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Scoring approach based on fish biomarkers applied to French river monitoring

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The aim was to apply a multimarker scoring approach as complementary to freshwater monitoring programmes carried out by the Water Agency Adour–Garonne. Fish (chub, barbel and trout) were collected in 11 sites in rivers in south-west France. Five biomarkers of response were measured either in muscle or brain for acetylcholinesterase (AChE) and in liver for glutathione *S*-transferase, catalase and 7-ethoxyresorufine *O*-deethylase. As a result of multivariate analysis, sites were clearly discriminated mainly by 7-ethoxyresorufine *O*-deethylase and acetylcholinesterase activities. According to the scoring approach, a multimarker pollution index was calculated for each sampling site as the sum of the response index of the five measured biomarkers (pollution index). Sorting was established by ranging the sites from lightly to highly contaminated locations.

Keywords: exposure biomarkers, fish, freshwater, scoring approach, pollution index.

Introduction

Thousands of chemical pollutants such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), heavy metals and pesticides have been produced and released into the environment.

Pollution monitoring programmes were developed in the early 1950s (Mussel watch in the USA, the French Survey Network in France RNO) based on chemical analysis. In the 1980s, measurement of biochemical parameters at molecular or cellular were proposed as sensitive 'early warning' tools for biological effects measurement in environmental quality assessment. The selected biomarkers should indicate: that the organism has been exposed to pollutant (biomarkers of exposure) and/or the magnitude of the toxic effects (biomarkers of effect or biomarkers of stress). Biomarkers are then defined as short-term indicators of long-term biological effects (McCarthy and Shugart 1990).

Biomarkers were selected among four clusters of early molecular mechanisms of action of contaminants as: phases I and II of drug metabolism, oxidative stress and neurotoxicity. Glutathione *S*-transferase (GST) and catalase (CAT) activities were modulated by metal or organic contaminants both under field and laboratory conditions (Pellerin-Massicotte 1994, Prahash and Rao 1995, Regoli and Principato 1995). Cholinesterase activities appeared as biomarkers of exposure for some

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pesticides and other pollutants on wildlife (Bocquene *et al.* 1990, Najimi *et al.* 1997). A multimarker study maybe useful to evaluate the various responses to natural or anthropogenic changes. The practical approach, carried out by the official agencies in charge of environmental monitoring, is to establish indexes of environmental quality taken into account chemical or biological criteria, in order to classify the sites being monitored in a scale from 'clean' to 'highly polluted'. In theory, any organism may be of use in a biomonitoring study. The abundance of potential test organisms should be considered, especially for river fish, commonly used as sentinel organisms for the detection of environmental pollution in freshwater. In this case, the distribution of fish species along the river is a major problem for sentinel species availability. Fish are collected by electric fishing, minimizing the initial stress, but the mobility and movement patterns of the test organism will also affect biomarker responses. The exposure levels of fishes to persistent contaminants are in part related to their level in a food chain (BAFs, i.e. bioacumulation factors). The aim of this study is to evaluate the biomarker responses in fish collected during the FISH BIO programme.

Materials and methods

Animals

Three omnivorous species were selected:

- Barbel (*Barbus barbus*) is the largest cyprinide in Europe. It is a strictly riverine fish (Huet 1949) living in rivers with fast flow, stony, gravely bottom and well-oxygenated water. The barbel distribution extends over the major part of Western Europe (the southern most extension limited to the basins of the Rhône and the Danube). Relatively sedentary apart from the spawning period, the barbel can move considerably (10 km) during reproduction period (Baras 1992).
- Chub (*Leuciscus cephalus*) is widely present in all the French rivers. Except for Scandinavia and islands in the Mediterranean Sea (Phillipart 1987), the Chub inhabits the whole of Europe, living in fresh as well as brackish waters, such as the Baltic Sea. Despite capability for adaptation to all water types, the chub has a marked preference for flowing water with a hard bed.
- Rainbow trout (*Oncorhynchus mykiss*) are know to colonize rivers, lakes and the sea. This species comes from in the west coast of the USA (Rocky Mountains) and was largely introduced during the 19th century all over Europe and specially in Western Europe where trout farming is strongly developed.

Sample collection and preparation

The three studied fish species were collected from 11 sites located in five rivers: Seudre, Dordogne, Garonne, Tarn and Gave de Pau (figure 2). These sites were selected according to previous studies conducted by the Adour–Garonne Water Agency. Pareloup lake is considered as a reference site. Fishes were sampled in late summertime. These sampling were carried out by electrofishing, involving a temporary paralysis of fish. The sampled animals were weighted and lengthened before being sacrificed. Animals were sorted according to the sex during dissection. Sex determination was made by examination of the colour and size of the gonads. Liver, bile, brain and muscle were taken out systematically. Bile was collected only when the size of the individual allowed removal. Tissues and organs were kept on dry ice for further analysis.

Preparation of subcellular fractions

All samples were homogenized at 4°C in 100 mM phosphate buffer, pH 7.4 (1/3 w/v) for 1 min using a Potter-Elvehjem, followed by centrifugation at 9000*g* for 30 min. Supernatants consisting of the sub-mitochondrial fractions (S9) were collected and stored at −80°C until use.

Bile was sampled in vials and then frozen in liquid nitrogen as pure bile without the gall bladder. For small bile volumes (<30 µl), an initial dilution was made at the time of sampling.

Biochemical assays

Enzymatic activities were measured with a dual-beam temperature-controlled Kontron Uvikon 932 spectrofluorimeter for AChE, GST and catalase activities. EROD activities and proteins concentrations were measured on a microplate-reader (BIOTEC FL600).

Assays were run in triplicate for each individual. AChE, EROD, GST and CAT and *in vitro* activities were measured in the post-mitochondrial fraction (S9) by using as substrates acetylthiolcoline (Ellman *et al.* 1961), ethoxyresorufine (Burke and Mayer 1974), 1-chloro-2-4-dinetrobenzene (Habig *et al.* 1974) and oxygen peroxide (Clairborne 1985), and were used as described (Michel *et al.* 1993a, Mora *et al.* 1999, Vidal *et al.* 2001).

Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as standard.

The measurement of PAH metabolites in fish bile were performed by direct fluorescence measurement (FF/SFS), followed by analysis in high-performance liquid chromatography coupled with fluorescence detection (HPLC-F) (Van der Oost *et al.* 1997, Aas *et al.* 2001).

Statistical analyses

Biomarkers data were analysed by carrying out ANOVA and Tukey tests. Discriminant analyses (DA) allowed the separation of the different sites using factor or discriminating functions as linear combinations of the original variables (Narbonne *et al.* 1999). In order to select the biomarkers with the most influence in distinguishing among site responses, a discriminatory power was calculated by ranking analysis as follows:

$$\mathrm{DP}_i = \sum \mathrm{R}_1\mathrm{V}_1 + \frac{\sum \mathrm{R}_2\mathrm{V}_2}{2} + \frac{\sum \mathrm{R}_n\mathrm{V}_i}{n}$$

where i is the biomarker, n is the number of the root, $\mathrm{R}_n\mathrm{V}_i$ is the rank number of the discriminatory variable for biomarker i and root n.

Discriminatory patterns were presented using two-dimensional graphs of the first two roots covering the largest fraction of variance. Data were analysed by using the Statistica 6.0 computer software package (Statsoft, Inc., US Headquarters, Tulsa, USA).

Pollution index

All the studied biomarkers were gathered in a global index in order to give a relative idea of the pollution level undergone by the animals. The multimarker pollution index (MPI) for each site was calculated as follows:

$$\mathrm{MPI}_i = \sum\nolimits_{j=1} \mathrm{BPI},$$

where i is site, j is the biomarker and BPI is the biomarker pollution index from the table of conversion (table 1) for individual mean (X_i), related to discriminatory factor (DF) of the measure:

$$\mathrm{DF} = (X_{\max} - X_{\min} + \mathrm{CI})/\mathrm{CI},$$

where $X_{\max}$ is the maximum mean, $X_{\min}$ is the minimum mean and CI is the confidence interval given by Tukey's test.

Finally, a pollution scale was established including five levels (from lightly to highly contaminated). The index level was then converted into colours (red, orange, yellow, green and blue from the highest to the lowest pollution effect) to map pollution levels.

Table 1. Index given for each biomarker response according to their rank in a scale related to the discriminatory factor.

	Discriminatory factors				
Number of levels	1	2	3	4	5
Index of response	4	10			
	3	6	12		
	2	4	7	12	
	1	2	4	8	14

The MPI classification scale from 1 to 5 (Narbonne *et al.* 1999) was first applied in European BIOMAR Programme.

The global biomarker index of each site investigated was calculated and converted to a pollution score level as described (Narbonne *et al.* 1999).

Results

Acetylcholinesterase activity in muscle and brain

For chub, the cholinesterase activity measured in muscle was significantly higher in animals from Le Fleix (75%) and from Tauriac (40%) compared with fish sampled in Pareloup. For barbel, AChE levels were lower at Bourret compared with Pareloup. Brain AChE activity measured in chub was lower in La Garde (-75%) than in Pareloup. No signification activity was observed for barbel and trout (table 2).

EROD activity

The three species show very different EROD activity from control site. Trout shows an activity 2.5 times higher than barbel and 6.5 times higher than chub. For chub, the activities found in La Garde and Lahontan were significantly higher (740 and 565%, respectively) compared with Pareloup (table 2).

For barbel, the highest activities were found in individuals sampled in Bourret and Rabastens. EROD activity measured in trout samples was significantly higher in Montespan than in other sites (table 2).

Catalase activity

Statistical tests show significant differences between the control site of Pareloup and St-André de Lidon (63%) for chub. Barbel, sampled in Rabastens, also showed a significant difference (75%) with the two other sites. No significant differences were observed for trout (table 2).

Glutathione S-transferase activity

Only barbel sampled on Bourret revealed a significant difference (167%) compared with Pareloup (table 2). Interestingly, the catalase activities were quite different in the three species sampled at Pareloup.

Analysis of fish from Lahontan demonstrated a clear presence of metabolites of naphthalene and pyrene compared with Pareloup. Moreover, a significant difference was observed in Millau for naphthalene metabolites compared with pyrene and benzo(*a*)pyrene metabolites (table 3).

Discriminant analysis

Given all the biochemical parameters investigated in the present study, EROD and AChEm were the best studied biomarkers to differentiate between the sampled sites (table 4). This finding was particularly true with respect to results for chub and barbel. In trout, EROD and GST were the best discriminant biomarkers.

For all analyses, the two main roots for discrimination between sites were EROD and AChE. For chub, sites can be ranged from Pareloup, St-André de

Table 2. Results of biomarker measurements for each site studied during the FISHBIO programme.

Sites	AChE activity in muscle (μmol min^{-1} mg^{-1} protein)	AChE activity in brain (μmol min^{-1} mg^{-1} protein)	CAT activity (μmol min^{-1} mg^{-1} protein)	EROD activity (pmol min^{-1} mg^{-1} protein)	GST activity (μmol min^{-1} mg^{-1} protein)
Chub					
Pareloup	0.119 $\pm$ 0.02	209.73 $\pm$ 37.2	1013.42 $\pm$ 254	27.43 $\pm$ 19.02	1.61 $\pm$ 0.36
St-André de Lidon	0.148 $\pm$ 0.028	214.04 $\pm$ 23.76	1602.77 $\pm$ 493.7*	40.38 $\pm$ 14.16	1.44 $\pm$ 0.47
Le Fleix	0.205 $\pm$ 0.04*	199.53 $\pm$ 37.6	804.48 $\pm$ 290.8	103.78 $\pm$ 42.23	1.37 $\pm$ 0.29
La Garde	0.15 $\pm$ 0.038	155.35 $\pm$ 9.5*	980.50 $\pm$ 267.6	199.39 $\pm$ 145.5*	1.77 $\pm$ 0.28
Lahontan	0.145 $\pm$ 0.025	180.49 $\pm$ 49.4	1031.41 $\pm$ 300.9	152.66 $\pm$ 70.2*	1.68 $\pm$ 0.58
Tauriac	0.174 $\pm$ 0.027*	205.22 $\pm$ 26.9	1235.41 $\pm$ 687.05	60.93 $\pm$ 39.87	1.4 $\pm$ 0.57
Barbel					
Pareloup	0.174 $\pm$ 0.042	149.52 $\pm$ 25.6	355.30 $\pm$ 164.65	72.07 $\pm$ 33.88	1.12 $\pm$ 0.38
Bourret	0.122 $\pm$ 0.026*	159.95 $\pm$ 29.23	462.64 $\pm$ 153.6	218.5 $\pm$ 78.28*	1.88 $\pm$ 0.64*
Rabastens	0.21 $\pm$ 0.039	162.62 $\pm$ 30.67	601.36 $\pm$ 294.18*	194.64 $\pm$ 122.37*	1.45 $\pm$ 0.67
Trout					
Pareloup	0.159 $\pm$ 0.04	106.33 $\pm$ 49	575.84 $\pm$ 263	176.64 $\pm$ 144.83	1.05 $\pm$ 0.43
Clarac	0.146 $\pm$ 0.03	77.14 $\pm$ 15.7	694.31 $\pm$ 207	161.4 $\pm$ 129.12	0.94 $\pm$ 0.33
Millau	0.151 $\pm$ 0.04	84.96 $\pm$ 18.5	774.76 $\pm$ 276	158.73 $\pm$ 126.01	1.13 $\pm$ 0.41
Montespan	0.128 $\pm$ 0.01	118.72 $\pm$ 67.2	794.84 $\pm$ 161.5	458.16 $\pm$ 200.57*	1.4 $\pm$ 0.53

Values are means $\pm$ SD (standard deviations, $n = 20$).
*Significantly different from reference site.

Table 3. PAH metabolites measured in bile from fish collected in sampled sites.

Sites	Naphthalene, FF290/335	Pyrene, FF341/383	Benzo(a)pyrene, FF380/430
Chub			
Pareloup	3287 ± 898	268 ± 86	79 ± 28
St-André de Lidon	4615 ± 5512	610 ± 852*	356 ± 306*
Le Fleix	2675 ± 2590	167 ± 138	58 ± 70
La Garde	2646 ± 1778	224 ± 145	79 ± 76
Lahontan	5826 ± 1876*	3187 ± 1400*	251 ± 153*
Tauriac	3136 ± 1640	4203 ± 383	147 ± 137*
Barbel			
Bourret	3186 ± 1437	477 ± 260	120 ± 107
Trout			
Pareloup	8581 ± 5130	790 ± 514	412 ± 309
Clarac	1816 ± 732*	280 ± 139*	144 ± 31
Millau	5373 ± 4461	578 ± 351	482 ± 447
Montespan	4670 ± 2659*	621 ± 461	138 ± 120

FF, fixed wavelength fluorescence.
For naphthalene: (μg naphthalene equivalents ml^{-1} bile); for pyrene: (μg pyrene equivalents ml^{-1} bile); and for benzo(a)pyrene: (μg benzo(a)pyrene equivalents ml^{-1} bile).
Values are means ± SD (standard deviations, $n = 20$).
*Significantly different from reference site ($p < 0.05$).

Lidon to La Garde. For trout, Montespan is clearly discriminated as a highly contaminated site among the others. For barbel, Pareloup is discriminated from Rabastens on the EROD basis and from Bourret on the AChE in muscle basis (figure 1).

Index calculation

The results of multimarker measurement expressed as MPI and BPI for each site are presented in figure 2. MPI is converted in five pollution levels associated with a colour from blue to red.

Pollution gradient was clearly indicated by mapping MPIs. Upstream sites (Clarac and Millau) exhibited a blue index; intermediate sites exhibited a green index (Montespan, Rabastens Tauriac, Le Fleix); and downstream sites (St-André de Lidon and Lahontan) exhibited a yellow or an orange index. Moreover, La Garde and Bourret areas exhibited orange and yellow index, respectively. The

Table 4. Ranking of the biomarker measured in the present study as listed according to their discriminant power.

Chub		Barbel		Trout	
Biomarkers	DP	Biomarkers	DP	Biomarkers	DP
EROD	5.70	AChEm	4.27	EROD	5.17
AChEm	7.17	EROD	6.71	GST	6.17
CAT	7.83	GST	9.15	CAT	7.17
GST	8.17	CAT	9.15	AChEb	7.67
AchEb	9.17	AChEb	9.15	AChEm	9.17

DP, discriminant power.

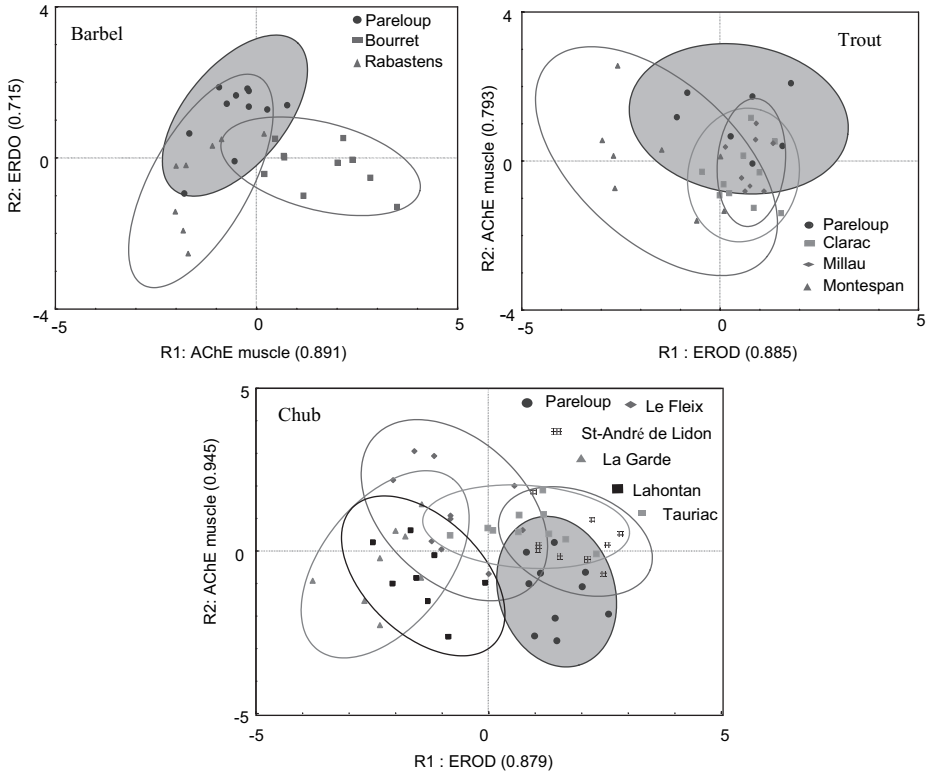

Figure 1. Discriminant analysis for barbel, trout and chub, respectively, sampling sites.

indices in Pareloup lake (selected as the reference site) were blue for trout, barbel and chub. The main contaminants, usually measured by the Water Agency in the sampled sites (Mora *et al*. 2003), indicated pollution by PAHs in Lahontan (there is chemical industry upstream in Lacq), contamination by heavy metals (specially cadmium) in the Tarn (Rabastens, La Garde), and high levels of pesticides in Bourret and St-André de Lidon. The gradient of PCB contamination was found in the Garonne from Clarac to Bourret.

Discussion

Enzymes activities

AChE is involved in the deactivation of acetylcholine at nerves ending, preventing continuous synaptic transmission, which is vital for normal functioning of sensory and neuromuscular systems. Many pesticides are effective AChE inhibitors and the inhibition of this enzyme has been used to assess the nature and the extent of exposure of wildlife to agriculture and forestry sprays. The effects observed in Bourret may indicate the presence of pesticides and/or heavy metals (Olson and Christensen 1980) in water. Pesticides used near the sampled sites are highly variable within the year and a better knowledge of agriculture and forestry practices will be useful to support biochemical data. For Le Fleix and Tauriac, a

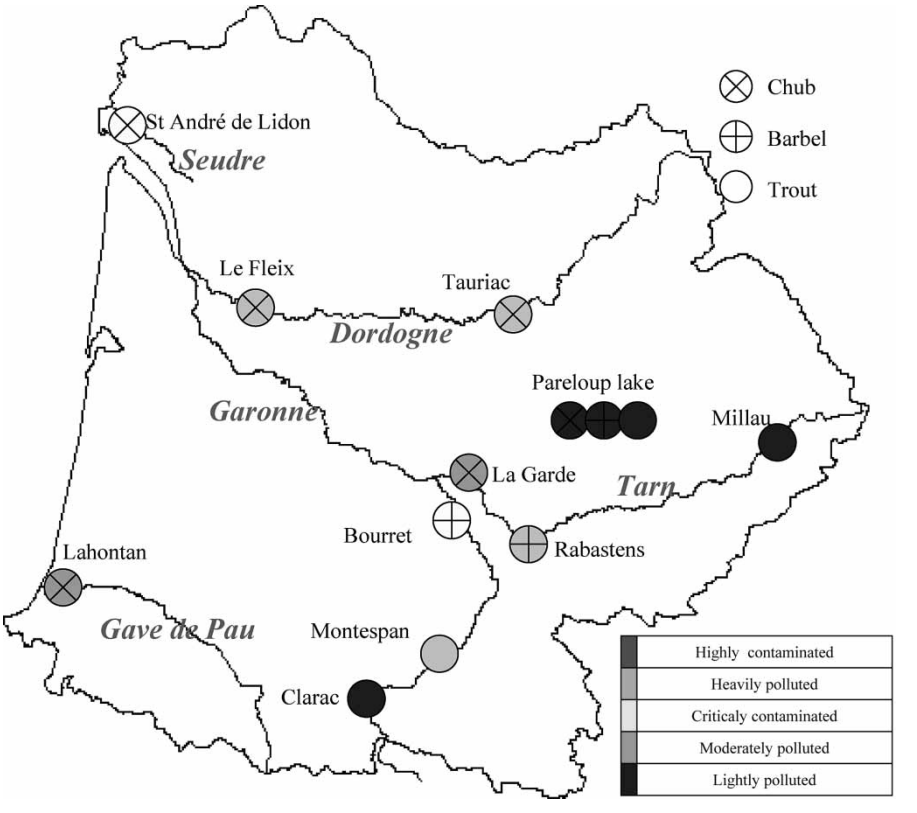

Figure 2. Biomarkers contribution for each station in the Dordogne–Garonne basin, South West France.

higher AChE activity in muscle was found compared with Pareloup, supposedly a less polluted site (Mora *et al*. 2003). Indeed, the temperature of the environment may also have a significant effect on the AChE activities (Bocquene *et al*. 1990).

The induction of CYP1A in fish, following exposure to certain classes of organic contaminants, was the basis of the use of the cytochrome P450 system as biomarker in pollution monitoring (Payne 1976). Hepatic EROD activity from all fish was significantly correlated ($p < 0.05$) to PCB body burden (estimated by concentration in the muscle). A number of field studies have shown a significant linear relationships between EROD induction and PCBs bioaccumulation in fish (Flammarion *et al*. 1998, Monod *et al*. 1988). Indeed, measurement of EROD activity in fish is a well-established *in vivo* biomarker of exposure to several planar halogenated/polycyclic aromatic hydrocarbons and many structurally related compounds (Lam and Gray 2003). However, some EROD values appeared to be lower especially in chub from Le Fleix. Such assumed inhibition of EROD has already been found on the chub in the presence of heavy metals (Flammarion *et al*. 2002).

Correlation between EROD activities in fish and concentration of potential EROD inducers have been reported (Garrigues *et al*. 1990, Narbonne *et al*. 1991) but, however, in certain *in situ* conditions inhibition of EROD activities by other

contaminations has been observed (Bucheli and Fent 1995, Bruschweiler *et al*. 1996, Klumpp *et al*. 2002). However, a direct correlation between single contaminant and EROD activity can hardly be expected in field situations because of generalized multiple contamination. Such an effect may derive from the influence of another natural factor that has not yet been taken into account in this study (Flammarion *et al*. 2002). However, it is known that many non-pollution variables may have an additional impact on the various enzyme systems, and may thus interfere with biomarker responses. Other confounding factors have to be taken into account such as health, condition, sex, age, nutritional status, metabolic activity, migratory behaviour, reproductive and developmental status, population density (Altenburger *et al*. 2003) and seasonal variations. The modulation of EROD during the year for the eelpout (*Zoarces viviparus*) shows highest activity in February–March and it decreased afterwards (Ronisz *et al*. 1999).

Despite its popularity and apparent success, the mixed-function oxidase (MFO) system itself is relatively non-specific. Many organisms possess this detoxification enzyme complex, which can be induced as a response to a wide variety of natural and xenobiotic compounds. Thus, MFO concentration in field collected samples are often difficult to interpret, especially in localities where no point sources exist (Lam and Gray 2003). Exposure to environmental contaminants involves many complex processes ending by oxidative stress, which can be evaluated by antioxidant enzyme activity such as catalase. The two responses observed in the barbel from Rabastens and the chub from St-André de Lidon are likely due to a higher presence of heavy metals than in the other sampling sites from each species (data not shown).

Elevated GST activity was measured in barbel found in Bourret. This site appeared to be contaminated by PCBs (Mora *et al*. 2003). Laboratory and field studies reported a strong GST induction in fish and mussels exposed to PCBs commercial mixtures or pure congener (Michel *et al*. 1993).

The major problem we could face in an *in situ* approach for continental contamination detection is the large number of compounds that could reach such ecosystem and could be able to interfere.

The pyrene-type bile metabolites are correlated to total PAHs and pyrene in liver and muscle ($p < 0.01$) (Mora *et al*. 2003). The particular pyrene-type bile metabolites were demonstrated to be relevant as an exposure marker to PAHs in fish (Aas *et al*. 2001).

Discriminant analysis

Discriminant analysis (DA) has already proven to be a very useful method for classifying the pollution status of different sites (Adams *et al*. 1994, 1996). It is performed in order to maximize the inter-site variance, thus helping to characterize the differences between the various sites. Recent examples of the use of DA on environmental quality data concerned the characterization of different stations along French rivers (Persat *et al*. 1985), in a Norwegian fjord (Beyer 1996) and in Amsterdam inland water sites (Van der Oost *et al*. 1997), which were based on the results of biomarker of exposure in fish provided an efficient tool for the statistical to discrimination between sampling sites. EROD appeared as the main discrimi-

nant biomarker in all fish species investigated. This result is according to the number of biomonitoring using biomarkers in fish (Wilson *et al*. 2000). In the VALIMAR programme (Behrens and Segner 2001), among the biotransformation index studied, only EROD activity (especially in trout) was distinguished between the study sites. In the present study, AChE measured in muscle was also able to discriminate sites in chub and barbel, while GST activity was a discriminant biomarker only for trout. The results of VALIMAR study indicated that GST was not a discriminant biomarker for loach and trout.

With the use of more than one biomarker in monitoring, traditional analysis of variance (or non-parametric equivalents) approaches to data interpretation are challenged to characterize and group the data. The multi-biomarker approach is similar to common procedures in human epidemiology where many responses are interpreted to diagnose disease (Handy *et al*. 2003). With measurement of multiple responses, more powerful multivariate statistics may be used to investigate the data and look for grouping or trends. Chèvre *et al*. (2003), for example, evaluated effects at the cellular and molecular levels in the clam *Mya arenaria* with discrimination methods. Rough set analysis was used to classify sites and identify important biomarkers for defining the groups. This type of analysis is particularly useful since it is a simple and efficient method for classifying multivariable biomarker data, and, furthermore, it is free from distributional assumptions.

As suites of biomarkers are more frequently used to evaluate the effects of contaminant exposure to assess environmental stress, constrains such as the availability of living material may limit the collection of data and thus hinder interpretation. Beliaeff and Burgeot (2002) describe a simple method summarizing biomarker responses, thereby aiding interpretation. They used star plots to display results for a range of biomarkers and integrated response was computed as the star plot area. The integrated responses were then used to investigate spatial and temporal variation in contaminant exposure. The approach was applied to Baltic Sea and English Channel sites and the integrated biomarker responses compared well with PAH and PCB levels measured in mussel and fish tissues (Beliaeff and Burgeot 2002). The scoring approach applied to the multimarker study was first developed for marine pollution monitoring (Narbonne *et al*. 1999). It seems a useful method for classifying the pollution level of different river sites when applied to freshwater fish species. The results indicate that biological monitoring based on biomarker exposure is an appropriate method providing reliable environmental risk assessment.

Conclusion

Fish appeared to be a useful species for freshwater monitoring. However, the problem of the presence of different species corresponding to different biotas along the rivers appeared to be limiting factors. While the specific enzymes activities may be different in the fish species studied, the scoring approach that takes into account the relative responses of biomarker of exposure provides an adequate tool for comparison between areas. The pollution gradient in some rivers from upstream to

downstream was mapped by using a colour scale, thus providing useful information for risk management.

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