The Combined Use of Chemical and Biochemical Markers to Assess Water Quality in Two Low-Stream Rivers (NE Spain)

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INTRODUCTION

Small rivers are ecologically important systems that provide diverse habitats for aquatic biota. Because of the physical size and often relatively low flows, they are particularly susceptible to human pressure and activities. Hence, overexploitation of water due to agricultural, industrial, or urban use can be important sources of stress to these systems (Coimbra et al., 1997). In Mediterranean areas, intensive water resource use is frequently linked to the lack of natural flow due to climatic constraints, and rivers can receive the effluents of cities, industries, and agriculture without any dilution. In this situation, water quality is extremely poor, and measures that are effective in humid countries, such as the building of sewage treatment plants (STPs), are failing to recover river water quality (Prat and Munne, 2000).

A combination of physical, chemical, and biological indicators are frequently used to assess water quality (Norton et al., 1992; Karr, 1993). But, in recent years, there has been a growing awareness of the need to detect and assess the adverse effects of contaminants in inhabiting fauna. Among the available techniques, the integrated use of chemical analyses and biochemical and cellular responses to pollutants is a sound procedure for detecting impact of anthropogenic contaminants in aquatic systems (Walker and Livingstone, 1992; Porte et al., 2001).

This study focused on the rivers Anoia and Cardener, two tributaries of the Llobregat river (northeast Spain), which supplies water to the city of Barcelona. They were selected because they are good examples of overexploited Mediterranean streams, receiving extensive urban and industrial waste water discharges and with low natural flows (0.68–6.5 m³/s). In a previous study the presence of endocrine disrupters in those waters and their effect on male carps in terms of vitellogenin induction was...
shown (Solé et al., 2000). However, there are no data available on the presence of other chemicals, such as persistent organic pollutants or metals, nor their toxicological impact on inhabiting fauna.

In this context, the present study was designed to integrate chemical analysis of selected contaminants in biota tissue (organochlorinated compounds, polycyclic aromatic hydrocarbons (PAHs)) with several biochemical responses (cytochrome P450 system, phase II enzymes), with the aim of investigating whether resident organisms are responsive to changes in water quality and therefore suitable for biomonitoring. Two organisms, the common carp (Cyprinus carpio) and the crayfish (Procambarus clarkii), were selected as sentinel species. The carp is a representative species of the fish communities of these rivers, whereas P. clarkii is an introduced species, nowadays widely distributed in eastern and southern Spain. The biochemical markers considered were the cytochrome P450 monooxygenase system, namely, total cytochrome P450, NADPH-cytochrome (P450)c reductase, 7 ethoxyresorufl O-deethylase (EROD), and CYP3A. Glutathione S-transferases (GTS) and UDP-glucuronyl transferases (UDPGT) were determined as phase II enzymes.

MATERIALS AND METHODS

Sample Collection and Preparation

Carps (C. carpio) were collected in the rivers Anoia and Cardener, two tributaries of the Llobregat river (northeast Spain), in May 1999 by electric fishing. Three stations were selected for each river (Fig. 1). Two stations were located upstream of the cities of Igualada (A1) and Manresa (C1), two stations were located 23 km (A2) and 4 km (C2) downstream of their respective STPs, and two stations (A3, C3) were located further downstream. In the Anoia river, it was not possible to find fish nearer the STP due to the high degree of pollution. For comparative purposes, carps were also sampled at the same time of the year in the Segre river, as an example of a medium-flow river (103 m$^3$/s), relatively less anthropogenized than the others. Samples were taken 15 km downstream the city of Lleida (L), and they were considered a reference. P. clarkii were collected only in the Anoia river (stations A1, A1#, and A3), A1# being located 5 km downstream of the STP plant, an area where no fish were available.

Just after collection, fish were killed by a blow to the head and the livers were immediately dissected, frozen in liquid nitrogen, and stored at $-80^\circ$C. The gall bladder was removed and stored in dark vials at $-20^\circ$C. A piece of dorsal muscle tissue was dissected, wrapped in clean aluminum foil, and stored at $-20^\circ$C for analysis of organochlorinated compounds. Crayfish were immediately dissected, the digestive gland was frozen in liquid nitrogen, and the muscle tissue was wrapped in clean aluminum foil and stored at $-20^\circ$C until use.

Chemical Analysis

Analysis of organochlorinated compounds. Fish samples were analyzed individually, whereas crayfish samples were pooled (four to six individuals...
per station). In both cases, 4–5 g of muscle tissue were homogenized with anhydrous Na₂SO₄ and Sox-hlet-extracted with n-hexane: dichloromethane (4:1) for 18 h. The solvent extract was evaporated to near dryness, the residue was dissolved in 3 ml of n-hexane and cleaned by vigorous shaking with 1–2 ml of conc. H₂SO₄. The resultant extract was diluted with n-hexane for GC-ECD analysis. The instrument was a Hewlett-Packard 5890 GC equipped with an ECD detector. The column, 50 m × 0.25 mm i.d. CP-Sil 5 CB fused silica (Chrompack, Middelburg, NL), was programmed from 80 to 180°C at 15°C/min and from 180 to 280°C at 3°C/min, keeping the final temperature for 15 min. The carrier gas was helium at a linear flow rate of 50 cm/s. The injector and detector temperatures were set at 280 and 300°C, respectively. Quantitation was performed using an external standard calibration mixture of selected congeners (I.U.P.A.C. Nos. 18, 28, 31, 44, 52, 101, 149, 118, 138, 153, 170, 180, 194) supplied by Promochem (Wesel, Germany). These congeners were quantified separately and the polychlorodibenzo- (PCB) phenyl (PCB) concentration was defined as its sum. p,p'DDT, p,p'DDE, hexachlorobenzene (HCB), and lindane were also determined.

Selected samples (one per tissue) were analyzed for confirmatory identifications, following a method involving fractionation of the organic extracts and GC-MS analysis in the negative ion chemical ionization mode, as described by Porte and Albaigés (1993).

**Hydroxylated PAHs in fish bile.** Bile samples were hydrolyzed by a modification of the method of Krahn et al. (1987) as described in Escartin and Porte (1999a). Briefly, 100 μl of bile were incubated for 1 h at 40°C in the presence of 0.4 M acetic acid/sodium acetate buffer, pH 5.0, containing 2000 U of β-glucuronidase and 50 U of sulfatase. Hydrolyzed metabolites were extracted with ethyl acetate, and the final extract was analyzed by gas chromatography-mass spectrometry electron impact mode (GC-MS-EI). Individual quantification of PAH metabolites was achieved by GC-MS-EI, using a Fisons GC 8000 Series chromatograph interfaced to a Fisons MD800 mass spectrometer. Metabolites were identified and quantified by comparison of retention times and spectra of reference compounds. Ions used for monitoring were m/z 144,115 for 1-naphthol, m/z 170,141 for 2-phenylphenol; m/z 182,152 for 9-fluorenone; m/z 194,165 for 9-phenanthrol, and m/z 218,189 for 1-pyrenol. 2,6-Dibromophenol (m/z 252,250) and hexamethylbenzene (m/z 162,147) were used as a surrogate standard and a GC internal standard, respectively, with their recoveries being higher than 95%.

**Analytical performance.** The above-reported methods included the processing of blanks, duplicates, and standard mixtures between each group of samples. The GC injections were performed with an automatic injector to improve reproducibility, which was < 14% for the whole procedure. The recoveries calculated from the surrogate standards were higher than 90%. The detection limits were 0.01–0.07 ng/g for DDT and PCB congeners in muscled tissue and 0.04–0.09 ng/ml for hydroxylated PAHs in bile, except for 1-naphtol and 1-pyrenol (0.7–0.9 ng/ml). The protocol for determination of organochlorinated compounds was validated through participation in intercalibration exercises (UNEP-IOC-IAEA).

**Biochemical Determinations**

Cytosolic and microsomal fractions were prepared by a modification of the method of Jewell and Winston (1989). Briefly, after weighing, livers were flushed with ice-cold 100 mM KH₂PO₄/K₂HPO₄ buffer, pH 7.4, containing 100 mM KCl, 1.0 mM ethylenediaminetetra-acid (EDTA) and supplemented with 1 mM dithiothreitol (DTT), 0.1 mM phenantroline, and 0.1 mg/ml trypsin inhibitor. Homogenates were centrifuged at 500 g for 15 min, the fatty layer was removed, and the obtained supernatant was centrifuged at 12,000 g for 20 min. The 12,000 g supernatant was further centrifuged at 100,000 g for 60 min, to obtain the cytosolic and microsomal fractions. Microsomal pellets were resuspended in a ratio of 0.5 ml buffer/g liver tissue in 100 mM Tris–HCl, pH 7.4, containing 1 mM EDTA, 20% w/v glycerol and supplemented with 1 mM DTT, 0.1 mM phenantroline, and 0.1 mg/ml trypsin inhibitor.

**Cytochrome P450 system.** Cytochrome P450 system components were measured in the microsomal fraction. Cytochrome P450 was determined by sodium dithionite-difference spectrum of carbon monoxide-treated samples, assuming an extinction coefficient of 91 mM⁻¹ cm⁻¹ for 450 nm and 105 mM⁻¹ cm⁻¹ for 420 nm (Estabrook and Werringloer, 1978). NADPH-cytochrome c reductase activity was measured by the increase in absorbance at 550 nm (extinction coefficient 19.6 mM⁻¹ cm⁻¹) after NADPH addition to the microsomal fraction (Shimakata et al., 1972). 7-Ethoxyresorufin O-deethylase (EROD) activity was determined at 30°C using 10-min incubation, essentially as
described in Burke and Mayer (1974); 10 μl of microsomes were incubated in a final volume of 1 ml containing 96.5 mM K2HPO4/KH2PO4, pH 7.4, 0.25 mM NADPH, and 4.15 μM 7-ethoxyresoruﬁn. The reaction was stopped by adding 2.0 ml of ice-cold acetone. Samples were centrifuged at 500g for 10 min, and 7-ethoxyresoruﬁn ﬂuorescence was determined using a Kontron Instruments SFM-25 spectroﬂuorimeter at 537/583 excitation/emission wavelengths.

For the immunodetection of CYP3A, microsomal fractions were boiled in SDS–PAGE buffer (Laemmli, 1970), and 25 μg of proteins was loaded on 10% SDS–polyacrylamide gels, as described in Porte et al. (2001). Proteins gels were transferred to nitrocellulose membranes (Trans-Blot; Bio-Rad) that were incubated overnight with rabbit-anti trout CYP3A Ab (gift from Professor M. Celander, Göteborg University) as primary antibody. Blots were incubated at room temperature overnight and rinsed three times with Tris-buffered saline containing 0.2% gelatine. The membrane was incubated for 1 h with alkaline phosphatase-conjugated anti-rabbit IgG; the excess of secondary antibody was removed, and the sites of binding of the antibody were visualized by incubation with the substrates p-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate. When the optimal signal–background ratio had been achieved, rinsing the membrane in water stopped the development reaction. Western blots were semiquantitatively scanned with a densitometer PhosphoImager (Bio-Rad), and the results were expressed as optical density units/mg protein.

**Phase II enzymes.** Glutathione S-transferase activity was measured in the cytosolic fraction using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, the final reaction mixture containing 1 mM CDNB and 1 mM reduced glutathione (Habig et al., 1974). UDPG activity was assayed by a modiﬁcation of Clarke et al. (1992). The assay, which contained 0.25 mg of microsomal protein (pretreated for 15 min with 0.2% Triton X-100 on ice), was initiated by the addition of 81 μM p-nitrophenol (pNP) and run for 30 min at 30°C in a shaking water bath. The reaction was stopped by the addition of 0.2 M ice-cold trichloracetic acid, centrifuged, and alkalized with 0.1 ml of 10 N KOH, and the remaining pNP was measured spectrophotometrically at 405 nm.

Cytosolic and microsomal protein contents were measured by a modiﬁcation the method of Lowry et al. (1951), using bovine serum albumin as standard.

**Statistical Procedures**

Biochemical activities were determined and chemical analyses were conducted individually in four to nine organisms per station and run by duplicate. Values are presented as means ± SE. Statistical significance was assessed using a one-way ANOVA test in a SPSS statistical package. A P value of less than 0.05 was considered statistically signiﬁcant. Pearson’s correlation coefﬁcients were calculated and only P < 0.05 was accepted as signiﬁcant.

**RESULTS**

**Biological Data of Samples**

Biological parameters of sampled ﬁsh are listed in Table 1. Individuals were rather homogeneous in size and weight, except for carps from station L and A1 which were bigger. The condition factor (CF) was higher in carps from L (2.06 ± 0.04), followed by carps from the Anoia (1.46 ± 0.78) and Cardener (1.19-1.37) rivers, indicating a better nutritional state in the former.

**Chemical Analysis**

The PCB load of the different organisms was estimated on the basis of 13 PCB congeners selected from the GC-ECD proﬁle. These congeners were quantiﬁed separately, and results are given as its sum (Table 2). The highest PCB residues were observed in carp from the Cardener river (stations C1

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Biological Data of Carps Sampled in the Rivers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>River</strong></td>
<td><strong>Station</strong></td>
</tr>
<tr>
<td>Anoia</td>
<td>A1</td>
</tr>
<tr>
<td></td>
<td>A2</td>
</tr>
<tr>
<td></td>
<td>A3</td>
</tr>
<tr>
<td>Cardener</td>
<td>C1</td>
</tr>
<tr>
<td></td>
<td>C2</td>
</tr>
<tr>
<td></td>
<td>C3</td>
</tr>
<tr>
<td>Segre</td>
<td>L</td>
</tr>
</tbody>
</table>

*Note. n, number of organisms analyzed.*
TABLE 2
Organochlorine Residues (ng/g, Dry Weight) in Carps (Cyprinus carpio), and Crayfish (Procambarus clarkii) from the Segre, Anoia, and Cardener Rivers

<table>
<thead>
<tr>
<th></th>
<th>Segre river</th>
<th>Anoia river</th>
<th>Cardener river</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L</td>
<td>A1</td>
<td>A2</td>
</tr>
<tr>
<td>Carps</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \Sigma )PCBs</td>
<td>4.1</td>
<td>49.9 ± 3.2</td>
<td>47.3 ± 5.3</td>
</tr>
<tr>
<td>( \Sigma )DDTs</td>
<td>2.0</td>
<td>13.2 ± 2.8</td>
<td>9.5 ± 1.7</td>
</tr>
<tr>
<td>HCB</td>
<td>0.2</td>
<td>0.6 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>( \gamma )-HCH</td>
<td>0.1</td>
<td>2.2 ± 0.7</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Crayfish</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \Sigma )PCBs</td>
<td>16.2</td>
<td>7.0</td>
<td>14.7</td>
</tr>
<tr>
<td>( \Sigma )DDTs</td>
<td>1.5</td>
<td>0.7</td>
<td>1.2</td>
</tr>
<tr>
<td>HCB</td>
<td>1.7</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>( \gamma )-HCH</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

and C3), up to 20-fold higher than those in the reference site (station L). Measurable levels of DDTs, HCB, and lindane were found in all the samples (Table 2), and no significant differences among Anoia and Cardener rivers were observed, apart from HCB residues, which were slightly higher in carps from the Cardener. These residues were between 3- and 18-fold higher than the amounts recorded in carps from station L. Comparatively, tissue residues of PCBs, DDTs, and lindane were lower (3- to 10-fold) in crayfish than in carps.

Generally, those organisms sampled immediately after STPs (crayfish from station A1 (5 km downstream) and carps from station C2 (4 km downstream]) showed lower PCBs and DDTs residues than the others.

When the global distribution of the identified PCB congeners was considered it was possible to observe further differences among the rivers. The distributions, from tri- to octachlorobiphenyls, of selected stations are summarized in Fig. 2. Hexachlorobiphenyls represented 35–40% of the whole mixture, followed by the penta- (22–26%) and heptachlorobiphenyls (12–21%). Carps from the Cardener river exhibited relatively higher levels of heptachlorobiphenyls (21%), whereas those from Anoia were relatively enriched in tetrachlorobiphenyls (13–20%) (Fig. 2).

The concentration of hydroxylated PAHs in fish bile is reported in Fig. 3. Considering the sum of hydroxylated PAHs, the highest concentrations were observed in station C1, located near the city of Manresa. Strong differences among sampling sites were observed in terms of individual PAHs. Hence, 1-pyrenol was particularly abundant in the bile of carps sampled along the Cardener river (63–91% of total detected metabolites), whereas those from the Anoia river, and particularly from stations A2 and A3, were highly enriched in phenylphenol (48–68% of total PAH metabolites). No data were available for station L.

Biochemical Markers

**Cytochrome P450 system.** Cytochrome P450 system components and activities are indicated in Fig. 4. Total cytochrome P450 ranged between 337 and 645 pmol/mg protein, the lowest values being detected in stations L and A1. Cytochrome P450 was elevated in C. carpio from stations A2 (Anoia river)
and C1 (Cardener river) (Fig. 2A), although differences were not statistically significant. P450 levels were well related with recorded levels of hydroxy PAHs in fish bile. NADPH cytochrome c(P450) reductase activity showed a pattern similar to that of total cytochrome P450 content in carp from Anoia river, the highest activity recorded in station A2 (Fig. 4). The same tendency was observed in crayfish; those organisms collected at station A1 # showed significantly higher cytochrome P450 content (718 pmol/mg protein) than the others (400 to 537 pmol/mg protein) (Table 3). Despite a similar cytochrome P450 content, P. clarkii had much lower NADPH cytochrome c reductase activity (2.3–2.8 nmol/min/mg protein) than carp (16–30 nmol/min/mg protein).

EROD activity allowed the detection of clear differences between stations and rivers. In accordance with total cytochrome P450, EROD activity was significantly elevated in carp from station A2 (located downstream of the STP of Igualada) in comparison with stations A1 and A3 (Fig. 4). This significant induction was also observed when compared with station L, where organisms had an activity of 49 ± 8 pmol/min/mg protein. Similarly, EROD activity in P. clarkii from A1 # was about 10-fold higher than that in organisms from A1 (Table 3). EROD activity was higher in organisms from Cardener river (343–548 pmol/min/mg protein) than in those from Anoia river (38–237 pmol/min/mg protein), and differences with respect to station L were statistically significant. EROD activity was elevated in carp from C2, 4 km downstream of the Manresa STP, although no significant differences were observed compared with C1.

Immunodetermination of CYP3A protein was carried out with anti-trout P450con IgG as the primary antibody (gift from M. Celander). Blots displayed an intense band of about 55 kDa in β-NF-treated trout (used as positive control). Two bands of approximately 54 and 57 kDa were obtained in carp. Both immunoreactive proteins were considered to be CYP3A, and they were quantified either together or separately, with no differences among stations being found in any case (Fig. 4). In contrast, crayfish blots displayed a faint band, if present, at approximately 55 kDa and a second band at approximately 70 kDa, which was observed in all the samples. The identity of this band is unknown, but Peters et al. (1998) reported a CYP3A immunoreactive band at a similar molecular weight (67 ± 1 kDa) in Mytilus edulis. Semiquantitative analyses of Western blots were performed and differences in presence and relative intensity of the 55-kDa band were visualized as a function of location; this band was present in 67% of samples from station A1 #, but only in 27 and 12% of samples from A1 and A3, respectively.

Phase II enzymes. In agreement with phase I data, the activity of cytosolic GST was higher in carp from the Cardener river. GST increased slightly downstream of the STPs, with the highest activity in stations A3 and C3 (Fig. 4). The same trend was observed in crayfish, whose GST activity was up to three-fold higher than that in carp from the same area (Table 3). Carps from A3 had significantly higher GST activity than those from A1, but this significant difference was not observed when compared with organisms from station L, which had an activity of 0.809 ± 0.076 μmol/min/mg protein.

Similarly, in the Anoia river, the lowest UDPGT activity was observed in A1 and the highest in A3 (Fig. 4B). This activity was elevated in all organisms sampled in Cardener river.
FIG. 4. Levels and activities of phase I and phase II related enzymes in the liver of *Cyprinus carpio* collected from Anoia and Cardener rivers. Each value is expressed as mean ± SE (n = 6–8). Significant differences from the respective reference site are marked with * (vs. station A1). Recorded values for station L are the following: cytochrome P450 = 337 ± 21 pmol/mg protein; NADPH-cyt c reductase = 24.8 ± 2.6 nmol/min/mg protein; EROD = 49 ± 8 pmol/min/mg protein; GST = 0.809 ± 0.076 μmol/min/mg protein; UDPGT = 191 ± 17 pmol/min/mg/protein.

**DISCUSSION**

**Xenobiotics**

This study confirms that both rivers are characterized by different pollutant loads and that biota inhabiting those areas are exposed to complex mixtures of contaminants; viz. carps from both rivers had up to 20-fold higher organochlorine residues than those from station L. A decrease in PCB residues was observed in those organisms collected a few kilometers downstream of the STPs (carps from C2 and crayfish from A1), probably as a consequence of the removal of hydrophobic contaminants during the waste water treatment process, by adsorption onto suspended solids, and by accumulation into the sludge. This decrease in PCB body burden was evident only in organisms collected near STPs (4–5 km), but not in those collected at a longer distance (see A2
TABLE 3
Levels and Activities of Phase I- and Phase II-Related Enzymes in Crayfish (Procambarus clarkii) collected from the Anoia River

<table>
<thead>
<tr>
<th></th>
<th>A1</th>
<th>A1 #</th>
<th>A3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome P450 (nmol/mg protein)</td>
<td>399.8 ± 48.3</td>
<td>718.0 ± 82.4*</td>
<td>536.8 ± 64.3</td>
</tr>
<tr>
<td>NADPH-cytochrome P450 reductase (nmol/min/mg protein)</td>
<td>2.32 ± 0.12</td>
<td>2.46 ± 0.87</td>
<td>2.76 ± 0.74</td>
</tr>
<tr>
<td>EROD (fmol/min/mg protein)</td>
<td>43 ± 14</td>
<td>426 ± 214</td>
<td>218 ± 118</td>
</tr>
<tr>
<td>GST (µmol/min/mg protein)</td>
<td>1.73 ± 0.12</td>
<td>1.94 ± 0.06</td>
<td>1.99 ± 0.04</td>
</tr>
</tbody>
</table>

Note. Each value is expressed as mean ± SE (n = 4-6). Significant differences versus station A1 are marked as *. In Table 2). Qualitative differences in terms of PCB patterns were observed for both rivers, as indicated in Fig. 2, and these patterns can be attributed to slightly different compositions of the PCB mixtures downloaded to the rivers.

Additionally, the determination of PAHs excreted through the bile allowed the detection of different exposure levels. High levels of phenylphenol were recorded in carps from stations A2 and A3. This compound is used as a disinfectant in paper industries and tanneries located in the area, and it is readily taken up by carps, metabolized and excreted through the bile. In contrast, carps from the Cardener river were exposed to high levels of pyrene, particularly those located in C1, near the city of Manresa (200,000 inhabitants), and these PAHs patterns are consistent with those found in sewage sludge from the area (Pérez et al., 2001).

Whereas the reported organochlorine concentrations are in the lower range of those described in cyprinids and freshwater fish from elsewhere (Schmitt et al., 1990; van der Oost et al., 1996; Stranberg et al., 1998; Viganó et al., 2000), levels of PAHs excreted through the bile are in the higher range of those reported in other studies (Escartín and Porte, 1999a,b). Additionally, previous studies have indicated the presence of high levels of alkylphenol polyethoxylates in river water (up to 15 µg/L of nonylphenol) and sediments (up to 645 µg/kg of nonlyphenol), particularly on those stations located downstream of STPs (M. Petrovic et al., unpublished).

Thus, carps inhabiting these waters are exposed to complex mixtures of pollutants that might have negative consequences for their fitness and reproduction. Some of these compounds are reported to exert estrogenic activity (Tyler et al., 1998), and other display carcinogenicity (Baumann, 1988); however, their combined effects are unknown.

Biomarkers

Elevated EROD activity (a measure of inducible CYP1A isofrom) was observed in carps from the Cardener river; the activity was over 10-fold higher than that detected in carps from station A1 (Anoia river) or station L (reference site), which confirms the presence of higher levels of hazardous compounds in that river. In addition, carps from the Cardener river had lower condition factors than those from the reference site or Anoia (Table 1), and a negative relationship \( r = -0.51, n = 19, P < 0.05 \) between EROD and CF was recorded, which all together supports the hypothesis that organisms from Cardener river are experiencing an adverse effect. Additionally, EROD activity was well related with total hydroxylated PAHs determined in bile of carps from Anoia river \( r = 0.92; n = 10, P < 0.05 \) This relationship was not observed in the Cardener river when considering total OH-PAHs excreted through the bile, although 9-hydroxyphenanthrene \( (r = 0.73, n = 9) \) and phenylphenol \( (r = 0.69, n = 9) \), individually, showed a statistically significant relationship with EROD activity \( P < 0.05 \).

A direct effect of the STPs in terms of EROD induction was observed only in the Anoia river, both in carps (A2) and crayfish (A1 #), although this increase was not statistically significant in the latter (see Table 3), due to high variability.

Generally, Inconsistent results have been reported during past years with regard to CYP1A induction in freshwater fish (Lindström-Seppä and Oikari, 1990; Van Veld et al., 1990; van der Oost et al., 1994; Viganó et al., 1998; Tuvikene et al., 1996, 1999). Increases in EROD activity in relation to PAHs or pulp and paper industry exposure did not occur in every study, and when the activity increased, the magnitude of the response was of the order of two- to threefold. It should be mentioned that most of the studies were designed to assess effects upstream and downstream of suspected pollution sources, and no ‘absolute’ reference sites were included. Hence, differences between putative contaminated and ‘clean’ areas are usually small. Additionally, the presence of unknown sources of contamination, the complex interaction between different contaminants, the variable sizes of fish territories, and the different sensitivities of different fish species are just some of the factors that can account for the lack of concordance of the results.
In the present study, both rivers, particularly stations 2 and 3, are characterized by a strong estrogenic load, (Solé et al., 2000), as they receive the effluents of their respective STPs with very poor dilution. This is not the case for station L, where the dilution factor of the STPs effluent (similar number of inhabitants) is between 15- and 150-fold higher. In this situation, we may have an underestimation of fish exposure to hazardous compounds when measuring EROD activity, as estrogenic compounds, and estradiol in particular, have been reported to inhibit this activity (Snowberger et al., 1991). Indeed, considering the complexity of the aquatic environment and the coexistence of inducing and inhibiting chemicals, this and other studies illustrate the difficulty of estimating risks in complex situations by using a single biochemical probe, even a widely accepted one, such as EROD activity.

The use of batteries of biomarkers can enhance the ability to identify areas/species threatened by chemicals. Hence, phase II enzymes (GSTs and UDPGT) were elevated in carps from Cardener and significantly elevated in station A3, in accordance with their role in conjugation of electrophiles produced by P450 monoxygenation.

Alternatively, hepatic microsomal cytochrome P450 content of C. carpio did not show a clear induction by pollutants as reported by other authors and for other species (Ueng et al., 1992; Haasch et al., 1993; Porte et al., 2002), probably due to the fact that the assay measures both constitutive and inducible forms of cytochrome P450. The content of the cytochrome P450 isoenzyme CYP3A was elevated in carps caught downstream at STPs (stations A2 and C2), but differences between stations were not statistically significant. CYP3A is involved in the 6β-hydroxylation of steroids, but with a broad substrate specificity (procarcinogens, therapeutic drugs, and dietary chemicals) (Celander et al., 1996).

P. clarkii collected at station A1# showed a series of biochemical alterations. Station A1#, located 4 km downstream of the STP, has an extremely poor water quality, and these organisms had significantly elevated cytochrome P450, higher EROD activity, and high GST. A CYP3A-like protein was also expressed in P. clarkii and particularly in those organisms sampled in station A1#.

Finally, it should be mentioned that not only the analyzed pollutants, but also many others, and complex interactions among them and with the biological systems, are responsible for the observed biological responses. Nonetheless, the set of biomarkers used in this and in a previous study (Solé et al., 2000) indicated high levels of stress in carps from both rivers, but particularly in those from the Cardener river. The lower condition factor and the higher content of organochlorinated pesticides and hydroxylated PAHs in bile, in conjunction with altered biochemical responses (EROD, phase II activities), are indicative of higher levels of stress in specimens from Cardener river.

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