Ecotoxicology, 11, 451–465, 2002

Biomarkers as Diagnostic Tools for Evaluating Effects of Unknown Past Water Quality Conditions on Stream Organisms

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Accepted 6 July 2002

Abstract. The following biomarkers were investigated in stream populations of juvenile brown trout (*Salmo trutta* f. *fario*) and gammarids (*Gammarus pulex*) to determine if crayfish mortality could have been confounded by pollutants: (1) alterations of fish liver ultrastructure, (2) fish gill and kidney histopathology, (3) stress protein (hsp70) expression in fish liver and gills and in gammarids, and (4) changes in various blood parameters of brown trout. In addition, the following measurements were conducted in parallel with the biological sampling: (a) chemical analyses including several pesticides, organochlorines, PCBs, and PAHs in sediment and tissue samples of brown trout and crayfish (*Astacus astacus*), and (b) limnochemical analyses of nutrients, electrolytes, dissolved oxygen content, temperature and pH. Biomarkers together with chemical and limnochemical analyses concomitantly indicated moderate pollution of the stream at all sampling sites. Biological data indicated a transient, episodic event at one sampling site resulting (a) in altered stress protein levels in gills and livers of trout and in whole gammarids as well as (b) in elevated numbers of macrophages in liver tissue. Biomarker responses provided spatial and temporal evidence that a contaminant release was associated with the crayfish mortalities observed in this stream system.

Keywords: trout biomarkers; crayfish mortality; ultrastructure and histopathology; stress proteins

Introduction

Ecosystem impairment in many cases is caused by exposure of organisms to multiple exogenous and endogenous stress parameters such as sublethal levels of chemical mixtures, suboptimal temperature, nutrient deficiencies or excesses, parasite infections, or stress related to reproductive activities. For organisms persisting under such conditions, survival strategies have evolved which may differ from one species to another, but which enable organisms to function in the gradient between homeostasis and death (Peakall,

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1994). Thereby, organisms are often capable of tolerating and compensating sublethal stress conditions up to a distinct species-specific degree. For organisms that are already sublethally stressed, however, any additional stressor such as an increase in temperature, additional pollution or even changes in the physicochemical nature of their habitat can result in physiological tolerance limits being exceeded followed by immediate mortality.

Generally, metabolic pathways and reactions that allow organisms to persist under adverse environmental conditions can be used as biomarkers to characterize either the exposure situation itself or effects induced by the stress in exposed organisms (Adams et al., 1989; Depledge and Fossi, 1994; Munkittrick and McCarty, 1995; Adams et al., 2000, 2001). Whereas established water quality criteria assessments are based on the mortality of species under stressed conditions (i.e., bioassay tests), the biomarker approach uses responses of feral organisms living under unfavourable conditions which, therefore, can be regarded as early warning sentinels in ecological risk assessment (Ham et al., 1997; Adams et al., 1999; Schramm et al., 1999; Adams, 2000).

In the present study, biomarker responses were investigated in feral brown trout (*Salmo trutta* f. *fario*) and gammarids (*Gammarus pulex*) sampled from a small stream (Katzenbach) and a tributary stream (Krebsbach). Both of these streams have been categorized as being slightly polluted by nutrients and populated by a large number of noble crayfish (*Astacus astacus*), a rare decapod species in central Europe (Gherardi and Holdich, 1999).

On 9 August 1997, a local newspaper (Schwäbisches Tagblatt, 1997a) reported sudden crayfish mortalities in the Katzenbach, observed for the first time on the 6th of August, 1997. Up to the 9th of August, about 100 crayfish (*Astacus astacus*) had died for unknown reasons, whereas other aquatic species were reported to have been unaffected. By 6 November 1997, more than 4,000 dead crayfish were collected along the Katzenbach/Krebsbach stream system (Schwäbisches Tagblatt, 1997b).

There were two possible reasons for the crayfish mortalities: (1) an outbreak of the crayfish plague (infection with *Aphanomyces astaci*), or (2) a spill of polluted water via a discharge not connected with the sewage plant system of the Katzenbach/Krebsbach region. Although limnochemical and chemical analyses of water sampled several days after the first occurrence of dead crayfish did not show any elevated levels of pollutants, it was obvious that the first dead crayfish were found a little downstream of the putative spill source. In order to provide evidence for the hypothesis of pollutant release, we measured various biomarkers in both feral brown trout and a benthic invertebrate (gammarid), which appeared, at least upon gross observation, to be unaffected by the putative spill. We conducted biomarker analyses in these two species under the premises that certain biological responses should be capable (1) of integrating the effects of exposure to contaminants over time (van Gestel and van Brummelen, 1996) and, (2) to indicate effects of a putative short-term event even after a considerable time span (Adams et al., 1999, 2001; Triebskorn et al., 1997, 2001). As biomarkers of sublethal stress we measured stress proteins in liver and gill, ultrastructure of liver and several blood parameters in brown trout. In addition, stress proteins in total body homogenates of gammarids were also measured. Biomarker responses in organisms collected upstream and downstream of the potential spill were compared at two different times, six days and four weeks after the putative event. In order to characterize the general status of pollution of the test stream, chemical and limnochemical analyses were conducted in parallel to the biological studies.

Methods

Sampling and sampling sites

Biological, limnological and chemical analyses were conducted along a longitudinal transect in the Katzenbach/Krebsbach system, which are two small streams about 20 km Southwest of Tübingen in SW Germany (Fig. 1). The sampling sites were chosen according to the location of potential sources of pollutants. Site 1 was located directly downstream of the putative spill source (identified as a discharge tube not connected to the sewage system), about 1 km from the mouth of the Katzenbach into the Neckar river; site 2 was 200 m upstream of the putative spill source and about 11 km downstream of the sewage plant at Bodelshausen; site 3 was 1 km upstream of the putative spill source and 10 km downstream of the sewage plant; site 4 was 1.5 km upstream of the putative spill source and 9.5 km downstream of the sewage plant; site 5 was about 9 km upstream the

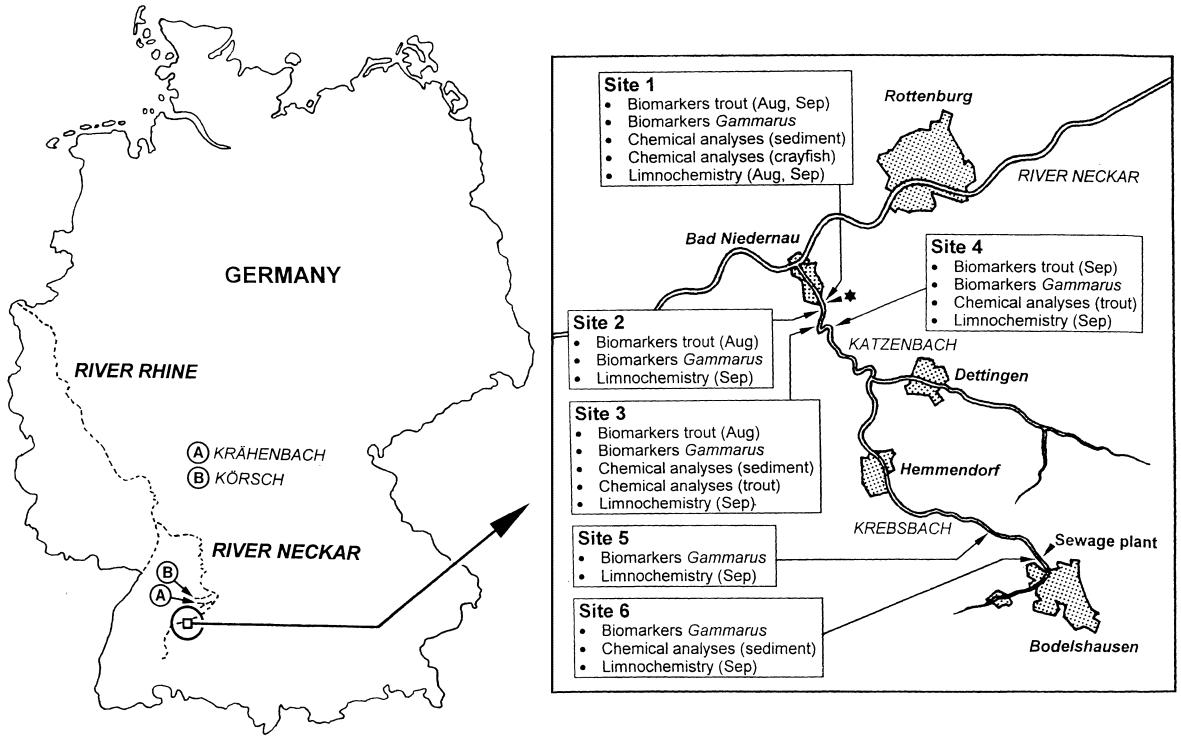

Figure 1. Location of the sampling sites in Southern Germany and investigated parameters. The asterisk indicates the putative spill south of Bad Niedernau.

putative spill source and 2 km downstream the sewage plant Bodelshausen; and site 6 was about 11 km upstream of the putative spill source and 50 m downstream of the sewage plant (Fig. 1).

Test organisms were about eight months old juvenile brown trout (*Salmo trutta* f. *fario*) and adult gammarid amphipods (*Gammarus pulex*). Trout were sampled on 12 August 1997 (sites 1, 2, 3) and on 3 September 1997 (sites 1 and 4). At each of the respective sampling sites, 16 fish of about same size were captured. Tissues of six specimens from each site were fixed in 1.5% gluardialdehyde for electron microscopy and 4% buffered formaldehyde for light microscopy. From the remaining 10 fish, blood samples were taken and tissues subsequently frozen in liquid nitrogen for protein analyses. In addition, four fish were caught for chemical analyses from sites 3 and 4. Gammarid amphipods were collected at sites 1–6 on 3 September 1997, and 12 specimens at each site were directly frozen in liquid nitrogen for stress protein analyses.

Chemical analyses were performed on (1) single sediment samples collected directly from the stream bed on 3 September 1997, at sites 1, 3 and 6, and (2) in trout caught from sites 3 and 4, and (3) not decomposed dead crayfish (cuticle and the rest of the body separately) collected at site 1 downstream of the putative spill source. Chemical analyses in trout (whole fish) and crayfish (cuticle, soft body) were conducted in pooled samples of four specimens each. Water samples for limnological analyses were taken on 12 August 1997 (site 1 only) and 3 September 1997 (sites 1–6).

Due to (1) the urgency to take samples as soon as possible after the first occurrence of dead crayfish, (2) limitations in trout available for investigations and (3) restrictions in analytical capacities, not all parameters could be measured at all sampling sites (see Fig. 1).

Chemical analyses

Sediment (particle size < 1 mm) and tissue samples were analyzed for pesticides, chlorobenzenes, PCBs (congeners 28, 52, 101, 138, 153, and 180) and PAHs by gas chromatography-coupled mass spectrometry

(GC-MS) as described in detail by Honnen et al. (2001). The samples were dried for 12 h at 40 °C, sieved (for sediment samples only) and the organic xenobiotics extracted with acetone in a Soxhlet extractor for 2.5 h. The solutions were cleaned with florisil and concentrated to a total volume of 1 ml prior to GC-MS analyses. In addition, the cadmium and lead concentrations in cuticle and the rest of the body of crayfish were quantified by graphite furnace atomic absorption spectrophotometry after tissue digestion in nitric acid.

Limnological analyses

On 12 August 1997, the following parameters were recorded for the stream water at site 1: temperature, pH, conductivity and the concentration of dissolved oxygen. On 3 September 1997, all these parameters plus the biological oxygen demand (BOD_5) and the concentrations of orthophosphate, ammonium, ammonia, nitrite, nitrate and chloride were determined at sites 1–6. Informations collected at this date were compared with a five year data set obtained for one minor polluted stream (Krähenbach) and one highly polluted stream (Körsch) impacted by sewage plant discharges and agricultural runoff. Both streams are located in the vicinity of the Katzenbach (Fig. 1) (Adam et al., 2001). A detailed description of these two streams is provided by Triebskorn et al. (2000).

Sampling, anaesthetization, and dissection of fish

Fish were captured along the Katzenbach/Krebsbach stream system by electrofishing at the sampling sites shown in Fig. 1. Fish were processed and dissected directly in the field. Prior to dissection, they were anaesthetized with 0.05% ethyl-4-aminobenzoate (benzocaine) for 1 min.

Stress protein analyses

After dissection, gills and liver samples were immediately frozen in liquid nitrogen. The gammarids were frozen and analyzed individually. Samples were homogenized in a buffer containing 250 mM sucrose, 5 mM MOPS (pH 7.4), 1 mM EDTA, 0.1% Ethanol, 200 mM phenylmethylsulfonyl fluoride (PMSF) in iso-propanol, 1 mM ε-amino-*n*-caproic acid, 0.2 mM mercaptoethanol and 0.02 mM dithiotreitol and analyzed by a standardized Western blotting technique and subsequent image analyses. Total protein concentration in the supernatant was determined according to the method of Bradford (1976). Constant protein weights (30 µg of total protein/lane for the fish tissues, 10 µg for *Gammarus*) were analyzed by minigel SDS-PAGE (12% acrylamide, 0.12% bisacrylamide (w/v), 15 min at 80 V, 90 min at 120 V). Protein was transferred to nitrocellulose by semi-dry blotting and the filter blocked for 2 h in 50% horse serum in TBS (50 mM Tris pH 5.7, 150 mM NaCl). After washing in TBS, monoclonal antibody (mouse anti-human hsp70; Dianova, FRG, dilution 1 : 5,000 in 10% horse serum/TBS) was added and incubated at room temperature (22 °C) overnight. After repeated washing in TBS for 2 min, the nitrocellulose filter was incubated in secondary antibody goat anti-mouse IgG (H + L) coupled to peroxidase (Dianova, FRG, dilution 1 : 1,000 in 10% horse serum/TBS) at room temperature (22 °C) for 2 h. After subsequent TBS washing, the antibody complex was detected by 1 mM 4-chloro(1)naphthol and 0.015% H_2O_2 in 30 mM Tris pH 8.5 containing 6% methanol. Grey value quantification of the Western blot protein bands took place with a densitometric image analyses system after background subtraction (absolute grey values).

Liver ultrastructure analyses

The anterior portion of the liver was excised immediately after fish had been anaesthetized. It was transferred into fixative (1.5% glutaraldehyde and 1.5% formaldehyde in 0.1 M sodium phosphate buffer (pH 7.6) containing 2.5% polyvinylpyrrolidone (PVP)) and cut into pieces of 1 mm length. After one hour, samples were rinsed three times in cacodylate buffer (0.1 M, pH 7.6). Then they were fixed in 2.5% glutaraldehyde dissolved in 0.1 M sodium cacodylate buffer (pH 7.6) containing 4% PVP and 0.05% calcium chloride for at least 24 h. After rinsing in cacodylate buffer, the tissue pieces were additionally postfixed in 1% osmium ferrocyanide (Karnovsky, 1971) for 1 h at 4 °C. After washing in 0.1 M cacodylate and 0.05 M maleate buffer (pH 5.2), samples were stained *en bloc* with 1% uranyl acetate in maleate buffer for at least 1 h at 4 °C. The specimens were dehydrated in a graded series of ethanol and embedded in Epon medium. Ultrathin sections (50–100 nm) were stained

with alkaline lead citrate (Reynolds, E.S., 1963) for 30 s and examined in a Zeiss CEM 9 electron microscope.

To characterize the integrity of the liver tissue, the ultrastructure of hepatocytes was classified (on the basis of ultrastructural descriptions by Triebskorn et al., 1997; Schramm et al., 1998; Gernhöfer et al., 2001) as belonging to one of the following three categories: (1) reference state, (2) slight deviations from the reference state with some pathologies visible, (3) major changes from the reference state with obvious pathologies and clearly visible damage. We assessed the integrity of compartmentalization, of the endoplasmic reticulum, mitochondria, peroxisomes, lysosomes, lipid and glycogen storage, and the degree of macrophage infiltration in the hepatocytes and.the ultrastructure of bile canaliculi and cell membranes adjacent to the Disse's spaces. The criteria upon which the classification of Ultrastructural symptoms is based have been described in detail by Triebskorn et al. (1997), Schramm et al. (1998), and Gernhöfer et al. (2001). After classification of ultrastructural symptoms, a mean value was calculated from these data for each test individual. Finally, a mean assessment value (MAV; Triebskorn et al., 1997) was calculated from all specimens caught at one site on one date. In addition, we calculated a separate MAV for the degree of liver tissue infiltration by macrophages, a parameter which was also classified into the three categories detailed above.

Histopathology of kidney and gills

Fish kidney and gill tissues were fixed in 4% buffered formalin and processed for paraffin embedding. Sections of 3 µm were cut at and stained with hematoxylin and eosin (H&E). Histopathological alterations were evaluated semi-quantitatively by ranking the severity of lesions (grades 1–3) as described in detail by Schwaiger et al. (1997) and Schwaiger (2001).

Hematological tests

Blood samples (1–2 ml) were taken with heparinized syringes from the caudal vein of trout (12 August 1997: sites 1, 2 and 3; 3 September 1997: sites 1 and 4). Blood smears were stained according to the Pappenheim method (Romeis, 1989) and analyzed for (1) percentage of lymphocytes, band and segmented granulocytes, and monocytes in a differential hematogram ($n = 100$ white blood cells per blood smear), and (2) the apparent erythrocyte 'area' ($2\pi r^2$) and size classes ($n = 50$ erythrocytes per blood smear) using light microscopy (magnification 1,000×).

Statistical parameters

In a first step, data were proved to be not normally distributed by the Shapiro-Wilk W-Test. To test for significance of differences for each biological and chemical variable from each sample site the Kruskal–Wallis test was used followed by a *a posteriori* comparison of means. Levels of significance were set to $p \leq 0.001$ (***, highly significant), $0.001 < p \leq 0.01$ (**, significant), and $0.01 < p \leq 0.05$ (*, slightly significant).

Results

Chemical analyses

Chemical analyses of sediment and biota revealed a moderate pollution of the entire stream system. Most of the chemicals which were analyzed were not detected in the sediment but had accumulated in the biota (*S. trutta*, *A. astacus*). A detailed summary of all chemical analyses is provided in Table 1.

Limnological analyses

Except for ammonium, ammonia and phosphate, the limnological data did not reveal an exceptional exposure situation for the Katzenbach/Krebsbach stream system. The water temperature varied between 14.8 °C (site 1, 12 August) and 18.5 °C (site 6, 3 September) with a trend to higher temperatures upstream. This range of temperatures is usual for Central European streams in summer. Also dissolved oxygen concentrations at sites 1–5 ranged from 94% to 128%, and only at site 6 a rather low oxygen concentration of 71% was recorded. Water pH on 3 September was 7.4 at upstream site 6, 8.7 at site 4, and 7.8 (8.1 on 12 August) at site 1. The water conductivity was rather constant (820–880 µS/cm) with a trend to higher values downstream. Also the nitrite (0.04 mg/l at a maximum), nitrate (29 mg/l at a maximum) and chloride (44 mg/l at a maximum)

Table 1. Concentrations of pesticides, chlorobenzenes, PCBs, PAHs and metals in the sediment and in trout and crayfish tissue samples (in µg/kg dry weight)

	Sediment			Trout		Crayfish, site 1	
Chemical	Site 1	Site 3	Site 6	Site 3	Site 4	Cuticle	Rest of body
Pesticides							
γ-HCH (lindane)	—	—	—	—	8	2	—
Triallat	—	—	—	n.d.	n.d.	n.d.	n.d.
Trifluralin	—	—	—	3	2	n.d.	n.d.
o,p-DDT	—	—	—	1	2	—	—
p,p-DDT	—	—	—	2	—	75	—
o,p-DDD	—	—	—	—	—	—	—
p,p-DDD	—	—	—	1	2	—	—
o,p-DDE	3.3	—	—	n.d.	n.d.	n.d.	n.d.
p,p-DDE	—	—	—	42	63	3	F4
Chlorobenzenes							
Dichlorobenzene	0.4	1	2	1	—	—	3
1,2,4-Trichlorobenzene	4.9	6	6	1	1	1	3
1,2,4,5-Tetrachlorobenzene	—	0.04	0.2	4	4	1	3
Pentachlorobenzene	—	—	0.4	—	0.4	—	1
Hexachlorobenzene	0.2	0.3	1	5	3	—	1
PCBs							
PCB 28	—	—	—	—	4	3	3
PCB 52	—	1	1	3	4	1	2
PCB 101	1	4	3	7	17	1	3
PCB 138	1	5	7	—	114	4	16
PCB 153	1	6	7	52	99	4	16
PCB 180	0.4	4	6	17	50	2	9
Sum of 6 PCBs	3.4	20	24	79	288	15	49
PAHs							
Naphthalene	3	5	10	28	12	7	35
Acenaphthylene	19	20	73	1	2	2	3
Acenaphthene	7	6	29	1	0.4	1	3
Fluorene	23	21	107	—	—	37	40
Phenanthrene	275	31	867	9	7	10	20
Anthracene	54	79	208	2	3	11	21
Fluoranthene	454	497	1,543	n.d.	17	13	27
Pyrene	343	263	1,046	3	2	12	20
Benzo[a]anthracene	179	236	712	—	—	—	6
Chrysene	177	204	616	—	—	2	10
Benzo[b]fluoranthene	107	—	—	—	—	3	5
Benzo[k]fluoranthene	n.d.	n.d.	n.d.	1	—	—	—
Benzo[a]pyrene	138	—	—	—	—	3	6
Indeno[1,2,3-c,d]pyrene	—	—	—	—	—	—	—
Dibenzo[a,h]anthracene	—	—	—	—	—	—	—
Benzo[g,h,i]perylene	—	—	—	—	—	—	—
Sum of 16 PAHs	1,779	1,362	5,211	45	43	101	196
Metals							
Cd	n.d.	n.d.	n.d.	n.d.	n.d.	0.1	0.3
Pb	n.d.	n.d.	n.d.	n.d.	n.d.	0.5	—

—: beyond detection limit (γ-HCH, triallat: 1; trifluralin: 0.2; DDT, DDD, DDE: 0.5; chlorobenzenes: each 0.1; sum of 6 PCBs: 0.1; sum of 16 PAHs: 1 [in µg/kg dry weight]).
n.d.: not determined.

concentrations as well as the BOD_5 (1.9 mg/l at a maximum) demonstrated little variation among the sites and were all comparable to unpolluted streams of the same size. However, elevated concentrations of orthophosphate (between 1.7 mg/l at site 6 and 2.8 mg/l at site 3) ammonium (between 0.1 mg/l at sites 1–5 and 0.2 mg/l at site 6), and ammonia (values ranging from 0.002 mg/l at site 1 to 0.015 mg/l at site 4) in the stream system were indicative of human impact.

Stress proteins

The hsp70 level in the gills of trout sampled at site 1 soon after the putative spill event (12 August) was significantly higher than the stress protein levels in gills of fish sampled from the other sites (2, 3, 4), and also significantly higher than in gills of trout captured at the same site four weeks later (Fig. 2a). Other than this exceptionally high value at site 1, the gill hsp70 levels and variability among individual fish from other sites and sample times were relatively low. Hsp70 levels in the liver significantly differed between first and second sampling at sampling site 1. In contrast to the responses observed in gills, however, hsp70 values in liver tissue were higher at the second sampling period (Fig. 2b) which can be explained by stress protein kinetics (Köhler et al., 2001, see discussion). The gammarid amphipod samples from site 1 had the highest hsp70 level. However, due to inter-individual variability, values were only significantly higher than those at sites 2. Stress protein levels for *Gammarus* at sites 5 and 6 (downstream the sewage treatment plant) approached site 1 values but were highly variable among individuals from the same site (Fig. 3). Only at sites 2 and 3, hsp70 values in gammarids were in the range of values from the moderately polluted stream Krähenbach.

Liver ultrastructure

In comparison to the ultrastructure of unaffected liver cells (Braunbeck and Völkl, 1993; Schramm et al., 1998), hepatocytes of trout caught in the Katzenbach/Krebsbach system were characterized by a poor compartmentalization, low glycogen storage, large amounts of vesiculated and dilated cisternae of the rough endoplasmic reticulum, high numbers of mitochondria and, particularly at site 1 (first

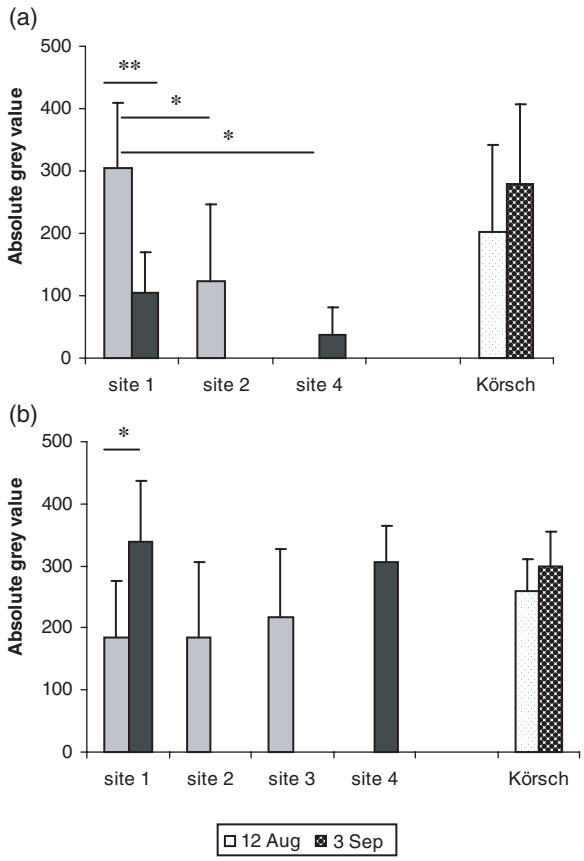

Figure 2. Hsp70 levels in (a) gills, and (b) liver of trout captured at 3 September and 12 August at the Katzenbach/Krebsbach stream system (normalized for 10 μg total protein). Reference values for the highly polluted Körsch are means over five years for July (light) and September/October (dark) (taken from Köhler et al., 2001). Means and standard deviations. Asterisks indicate significant differences at the level of $0.01 < p \leq 0.05$ (∗), and $0.001 < p \leq 0.01$ (∗∗).

sampling), a large number of macrophages. According to the assessment method described by Triebskorn et al. (1997), Schramm et al. (1998), and Gernhöfer et al. (2001) mean assessment values (MAV) between 1.7 ± 0.1 (site 2, 12 August) and 2.0 ± 0.3 (site 3, 3 September) were recorded for the liver cells of trout (Fig. 4a). There were no significant differences of MAVs among sites or between sample periods at the same site. However, when data were compared to the MAVs of brown trout held in unpolluted water under laboratory conditions, the field levels were significantly elevated above lab values and resemble values obtained in trout of the highly polluted Körsch

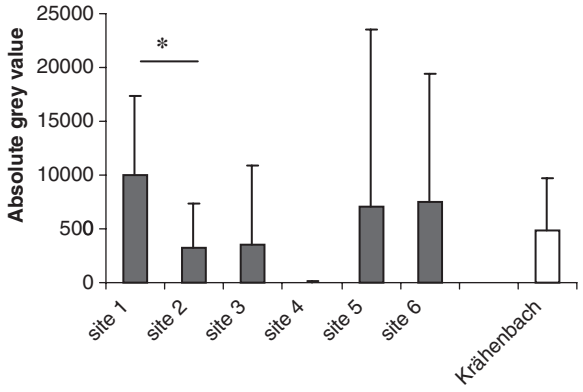

Figure 3. Hsp70 levels in gammarids sampled at the 3 September Katzenbach/Krebsbach stream system. The reference value is a mean for 12 gammarids sampled in parallel at the moderately polluted stream Krähenbach at 3 September. Means and standard deviations. Asterisks indicate significant differences at the level of $0.01 < p \leq 0.05$ (∗).

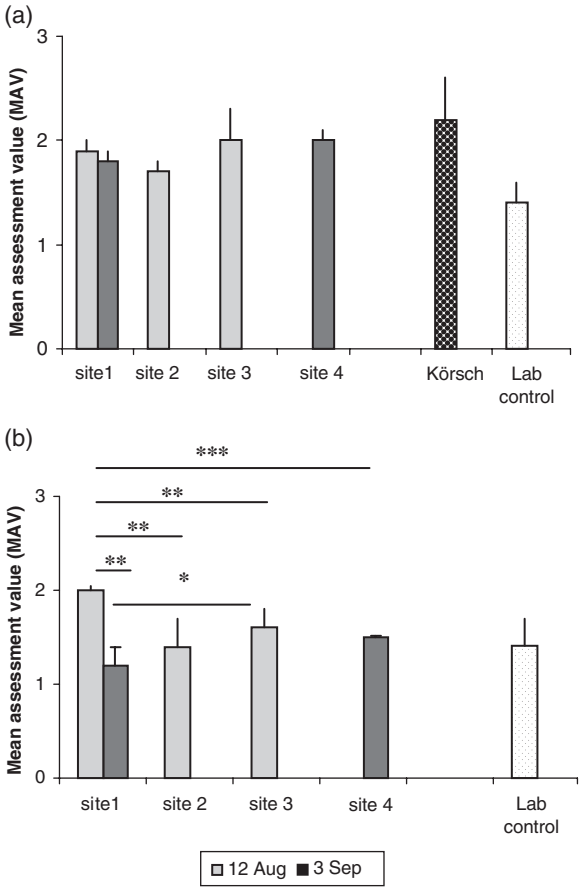

Figure 4. Mean assessment values for (a) ultrastructure of liver tissue and (b) infiltration of liver with macrophages. Reference values for the lab control and the highly polluted Körsch are means over five years (taken from Gernhöfer et al., 2001). Means and standard deviations. Asterisks indicate significant differences at the level of $0.01 < p \leq 0.05$ (∗), $0.001 < p \leq 0.01$ (∗∗), and $p \leq 0.001$ (∗∗∗).

(Fig. 4a) indicating pollution effects along the entire Katzenbach/Krebsbach stream system. When evaluating effects on different organelles, however, the results for the macrophages differed significantly between site 1, 2 and 3 for the first sampling period, with highest values at site 1 (Fig. 4b). Four weeks later, values for site 1 were similar to those obtained for all other sites. These data on liver tissue infiltration by macrophages are higher than values for trout held under laboratory conditions and indicate an exceptional situation for site 1 one week after the first crayfish mortalities.

Histopathology of kidney and gills

The observed histopathological kidney alterations were closely related to the symptoms of proliferative kidney disease (PKD), which occurred in most of the fish to varying degrees. While in some individuals kidney pathologies were restricted to a distinct proliferation of the interrenal tissue (Fig. 5a) other individuals displayed all the morphological characteristics of PKD, such as necrotic changes within the hematopoetic and excretory renal tissue, and the occurrence of PKX cells (Fig. 5b). Individuals from different locations did not differ significantly with regard to the severity of the kidney lesions (Fig. 6). The observed symptoms resembled those obtained in trout exposed to stream water heavily influenced by sewage effluents, and indicate a moderate background pollution of the entire Katzenbach/Krebsbach system. Also in the gills, histopathological alterations were prominent at all sampling sites, including marked hyperplasia of chloride cells along the surface of the secondary lamellae. Furthermore, a focal hyperplasia of interlamellar epithelial cells was evident which, in a few cases, had resulted in a complete fusion of the secondary lamellae. In addition, single cell necrosis of epithelial cells and distinct inflammatory reactions were observed. There were no significant differences between sample sites with regard to the incidence and severity of gill alterations

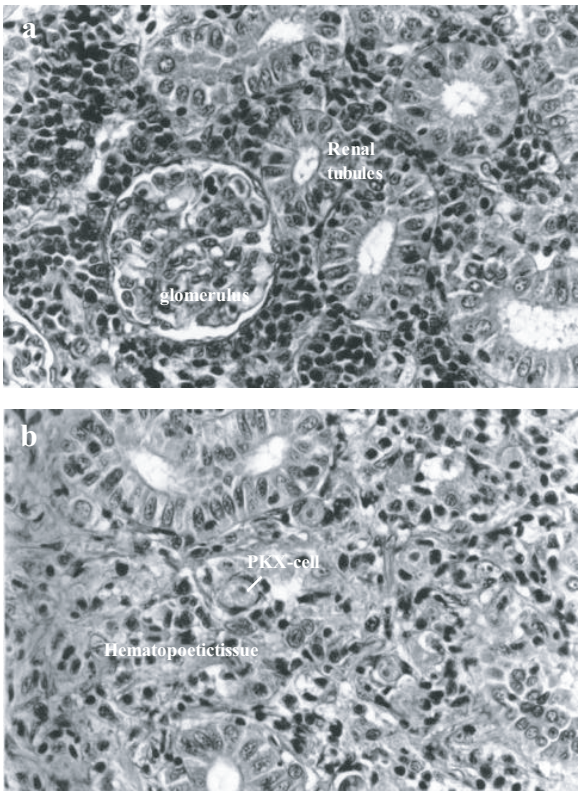

Figure 5. Kidney of brown trout (a) without pathological alterations, and (b) showing all signs of Proliferative Kidney Disease including a loss of kidney tubules, necrotic changes of the hematopoetic tissue and the appearance of PKX-cells. ×1,400.

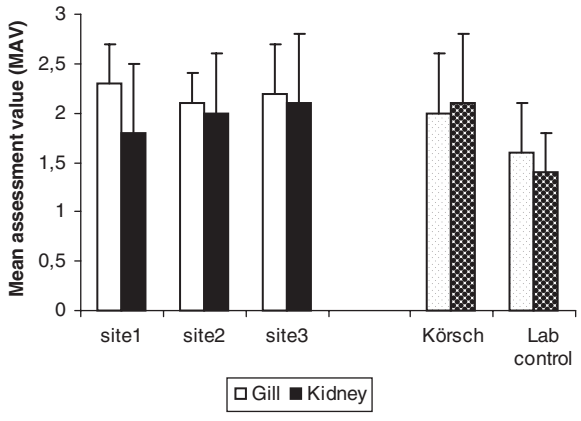

Figure 6. Mean assessment values for histopathological changes in kidney and gills of trout from the Katzuenbach/Krebsbach stream system. Reference values for the lab control and the highly polluted Körsch are means over five years (taken from Schwaiger, 2001).

(Fig. 6), though symptoms were slightly more pronounced at site 1.

Hematology

The differential hematograms did not show significant differences among sample sites for trout caught upstream and downstream of the putative spill source. In all cases, an average of $97.4 \pm 2.6\%$ (downstream, site 1) to $97.5 \pm 0.7\%$ (upstream) of all white blood cells were lymphocytes, $1.9 \pm 1.4\%$ (upstream) to $2.0 \pm 2.2\%$ (downstream) were band granulocytes, and about 0.6% ($\pm 0.8\%$, upstream; $\pm 0.9\%$, downstream) were segmented granulocytes. Monocytes occurred only occasionally. The apparent erythrocyte 'area' also did not differ significantly between the sites but had a maximum ($96.2 \pm 5.1\ \mu m^2$) at site 1 (12 August) and a minimum (87.5 ± 3.5) at site 5.

Discussion

Biomarker science is a relatively new field in environmental research, which is currently enjoying a rapid expansion and development (summarized by Adams et al., 2001). Biomarkers have been successfully used not only as effective tools for monitoring of environmental pollution, but also as most efficient sentinels in early warning in ecological risk assessments (McCarthy and Shugart, 1990; Depledge and Fossi, 1994; Peakall, 1994; Munkittrick and McCarty, 1995; Adams et al., 1999, 2000). Various advantages of the biomarker approach have been identified over the classical methods of hazard assessment (Peakall and Walker, 1994), in particular: (1) A high sensitivity which qualifies them to be used as early warning systems (before populations and communities are severely damaged), and (2) the ultimate capability to integrate effects of animals exposure to contaminants and confounding factors over time (van Gestel and van Brummelen, 1996; Triebskorn et al., 1997, 2001).

In the present study, we used a set of biomarkers with different sensitivities and different temporal integration capacities (Triebskorn et al., 1997, 2001) in order to prove whether crayfish mortality in the Katzenbach/Krebsbach stream system could have been caused by a pollution spill. In the following, advantages and disadvantages of these biomarkers for risk assessment purposes will be discussed.

Biomarkers which did not indicate stress at site 1: Blood parameters in trout

Blood parameters are known to be moderately sensitive biomarkers for acute and strong chronic stress (Buckley, 1977; Jülich, 1979; Schramm, 1998). In the present study, there were no significant differences in the blood parameters of trout among sample sites. The differential haematograms were comparable to those obtained for laboratory-reared brown trout of the same age (Schramm, 1998), whereas the apparent surface area of erythrocytes seemed to be slightly but not significantly reduced compared to reference data given by the same author. A size reduction of erythrocytes was found in rainbow trout after exposure to sublethal doses of different pesticides (Jülich, 1979). Based on the results of the blood parameters alone there was no indication that trout were extremely stressed at the Katzenbach 6 days after the putative spill.

Even though evidence of stress in trout was not indicated by the response of these blood parameters, it cannot be excluded that (1) a moderate background pollution, not detectable by these markers, had previously existed, and (2) effects on blood parameters had already been compensated for and down-regulated by fish 6 days after the putative spill. To further investigate these possibilities, we have examined histological and ultrastructural effects in liver, kidney and gills of trout which are generally regarded as good indicators of sublethal environmental (Braunbeck and Völkl, 1993; Schwaiger et al., 1997; Schramm et al., 1998; Pawert et al., 1998; Schmidt et al., 1999).

Biomarkers which indicated background pollution at all sampling sites: Ultrastructure and histology of liver, gills and kidney, stress protein hsp70 in liver of trout

At all sampling sites, liver ultrastructure in general as well as gill and kidney histopathology of trout indicated an exposure situation comparable to that described by Schwaiger et al. (1997), Triebskorn et al. (1997), Schramm et al. (1998) and Schmidt et al. (1999) for complexly polluted streams heavily influenced by sewage effluents. The level of histopathological damage in response to sewage effluents appears to depend on the quality and dilution of these effluents (Schmidt et al., 1999), whereas symptoms such as chloride cell proliferation in the gills or glycogen reduction in the liver may generally indicate impairment of tissue integrity (Bucher and Hofer, 1993). The occurrence of proliferative kidney disease (PKD) in kidney tissue also corroborates the findings for gill and liver damage, since it is well known that an outbreak of this disease is favored by poor water quality (Hoffmann and Dangschat, 1981; Clifton-Hadley et al., 1984). Each of the histological and ultrastructural biomarkers, however, were not able to distinguish between stress levels of trout at the different sampling sites. Instead, all these biomarkers revealed that trout were exposed to some background pollution in the entire Krebsbach/Katzenbach system. Also the pattern of stress protein hsp70 in liver samples recorded at all sampling sites resembles the situation of fish from the polluted stream Körsch (Köhler et al., 2001).

In contrast to the hsp70 levels in gills which were elevated at the first sampling at site 1, the hepatic stress protein levels did not indicate any exceptional situation at sampling site 1. The transience and the chemical character of the assumed spill may have prevented additional proteotoxic action in the liver but caused a short-term elevation of proteotoxicity in the gills. However, the pattern recorded for hepatic hsp70 (lower levels in August and increased levels in September) not only at site 1, but also, tendentially, at site 4 at the Krebsbach/Katzenbach mirrored the situation of brown trout exposed to water from the polluted stream Körsch (Köhler et al., 2001). In the Körsch, this pattern was interpreted as resulting from an overwhelming of the hsp70 response in the presence of high background pollution specifically in summer. This interpretation is based on the general kinetics of hsp70 induction which follows an optimum curve (Guven et al., 1994; Eckwert et al., 1997; Pyza et al., 1997; Vijayan et al., 1998; Köhler et al., 1999, 2001). With increasing intensity of a stressor (e.g. the concentration of a chemical), the hsp70 level increases at first, reaches a maximum and finally declines beyond a certain threshold limit. In addition, Fader et al. (1994) have shown for a series of fish species including brown trout that even under unpolluted conditions, seasonal variability resulted in highest hepatic hsp70 levels in late spring and summer. They also demonstrated that this seasonality was independent from temperature. Köhler et al. (2001) have shown for brown trout that in spring and summer, additional stressors, such as extreme temperature or pollution, are more likely to induce overwhelming

of the stress response (resulting in low stress protein values) than in fall and winter.

Concomitantly to the data recorded for brown trout in the highly polluted Körsch (Köhler et al., 2001), also trout from the Krebsbach/Katzenbach exhibited higher hepatic hsp70 levels in September than in summer (at both upstream and downstream sites). This effect can be interpreted as a 'recovery' from the summer depression of hsp70 values in an environment with background pollution. However, the significant increase in hepatic hsp70 in September at site 1 may have been additionally triggered by accumulated toxicants possibly introduced into the stream system by the putative spill. As will be explained below, stress responses which require accumulation of toxicants (e.g. in the liver) are temporally delayed in comparison to responses of directly exposed tissues (e.g. gills).

Thus it appears that trout residing in the Krebsbach/Katzenbach system were incurring some level of sublethal stress as indicated by these biomarkers at different levels of biological organization (molecules, cells, tissues).

Biomarkers which indicated a transient, episodic event at sampling site 1

In addition to these findings, significant differences in biomarker responses between the downstream and the upstream sampling sites could be shown for the stress protein level in gills of fish and in gammarids and also for the intensity of infiltration of the liver with macrophages. These two parameters, therefore, might be characterized as sensitive markers for retrospective stress assessment even within a few days following exposure to a pollutant stress.

The magnitude of response of the hsp70 level in gills six days after the putative spill might be due to the high sensitivity of this exposed organ for certain types of environmental pollutants (Pawert et al., 1998). These include predominantly toxicants which affect outer surfaces of animals (e.g. tensides, acids) or particle burdens, but do not accumulate to a great extend in the liver. Thus, the differences in the kinetics of hsp70 induction in the gills and the liver can be explained mechanistically. The hepatic stress response requires accumulation of proteotoxic chemicals in the liver and, therefore, occurs with some temporal delay to defined toxic spills. In contrast, the gill tissue responds immediately even to short-term events due to its close contact with the outer environment. The low stress response in gills at the second sampling 28 days after the putative spill indicates a recovery of this organ and corroborates the hypothesis that a short-term spill event may have occurred on 6 August 1997.

Despite their high variability, also hsp70 responses in gammarids were useful for discrimination between the different sampling sites. This result suggests that biomarker responses in limnic invertebrates should be considered in the design of future research programmes.

Limnochemical and chemical analyses

In addition to the biomarkers measured in this study, limnological and chemical analyses of environmental pollutants also revealed the Katzenbach/Krebsbach stream system to be moderately influenced by environmental contaminants. The concentrations of ammonia, especially at sites 3 and 4, were about 25% of the LC50 (96 h) value for fish, which ranges between 0.05 and 3 mg/l (Schwoerbel et al., 1991), thus probably representing a sublethal stressor for the investigated trout. From site 1 to 6, ammonia concentrations increased and decreased in parallel to increasing and decreasing pH values. This is expected since pH and temperature are parameters responsible for the formation of ammonia from ammonium. Also the phosphate concentrations (2.7 mg/l at a maximum) indicate an insufficient treatment of sewage discharge, which is classified as 'polluted' with phosphate levels between 0.2 and 1.5 mg/l (Klee, 1991).

Available data for pesticides, PCB and PAH in sediment, trout and crayfish also reflect a moderate background of pollution in the Katzenbach/Krebsbach system which is generally comparable to the background pollution in the slightly polluted Krähenbach (Honnen et al., 2001). PAH in sediment were at the highest levels at site 6. These concentrations were about double the concentrations in the Krähenbach and half those in the highly polluted stream Körsch (Honnen et al., 2001). Crayfish at site 1 accumulated four times as much total PAH than trout from sites 3 and 4, and resembled trout from the Körsch stream in this respect. Also Masterson and Bannerman (1994) found elevated PAH levels in crayfish but not in fish of the polluted Lincoln creek, which were in the range of PAH burdens in crayfish from the Katzenbach/Krebsbach. In crayfish

from the Katzenbach/Krebsbach, values for anthracene, chrysene, and fluoranthene are in the range of values found in crayfish of the polluted East Fork Poplar Creek (EFPC) in Oak Ridge, Tennessee (Rao et al., 1996). Values for fluorene and pyrene were higher in crayfish of the Katzenbach/Krebsbach than in crayfish of the EFPC. The PCB congener 138 preferentially accumulated in trout of site 4 and was also present in crayfish. Rao et al. (1996) showed that the mean body burdens for PCBs and pesticides were similar for fish and crayfish from the EFPC. Accumulation of PAHs and inorganics, however, differed between fish and crayfish, whereas body burdens of PAHs in fish were generally higher than in crayfish (except for acenaphthalene and acenaphthene). The accumulation of cadmium and lead in crayfish was very low compared to metal accumulation in trout of the Krähenbach and Körsch.

To summarize, this study has demonstrated that the combined application of biomarkers with different sensitivities in brown trout was a reliable tool not only to detect the effect of background pollution in the Katzenbach/Krebsbach system, but also to provide evidence for a episodic event, presumably a spill of chemicals into the stream system. The first occurrence of dead crayfish in the Katzenbach coincided in time and space with this event. For trout, the biomarker responses can be regarded as early warning signals of impending biological and ecological impairment. Similar biomarker responses were observed in the highly polluted Körsch, where these responses could be correlated with a severe impact on fish reproduction (Luckenbach et al., 2001; Siligato et al., 2001). In the Katzenbach, trout apparently are (still) able to maintain successful reproduction under slightly polluted conditions, even though impacts at the population level cannot be totally excluded. In any case, feral trout populations appear able to compensate for a permanent background as well as transient peaks in pollution. For crayfish, however, this level of pollution apparently caused physiological tolerance limits to be exceeded, resulting in death. Stressed organisms are typically in a weakened status and are therefore more vulnerable to infection and disease (Rice et al., 1996).

In the case of crayfish, the fungal pathogen *A. astaci*, even though not identified by pathologists, is known to have wiped out numerous crayfish populations in Europe since the middle of the 19th century (Söderhäll and Cerenius, 1999). Despite knowledge of the extreme virulence of this fungus for noble crayfish, care should be taken when considering the infection itself to be the only reason for mass mortalities of crayfish, assuming that, independent of any environmental circumstances, the infection of a single *A. astacus* would without exception wipe out the entire population. Infection of *A. astacus* could have occurred via infected alien North American crayfish which are commonly considered to be tolerant vectors of crayfish plague, but, up to 1997, have never been detected in the Katzenbach/Krebsbach. Cerenius et al. (1988) reported on environmental stressors which triggered growth and sporulation of the fungus even in American crayfish. The latter can then even die of crayfish plague via suppression of the immune system (Cerenius et al., 1988; Vogt, 1999). Variation in disease resistance has generally been shown to represent a sensitive indicator for toxic insult (Zelikoff, 1998).

Considering (1) that the risk of European crayfish to become infected by spores released by more tolerant crayfish species can be increased by environmental stressors and (2), that some populations of *A. astacus* were able to withstand an outbreak of crayfish plague for about half a year even though some individuals were infected (Oidtmann et al., 1999), we must conclude that a single causal explanation for mass crayfish deaths underestimates the complexity of the biotic and abiotic interactions regulating epidemiologic interrelationships of parasites and hosts.

Conclusions

Although it is generally difficult to draw any firm conclusion concerning causal relationships in a complex natural system like the Krebsbach/Katzenbach stream system influenced by a series of dependent and/or independent parameters, as e.g. background pollution, episodic events, parasitism, the present study revealed cellular and biochemical biomarkers in fish and gammarids to be useful as tools for retrospective assessment of short-term pollution events which generally cannot be detected by chemical analyses. Biomarker responses provided information on long-term background pollution of the investigated stream system. Although the investigated trout were still able to maintain stable populations in the Katzenbach/Krebsbach stream system histological and ultrastructural effects in different organs of trout and stress protein responses in trout and gammarids

indicated an elevated stress level of these organisms which might result in impaired population parameter in the future, as it has been shown for feral trout of the neighboured Körsch stream (Triebskorn et al., 2001). Moreover, the study provided evidence for crayfish mortality being confounded by background pollution in the entire stream system as well as by an episodic spilling event at sampling site 1.

Acknowledgements

The authors are grateful to Thomas Schlegel and Pia Wohland who assisted in the chemical analyses, and to Thomas Hülsmann for his help in the field. Our thanks also go to the Bad Niedernau Fishery Club and particularly to Ulrich Baderschneider for permission and help with the sampling and for a financial contribution to our expenses. Laboratory facilities were provided by E. Müller, Tübingen. Many thanks are also due to Robert Paxton for the detailed language revision and to S. Marshall Adams for the careful revision of the manuscript.

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