Determinant and Multivariate Statistical Analysis of Biochemical Responses to Environmental Contaminants in Feral Freshwater Fish Leuciscus Cephalus L.

Miroslav Machala,*† Ladislav Dusék,‡§ Klára Hilscherová,¶ Renata Kubínová,** Pavel Jurajda,*** Jiří Neča,† Robert Ulrich,‡ Milan Gelnar,‡ Zdena Studničková,‡‡ and Ivan Holoubek‡‡§

†Veterinary Research Institute, 62132 Brno, Czech Republic
‡Masaryk University, Faculty of Science, 61137 Brno, Czech Republic
§RECETOX-TOCOEN, 61137 Brno, Czech Republic
¶Veterinary and Pharmaceutical University, 6200 Brno, Czech Republic
**Landscape Ecology Institute, Academy of Sciences of the Czech Republic, Brno, Czech Republic
***To whom correspondence may be addressed (machala@vri.cz).

Abstract—Modulations of 11 prospective biochemical markers of impacts of aquatic pollutants in liver tissue of chub (Leuciscus cephalus), caught at several sampling sites of a river with various pollution types and rates, were matched against analytical data of concentrations of organochlorine compounds, polycyclic aromatic hydrocarbons (PAHs), and heavy metals. Multivariate principal component analysis (PCA) of the field data showed general patterns of biochemical responses to different types of pollutants and relationships among the biomarkers. Cytochrome P4501A-dependent 7-ethoxyresorufin O-deethylase (EROD) activity, inducible by 2,3,7,8-tetrachlorodibenzop-dioxin and structurally related planar compounds, was strongly enhanced in the more contaminated areas. Compared with polychlorinated aromatic hydrocarbons, PAHs did not contribute so significantly to EROD induction. Testosterone 6β- and 16α-hydroxylase activities, as an expression of the cytochrome P4503A27, were slightly increased at several sites but were significantly decreased in samples from some heavily polluted areas. Recently, these activities have been suggested as potential biomarkers of exposure to contaminants that do not induce cytochrome P4501A. In this study, their inhibition or induction was not associated with a specific class of monitored contaminants, and selectivities of these modulations are still to be investigated. Similar modulations of the prospective biochemical indicators of oxidative stress, including microsomal glutathione S-transferase activity, cytosolic glutathione S-transferase with ethacrynic acid, and glutathione reductase, were demonstrated by PCA. The pattern of the modulations of the microsomal nicotinamide adenine dinucleotide phosphate (NADPH)-dependent lipid peroxidation in vitro differed from the responses of the rest of oxidative stress parameters at some sampling sites. Further biochemical markers of oxidative stress under study, including in vivo lipid peroxidation, in vitro production of reactive oxygen species, and the concentration of metallothioneins did not correlate well with the concentrations of the contaminants. Principal component analysis demonstrated that the EROD activity, glutathione-dependent enzymes, and Fe(II)-enhanced lipid peroxidation formed a suitable battery of biomarkers of exposure.

Keywords—CYP1A Testosterone Glutathione enzymes Oxidative stress Biomarker

INTRODUCTION

Biochemical markers of impacts of aquatic pollutants are defined as parameters, the changes of which can be related to exposure to or toxic effects of chemicals [1]. Alterations in biochemical markers appear to be the responses reflecting the mechanisms of adverse effects rather than an exposure of organisms to a specific class of compounds. The concept of biochemical markers of exposure, based on investigations of major mechanisms of toxicity such as dioxin-like toxicity and oxidative stress, has been generally accepted [1,2]. However, a suite of assays using biochemical markers for ecological risk assessment has not yet been established.

The biochemical responses associated with the major mechanisms of adverse effects of aquatic contaminants that have been studied the most extensively as potential biomarkers are (1) the Ah receptor-mediated induction of cytochrome P4501A (CYP1A), a specific biomarker of dioxin-like toxicity; (2) other CYP activities, especially steroid hydroxylations, which are probably not modulated by CYP1A-inducing agents but rather by other persistent contaminants; (3) various biomarkers of oxidative stress; and (4) metallothioneins as potential biomarkers of the impact of heavy metals [3,4]. Simultaneous determination of more than one biomarker is necessary for in situ bioindication of adverse effects of pollutants [3,5]; this necessity implies problems with unclear correlation structure of the data or even with partial redundancy of parameters. Multivariate statistical analyses were used in several recent papers to interpret the modulations of biochemical markers in fish [6–8]. The main advantage of multivariate data treatment is the possibility of recognizing simultaneously different or similar patterns of changes in biomarkers affected by environmental stress. The principal component analysis (PCA), combined with discriminant analysis, appears to be very helpful in a reliable interpretation of the observed changes and optimization of the choice of the biomarkers, as demonstrated recently [6].

This study investigated the responses of a suite of biochemical parameters to environmental mixtures of contaminants in the liver of chub collected from seven sampling sites differing in the level of contamination. Chub (Leuciscus cephalus L.) is a carnivorous cyprinid inhabitant of rivers of central and south Europe surviving also in more polluted water; chub has been used previously in biomarker field studies [9,10]. This paper combines analytical data on major classes

Markers of oxidative stress, including in vivo lipid peroxidation, in vivo production of reactive oxygen species, and the concentration of metallothioneins did not correlate well with the concentrations of the contaminants. Principal component analysis demonstrated that the EROD activity, glutathione-dependent enzymes, and Fe(II)-enhanced lipid peroxidation formed a suitable battery of biomarkers of exposure.
of contaminants in sediment, biochemical data (i.e., determination of CYP1A-dependent 7-ethoxyresorufin-O-deethylase activity, testosterone hydroxylases, a series of oxidative stress parameters, and metallothioneins in fish liver), and multivariate statistical analysis to reveal similarities and distinct patterns of biochemical responses in fish. The principal component analysis was used as a tool for the assessment of the applicability of potential biochemical markers.

MATERIALS AND METHODS

Chemicals

Both 7-ethoxyresorufin and 2',7'-dichlorofluorescein diacetate were purchased from Molecular Probes (Eugene, MI, USA); resorufin, nicotinamide adenine dinucleotide phosphate (NADPH), reduced and oxidized glutathione, 1,1,3,3-tetraethoxypropane, 1-chloro-2,4-dinitrobenzene, ethacrynic acid, bicinechonic acid, and monohydroxylated testosterone metabolites were purchased from Sigma-Aldrich (Prague, Czech Republic). Sixteen U.S. Environmental Protection Agency priority polycyclic aromatic hydrocarbons, the organochlorines DDT, hexachlorocyclohexane, and polychlorinated biphenyl (PCB) congeners, and isomers were obtained from Dr. Ehrenstorfer (Darmstadt, Germany).

Sampling and processing of fish tissues

Adult three- to eight-year-old male chubs (Leuciscus cephalus L.) were caught at seven stations in the Morava river basin, Czech Republic (Fig. 1). The fish were killed by a blow to the head and cervical dislocation; liver tissues were excised and immediately frozen in dry ice. Hepatic microsomes and cytosolic fractions were prepared by homogenization and ultracentrifugation as described previously [7]. Microsomes were washed twice with 0.05 M Tris-HCl buffer containing 20% glycerol and 0.1 mM ethylenediaminetetraacetic acid, pH 7.5, and stored at −70°C. Top layers of river sediments were sampled from the same locations, freeze dried, and fractionated using a set of separation screens.

Chemical analyses

Concentrations of PAHs in the river sediments were determined by a conventional reverse phase high-performance liquid chromatography using a Waters 600 system (Waters, Fairchold, OH, USA) coupled with a Model 470 fluorescence detector in extracts obtained through Soxhlet extraction for 3 h with dichloromethane and a size exclusion chromatography using Bio-beads SX-3 (J.E. Merck, Darmstadt, Germany). An aliquot of the fractions collected by the size exclusion chromatography was further fractionated in a Hypercarb PCB column (50 × 4.6 mm, 7 μm; Shandon, Runcorn, UK); DDT and hexachlorocyclohexane isomers and di-ortho-, mono-ortho-, and non-ortho-PCBs were determined in the fractions by a high resolution gas chromatography (HRGC/ECD) (Varian Star 3600, Walnut Creek, CA, USA) in a two-dimensional system using the DB-5 and DB-1701 columns (60 m × 0.25 mm, 0.25 μm; Quadrex, New Haven, CT, USA). Heavy metals were assayed by conventional atomic absorption spectrometry. The analyses for polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) in the sediments were made to order by Axys-Varilab (Prague, Czech Republic) using a HRGC system.

Biochemical assays

The EROD activity was determined with a Perkin-Elmer spectrofluorometer LS-50 (Norwalk, CT, USA) as described by Prough et al. [11]. The final concentration of 7-ethoxyresorufin was 2 μM. Microsomal testosterone hydroxylase activities were measured by high-performance liquid chromatography. Hepatic microsomes were incubated with 250 μM testosterone in 50 mM phosphate buffer (pH 7.4). The reaction was started by the addition of 10 μM NADPH, run for 10 min, and stopped by the addition of 4 ml dichloromethane. After the evaporation under a stream of nitrogen, the sample was dissolved in methanol and the metabolites were determined in the Waters 600/ NovaPak C18/DAD600 system (Milford, MA, USA) as described by Reinerink et al. [12].

The cytosolic glutathione S-transferase activity towards 1.25 mM ethacrynic acid (GST-ETHA) and the glutathione reductase activity were measured spectrophotometrically [13,14]. The microsomal glutathione S-transferase (mGST) activity was measured toward 1.25 mM 1-chloro-2,4-dinitrobenzene [7]. All enzyme activities were assayed at 30°C. The protein concentration was determined using the bicinechonic acid assay [15] in a 96-well microplate modification. Determination of thiobarbituric acid reactive substances was used to assess the extent of lipid peroxidation in microsomes. The thiobarbituric acid reactive substances products were measured spectrophotometrically [16]. Both nonstimulated lipid perox-
Biomarkers of contamination in feral fish

Environ. Toxicol. Chem. 20, 2001 1143

Table 1. Location of the sampling sites, relative levels of contamination based on chemical data in sediments, and sample sizes of fish (n = number of fish; fish data represent mean values and range in parentheses)\(^a\)

<table>
<thead>
<tr>
<th>Site</th>
<th>Location</th>
<th>Qualitative pollution characteristics (based on chemical analysis of sediments)</th>
<th>Characteristics of fish in which biomarkers were determined</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Moravská Šázava</td>
<td>Reference site</td>
<td>n</td>
</tr>
<tr>
<td>II</td>
<td>Kroměříž</td>
<td>High concentrations of DDT, PCDD/Fs, Cd</td>
<td>6</td>
</tr>
<tr>
<td>III</td>
<td>Malenovice</td>
<td>Moderate contamination (higher PCBs, Hg)</td>
<td>8</td>
</tr>
<tr>
<td>IV</td>
<td>Spytihněv</td>
<td>Low contamination</td>
<td>7</td>
</tr>
<tr>
<td>V</td>
<td>Rajhradice</td>
<td>Highest contamination by PCDD/Fs, PCBs, HCH; high concentrations of PAHs, DDT, Cd, Hg</td>
<td>8</td>
</tr>
<tr>
<td>VI</td>
<td>Val. Meziříčí</td>
<td>Highest concentrations of PAHs, Hg, and Cd; high concentrations of PCDD/Fs</td>
<td>8</td>
</tr>
<tr>
<td>VII</td>
<td>Uherské Hradišť</td>
<td>High contamination by PAHs, DDT, PCBs, Cd</td>
<td>8</td>
</tr>
</tbody>
</table>

\(\text{PCDDs/Fs} = \text{dibenzo-p-dioxins and dibenzofurans; PCBs = polychlorinated biphenyls; PAHs = polycyclic aromatic hydrocarbons; ND = not determined.}
\)
\(\text{n = number of fish; fish data represent mean values and range in parentheses.}
\)
\(\text{a = CF = Fulton’s condition factor (body weight (g) × 100/standard length (cm))}^b\)
\(\text{b = Numbers within the column marked by the same capital letter are not significantly different (Kruskal–Wallis analysis followed by Mann–Whitney test, p = 0.05).}
\)

\(^a\) PCDDs/Fs = dibenzo-p-dioxins and dibenzofurans; PCBs = polychlorinated biphenyls; PAHs = polycyclic aromatic hydrocarbons; ND = not determined.
\(^b\) No significant difference between sites (Kruskal–Wallis analysis, p > 0.3).
\(^c\) CF = Fulton’s condition factor (body weight (g) × 100/standard length (cm)).
\(^d\) Numbers within the column marked by the same capital letter are not significantly different (Kruskal–Wallis analysis followed by Mann–Whitney test, p = 0.05).

Statistical analyses

Sample frequency distributions were examined prior to any further statistical analyses. Provided the log-normal character of the chemical or biological parameters had been detected, log transformation (\(\ln[X + 1]\)) was verified to be effective in reaching the normality (\(\chi^2\) goodness-of-fit test or Shapiro–Wilk’s test). The transformation function also sufficiently stabilized the variability of a specified parameter (Bartlett’s test, Levene’s test) within more than two objects (sites) prior to their comparison in parametric analysis of variance tests. Under verified assumptions for the analysis [20], the comparison of chemical parameters of sediments and biochemical markers in fish in different sites was based on one-way analysis of variance models followed by Tukey’s multiple-range test. All the parametric analyses were simultaneously controlled by the appropriate nonparametric alternatives (Kruskal–Wallis analysis, Mann–Whitney test). Where possible, the results of multiple comparisons were presented using multiple box-and-whisker plots.

The Spearman rank correlation coefficient \((r_s)\) was applied as a measure of relations among the compared parameters [20]. Multivariate variation of 11 tested biomarkers was further summarized in the PCA as an effective technique simplifying the correlation structure through linear transformation of the original variables [21]. Principal component analysis was done to provide component loading vectors sufficient for the explanation of relationships among the biomarkers and their roles in the evaluation of the sites and to provide component score vectors as pairwise uncorrelated variables, which were used for the final exploratory survey of the data from the examined sites. The most informative bilinear projections showing the relation between objects (fish from examined sites) and variables (more or less correlated biomarkers) were reached if logarithmically transformed variables directly entered the PCA, i.e., analysis based on covariance matrix [22]. Component weight vectors were scaled to the length one. Biplots were used as a graphical tool representing not only projections on extracted principal components but also the two-dimensional loadings of original variables by lines. The length and direction of the lines represent the significance of the associated variables for the plotted components and for the discrimination of the sites based on component scores [23,24].

RESULTS

Concentrations of aquatic contaminants

The results of analyses for PCBs, PAHs, organochlorine pesticides, PCDD/Fs, and heavy metals in the river sediments are shown in Figure 1. The highest rates of pollution by PCBs and PCDD/Fs were unambiguously demonstrated at sampling site V. High concentrations of all monitored chemicals were determined also at site VII. High levels of organochlorine compounds, especially DDT, and PCDD/Fs were detected at site II; the highest concentrations of PAHs and heavy metals were found in the sediment collected at site VI (Fig. 1). The locations of the sampling sites, their pollution characteristics as revealed by chemical analyses of sediments, and sample sizes of fish are shown in Table 1.
Biochemical responses to aquatic contaminants in fish liver

The biochemical parameters investigated as potential indicators of exposure to contaminants were EROD activity as the measure of 2,3,7,8-TCDD-like toxicity [25], testosterone hydroxylase activities as possible biomarkers of exposure to organochlorine chemicals and some other classes of contaminants [26±28], modulations of glutathione-dependent enzymes involved in the antioxidant defense and phase II bio-transformation [3,4,7,28], in vitro production of reactive oxygen species and breakdown products of lipid peroxidation as parameters of susceptibility to oxidative damage [5,29], and metallothioneins as potential biomarkers of exposure to heavy metals [19]. The biochemical data are presented in Figures 2 and 3.

The CYP1A-dependent EROD activity was strongly induced in chub caught at site V, and only a slight increase of the activity was found at sites II, III, IV, and VII (Fig. 2a). Modulations of testosterone hydroxylase activities were distinct from the EROD activities. The patterns of responses of testosterone 6ß- and 16α-hydroxylase activities and partly also the 16ß-hydroxylation were much alike, i.e., there was significant elevation at sites II and IV and depression at heavily contaminated sites V, VI, and VII (Fig. 2b through d). Only small differences in concentrations of cytosolic metallothioneins were detected among all sites (Fig. 2e).

Three glutathione-dependent activities were analyzed in the hepatic subcellular fractions (Fig. 3a through c). The microsomal GST activity showed increases similar to the cytosolic GST-ETHA at sites II and V; cytosolic glutathione reductase activity (GR) was increased also in samples collected at sites III and VI. The NADPH/Fe(II)-dependent in vitro production of reactive oxygen species, detected by dichlorofluorescein probe (DCF), and NADPH/Fe(II)-enhanced in vitro lipid peroxidation (LPin vitro ) were measured in the hepatic microsomal fraction. An elevation of these prooxidative processes were found only at site II, while LPin vitro was also significantly depressed at sites VI and VII (Fig. 3d and f). In vivo lipid peroxidation was poorly modulated by chemical contamination (Fig. 3e).

Multivariate statistical analysis

An exploratory survey of the type of frequency distribution and variability of measured responses was necessary to characterize the tested biomarkers. With the exception of metallothioneins, all the biochemical variables showed positively skewed frequency distribution of the log-normal type that was effectively normalized using the transformation ln(X + 1). In the case of the relatively sensitive biomarkers (EROD, LPin vitro , GST-ETHA, mGST, GR, T6ß, T16α), a weekly bimodal or multimodal pattern occurred in distribution function due to significantly changed activities at the polluted sites. In terms of these sensitive parameters, the differences among the sites accounted for more than 70% of the total variability in the data set. On the other hand, levels of metallothioneins, the
DCF-detected production of reactive oxygen species, and the in vivo lipid peroxidation revealed homogeneous and fully continuous distribution with rather a low potential to discriminate among the sites (Figs. 2 and 3).

The covariance structure of the data was subjected to multivariate PCA (Figs. 4 through 6) to interpret relationships among biomarkers and to define general patterns of their responses to the pollution. As a dimension-reducing technique, the PCA led to three relevant components that altogether sufficiently accounted for nearly 90% of the overall variability of the original data. The other components were neglected because they did not provide meaningful interpretation and did not contribute significantly to the discrimination among the sites (the scree test [24]).

The first principal component (PC1) was clearly associated with the EROD activity as the leading variable, which was slightly supported by GR and mGST (Fig. 4a). The knowledge of the sites allowed us to apply the analysis of variance on the component score and thus to extract the response pattern belonging to the variables. It is apparent that PC1 reflected mainly the EROD-like response to the pollution (Fig. 5a). The biomarkers T6β, T16α, and LP in vivo were the major contributors to the second component (PC2), with a minor additional influence of DCF (Fig 4a). The main pattern of responses associated with these variables involved increased activity at sites II and IV and relatively reduced levels at sites V, VI, and VII (Fig. 5b). The association of the three variables was also confirmed by bivariate rank correlations (r = 0.623–0.875) computed on original untransformed data.

In contrast with PC1 and PC2, no variables clearly associated with PC3, which accounted for 6.15% of the overall variability, could be identified. However, this component stressed another significant feature in the data, i.e., different reactions of mGST, GST-ETHA, GR, and LP in vivo as one group and T6β and T16α as the other group of biomarkers (Fig. 4b). Analysis of variance analysis of PC3 scores revealed clearly increased responses at site II and significantly decreased activity at site IV, which corresponded to the negative weights of T6β and T16α (Fig. 5c). As already proven by one-dimensional analyses, modulations of metallothionein concentrations as well as in vivo
l lipid peroxidation appeared to be of minor relevance in the explanation of the data structure (Fig. 4a and b).

It should be emphasized that clear prevalence of EROD activity could have partially masked the relations among the other biomarkers in the PCA analysis. To demonstrate more clearly the pattern of relations among all the biomarkers under study, the multivariate statistical analysis was repeated excluding the EROD activity. Figure 6 shows a biplot from this analysis representing projections on newly extracted PC1 and PC2 (both accounted for 89% of the overall variability) and also the two-dimensional loadings of biomarkers by lines. The biplot clearly demonstrated profound stimulation of LP in vitro at site II and increased activity of T6β and T16α at site IV as three variables associated with PC2 (62.16% of the overall variability). Parameters GR, mGST, and GST-ETHA were apparently associated with PC1, with positive indication mainly at sites V and II. Biplot presentations of PC2 also involved the opposite response of T6β and T16α at sites V, VI, and VII in comparison with the other biomarkers correlated with this component, i.e., GR, mGST, GST-ETHA, and partially with LP in vitro. Quadrants formed by component scores of PC1 and PC2 clearly separated biomarker values at site V from sites I and IV; PC2 further contributed to significant distinguishing of sites II and V from sites I and IV. The similarity observable for component scores at sites I and IV at sites III, VI, and VII corresponded to the results of sediment analyses.

**DISCUSSION**

*Modulations of hepatic microsomal CYP activities*

An increase in fish hepatic CYP1A-dependent EROD activity represents specifically the Ah receptor-mediated toxicity of planar PCBs, PCDD/Fs, several polycyclic aromatic hydrocarbons, and other planar aromatic contaminants [25]. However, specific biochemical responses to organochlorines, nonplanar PCBs, and phenolic contaminants in fish have not yet been sufficiently characterized, although these chemicals are known to cause oxidative stress, reproductive failures, or other toxic effects. From this point of view, an interesting parameter is the steroid hydroxylase activity, especially hydroxylation of progesterone or testosterone in the 6β-position mediated by cytochrome P450A27 [26–28] and perhaps also by fish cytochrome P4502K [8]. In rainbow trout, the activity represents specifically the Ah receptor-mediated toxicity of membrane lipids and oxidative damage to other key cellular components can lead to genotoxicity, promotion of carcinogenesis, and perturbation of several other signal and metabolic pathways [30]. In the last decade, a series of antioxidants and antioxidant enzymes have been tested as potential biomarkers of oxidative/antioxidative imbalance in fish [3,5]. Often, contradictory modulations of potential biochemical indicators of oxidative stress in various fish species, including glutathione peroxidase, catalase, superoxide dismutase, and in vivo lipid peroxidation, have been reported [31–35]. Conflicting results were also obtained when total levels of the cytosolic GST, measured as GST activity toward 1-chloro-2,4-dinitrobenzene, were used as a biomarker of exposure to aquatic contaminants [32,35–38]. However, differential induction of the hepatic GST isoenzymes can play an important role in the antioxidant defense [39]. Therefore, more selective GST activities, including GST-ETHA associated with a Pi class of GST enzymes [40], mGST, and GR activity, appeared to be additional potential biomarkers, as shown in both field and short-term laboratory studies [28,32,35,41].

In this study, similar patterns of modulations of glutathione-dependent enzymatic activities were demonstrated; the GST-ETHA, mGST, and GR activities were strongly increased at sites heavily polluted by organochlorine pesticides and PCBs, which are known to induce oxidative stress (Fig. 3a through c). However, detailed studies of specific responses of glutathione-dependent enzymes to mixtures of contaminants such as heavy metals and persistent chlorinated compounds are necessary.

Further oxidative stress parameters showed distinct modulations; while LP in vitro, enhanced by Fe(II) and NADPH, was found to be another potential biomarker reflecting chemical stress, the production of reactive oxygen species, detected by dichlorofluorescein, and in vivo lipid peroxidation showed similar but nonsignificant responses (Fig. 3d through f). It should be noted that the highest DDT concentration was found at site II, where LP in vitro was also significantly elevated.

Unlike other authors [42], we did not find the concentration of metallothioneins in the cytosolic fraction of chub liver to be a suitable biomarker of pollution by heavy metals. This discrepancy was perhaps due to the relatively high levels of heavy metals at all the sampling sites under study. However, our results are in accordance with the findings of Palace and Klaverkamp [43] that feral cadmium exposure did not increase the metallothionein protein in three freshwater fish species.

**Multivariate statistical analysis**

As expected, the tested biomarkers responded differently to the pollution, as they differed remarkably in their discrimi-
Biomarkers of contamination in feral fish

Environ. Toxicol. Chem. 20, 2001 1147

...ination power (comparing the sites with respect to the within-site variability) (Figs. 2, 3, and 6).

Although a ranking of the sites was possible on the basis of unidimensional analyses, the results were strongly parameter dependent and detailed description of single parameters need not necessarily agree with the required description of basic patterns associated with the stressors. Multivariate treat-

...ent of the data and particularly reasonable reduction of dimensions in the PCAs were therefore necessary. Generally, the principal components can be generated from the correlation or covariance matrix of the original data and the results of the two analyses cannot be mutually converted because the PCA is not scale invariant. If the variables are grossly different in their scales, the correlation matrix input, which implies the analysis on standardized original data, is more reasonable [44].

Van der Oost et al. [6] applied PCA to define the association among 46 variables involving biochemical biomarkers, physiological and morphological parameters of fish, and chemical analyses of fish tissues in a data set from 58 eel.

In this study, the multivariate analysis treated more homogeneous data with the main aim of recognizing relationships among 11 biochemical biomarkers and of interpreting the resulting patterns with respect to the polluting chemicals. Logarithmic transformation of the input variables stabilized their variability and gave them approximately the same weight. Therefore, the PCA could be performed on the covariance matrix of log-transformed original data.

On the basis of their correlations, the investigated biomarkers could be divided into three categories with internally consistent response patterns, and differences among the groups should be related to some type(s) of environmental stressors. The first principal component was dominated by EROD activity as a specific indicator of TCDD-like toxicity, which was markedly stimulated mainly at site V. This response was slightly supported by the parameters GR and mGST (Figs. 4a and 5a). Apart from its nonsignificant character, the similar behavior of GR and mGST activities, as indicated by the composition of PC1, could be mainly related to the increased concentrations of prooxidative stressors at site V (rank correlation of EROD vs GR or mGST, 0.39–0.45). The second type of biointerpretative reaction was represented by the component score of PC2 and comprised increased responses at sites II and IV while the values that belonged to sites VI and VII declined. Biomarkers T6β, T16α, and LP<sub>in vitro</sub> were the main contributors to this pattern, with minor additional influence of DCF (Figs. 4a and 5b). The component PC3 mainly demonstrated the distinct behavior of the group of oxidative stress parameters GR, GST-ETHA, and mGST compared with T6β and T16α at sites I and IV. Furthermore, the loadings of PC3 emphasized distinct modulations of LP<sub>in vitro</sub> and glutathione-dependent enzymatic activities (Figs. 4b and 6). We concluded that, compared with the adaptation induction of antioxidant glutathione enzymes, the in vitro lipid peroxidation better reflected the susceptibility to oxidative damage in fish collected from more contaminated locations [31].

In conclusion, multivariate analysis demonstrated that the measurement of the EROD activity, selected glutathione-dependent enzyme activities, and in vitro lipid peroxidation represent currently a suitable battery of biomarkers of toxicity on the biochemical level. The modulations of the testosterone 6β-hydroxylase activity were clearly distinct from those of the other potential biomarkers under study. However, its toxicological relevance and specificities of responses should be investigated in detail.

Acknowledgement—This work was supported by the Ministry of Agriculture (RE 5563) and the Ministry of the Environment (VaV 340/196) of the Czech Republic.

REFERENCES

...transduction enzymes in Atlantic salmon (Salmo salar) liver treated with an estrogenic compound, 4-nonylphenol. Environ Toxicol Chem 16:2576–2583.

