained at the San Colombano area. In this report, chub samples collected downstream the River Lambro confluence to the River Po were analyzed for the EROD activity, indicating a significative increase caused by pollutants carried in this river. However, other biomarker responses tested in this fish, such as cytosolic responses, were lower than expected.

Even though the Lambro River is known to transport CYP1A1 inducers and genotoxic chemicals to the river Po (Vigano et al. 1998), concentrations of these class of pollutants has not been previously described in fish inhabiting the Lambro River. Muscle tissue was analyzed for PCBs and results for each individual PCB congener are shown in Figure 3. Several congeners (34) were analyzed, from which PCBs 153 and 138 were detected in all three sampling stations with the highest concentrations. Both congeners are not coplanar, but it is interesting to note that mono ortho PCBs 118 and 66 have been described as able to induce induction of CYP1A1 in other fish species, and a Toxicity Equivalent Factor has been stablished for that congeners (Erik et al, 1995). In addition, a similar congener fingerprint was observed among the three sampling sites, that is major differences basis was only in concentration ranges, San Colombano being the highest. These results show a clear gradient of pollution from Pusiano to San Colombano station.

The total concentration of PCBs was 610, 1014 and 1456 ng/g dry weight for Pusiano, Monza and San Colombano stations, respectively. In fact, Galassi et al. (1981) analyzed samples of *L. cephalus* for PCBs within the reach of the River Po which received discharges from the Lambro River. In this study, levels of Arochlor 1254 and 1260 of about 15 and 8 mg/kg when expressed on a neutral lipid basis were found. From these results, it appears that levels of PCBs have decreased during the last few years, indicating a trend to recovery. Experiments using caged organisms upstream and downstream of the confluence of the river Lambro with the river Po showed higher levels of PCBs in fish caged downstream the river Lambro reaches the river Po, demonstrating that loading of pollutants from the Lambro River represents an important point source of bioavailable PCBs for the River Po below its confluence with the Lambro River (Vigano et al, 1994).

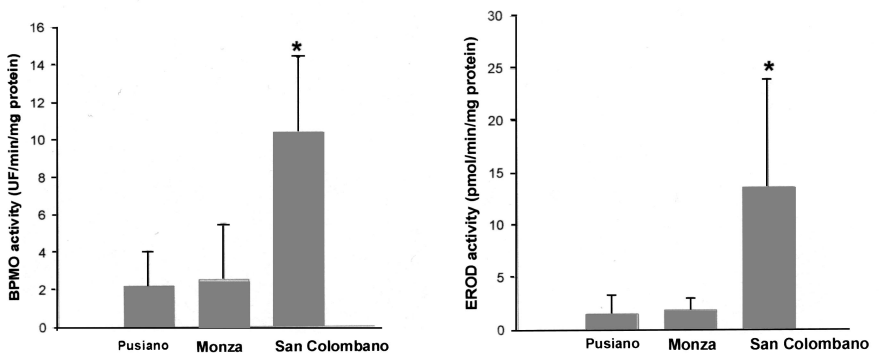

Figure 2. Mean (± SD) cytochrome P4501A1 activities expresed as Benzo (a) pyrene monoxigenase BPMO and 7-ethoxresorufin-O-deethylase dealkylation EROD, in *L.cephalus* at the Lambro river. BPMO and EROD activities

significantly different ($p < 0.01$, Mann Whitney U test) from the reference area (Pusiano) are indicated with an asterisk.

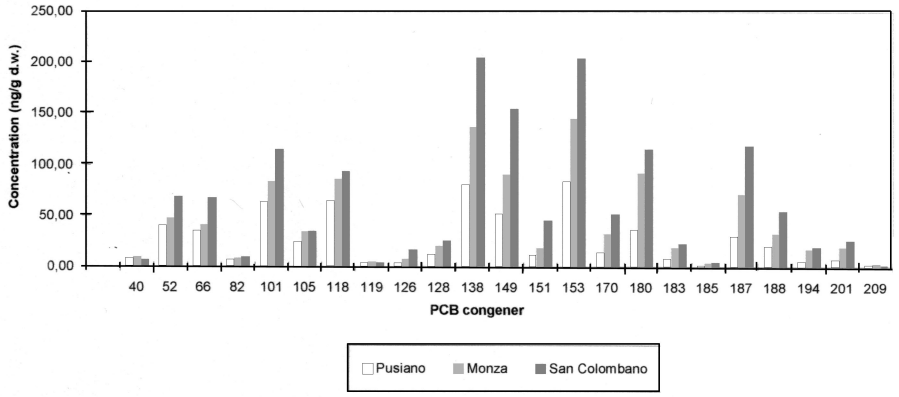

Figure 3. Mean Concentration of PCB congeners (ng/g dry weight) in muscle tissue of *L. cephalus* collected from three stations along the River Lambro

The organochlorine content in fish demonstrates the same pattern in concentrations among sample sites and did the cytochrome P450 data. Total DDT concentrations ranged from 120 to 726 ng/g from Pusiano to San Colombano, while α-HCH and γ-HCH ranged from 6-47 ng/g dw and 5 –48 ng/g dw, respectively, in the same stations. HCB was detected in all muscle samples ranging from 128 to 460 ng/g dw (Figure 4).

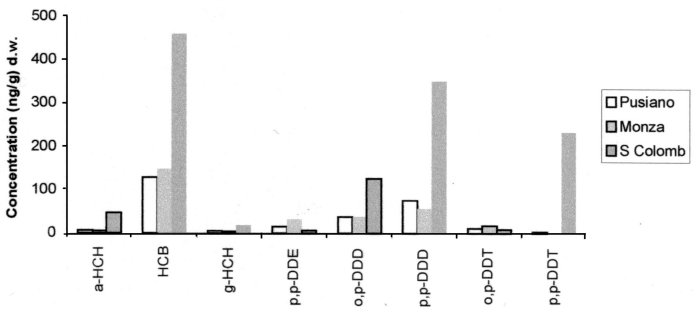

Figure 4. Mean concentrations of organochlorine pesticide content in muscle tissue of *L.Cephalus* collected along the Lambro River.

Inhibition of acetylcholinesterase (AchE) activity is another biomarker widely used to assess the exposure to and effects of compounds such as organophosphorus and carbamate pesticides. In the River Lambro case, a statistically significant inhibition of about 40% ($p<0.05$, Mann Whitney U-test) was observed in brain AchE activity of fish collected at San Colombano station in comparison to those

collected upstream, suggesting the presence of neurotoxic compounds at the basin outlet (Figure 5). AchE is specifically inhibited by organophosphorous or carbamate compounds, probably draining from the agricultural areas located around the lower basin.

In conclusion, the induction of CYP4501A1 appears to be due in part to chlorinated compounds that occurs as a concentration gradient from the upper to the lower reached of the River. Induction of MFO system, however, not necessarily implies a PCB exposure only, since another class of organic pollutants can act as inducers (e.g., PAHs or dioxins). Our study, therefore, did not exclude fish exposure to a mixture of organic pollutants occurring in the Lambro River water with induction effect on the MFO activity, above all considering the industrial effluent discharges and the agricultural use of catchment areas of Lambro River. Both Liver EROD and BPMO activities provide evidence of a correlation between biochemical responses in fish and pollutant levels in muscle tissue. In addition, other anthropogenic stressors such as organophosphate or carbamate compounds are probably affecting the system, because their spatial pattern in the river are similar to PCB levels.

Therefore, based on the relationships between contaminant level in the river and the biomarker responses observed, *L.cephalus* is proposed as a good bioindicator species of pollutant levels within the system and should be used in future biomonitoring programs that assess the recovery of this polluted system

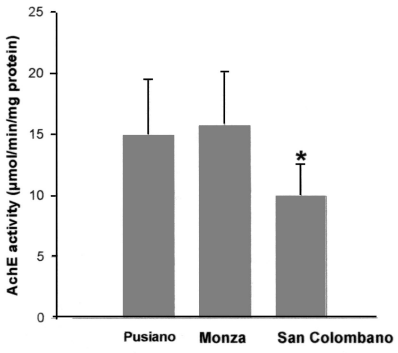

Figure 5. Mean brain AchE activity of *L.cephalus* (n=13,13 and 4 from Pusiano to San Colombano respectively) at the three sampling stations (* significant differences at $p<0,05$)

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REFERENCES

Adams SM (1990) Status and use of biological indicators for evaluating the effects of stress in fish. Amer Fish Soc Symposium 8:1-8.

Andersson T, Förlin L (1992) Regulation of the Cytochrome P450 enzyme system in fish. Aquat Toxicol 24:1-20

Battegazzore M (1994) Evaluation of the water quality of the river Lambro based upon the benthonic macroinvertebrates communities Att Soc It Sci Nat 135: 388-400

De Flora S, Viganó L, D'Agostini F, Camoirano A, Bagnasco M, Bennicelli C, Melodia F.and. Arillo A (1993) Multiple genotoxicity biomarkers in fish exposed in situ to polluted river water. Mutat Res 319: 167-177

Ellman L, Courtney KD, Andreas V and Featherstone RM (1961) A new rapid colorimetric determination of cholinesterase activity. Biochem Pharmacol 7: 88-95.

Erik W, Zabel P, Cook M, Peterson RE (1995) Toxic equivalency factors of polychlorinated dibenzo-p- dioxin, dibenzofuran and biphenyl congeners based on early life stage mortality in rainbow trout (*Oncorhynchus mykiss*), Aquatic Toxicol 31: 315-328

Fossi MC and Leonzio C (1994) Non destructive biomarkers in vertebrates. Lewis Publishers, Chelsea, MI. USA.

Galassi S, Gandolfi G and Pacchetti G (1981) Chlorinated hydrocarbons in fish from the river Po (Italy). Sci Tot Environ 20: 231-240

Galassi S, Viganó L and Sanna M (1996) Bioconcentration of organochlorine pesticides in rainbow trout caged in the river Po. Chemosphere 32:1729-1739

Gandolfi G, Zerunian S, Torricelli P and Marconato A (1991) I pesci delle acque interne italiane. Ministero dell'Ambiente - Unione Zoologica Italiana, Istituto Poligrafico e Zecca dello Stato, 616 pp.

Grimaldi E, Manzoni P (1990) Enciclopedia illustrata delle specie ittiche d'acqua dolce di interesse commerciale e sportivo in Italia. Istituto Geografico De Agostini, Novara, 141 pp.

Kurelec B, Britvic M, Rijavec M, Miller WE and Zahn RK (1977) Benzo(a) pyrene monooxigenase induction in marine fish. Molecular response to oil pollution. Marine Biology 44:211-216.

Lubert RA, Nims RW, Mayer RT, Cameron JW and Schechtman LM (1985) Measurement of cytochrome P450 dependent dealkylation of alkoxyphenoxazones in hepatic S9s and hepatocyte homogenates: effects of dicumarol. Mutat Res 142:1 27-131.

Pettine M, Bianchi M, Martinetti W, Muntau H, Renoldi M and Tartari G (1996) Contribution to te Lambro river to the total pollutant transport in the Po watershed Italy. The Sci Tot Environ 192:275-297

Schoor WP, Williams DE, Takahashi N (1991) The induction of cytochrome P450 1A1 in juvenile fish by creosote contaminated sediment. Arch Environ Contam Toxicol 20:497-504

Viganó L, Arillo A, Melodia F, Arlati P and Monti C (1998). Biomarker responses in cyprinids of the middle stretch of the river Po, Italy. Environ Toxicol Chemistry 17: 404-411.

Viganó L, Arillo A, Melodia F, Bagnasco M, Bennicelli C, and De Flora S (1996b). Hepatic and biliary biomarkers in rainbow trout injected with sediment extracts from the river Po (Italy). Chemosphere 30:2117-2128

Vigano L, Bassi A and Garino A (1996) Toxicity evaluation of waters from a tributary of the river Po using the 7 day ceriodaphnia test. Ecotoxicol Environ Safety 35:199-208

Viganó L, Galassi S, and Arillo A. (1994) Bioconcentration of polychlorinated biphenyls (PCBs) in rainbow trout caged in the river Po. Ecotox Environ Saf 28: 287-297

Vindimian E, Namour P, Migeon B and Garric J (1991) In situ pollution induced cytochrome P450 activity of freshwater fish: barbel (*Barbus barbus*), chub (*Leusciscus cephalus*) and nase (*Chondrostoma nasus*). Aquatic Toxicol 21: 255-266.