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Laboratory simulation of a mining accident: acute toxicity, hsc/hsp70 response, and recovery from stress in *Gammarus fossarum* (Crustacea, Amphipoda) exposed to a pulse of cadmium

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Abstract

The rate of survival and stress protein (hsc/hsp70) response were investigated in the freshwater amphipod, *Gammarus fossarum* Koch, 1835, during a 20-day stress and recovery experiment. Adult females and males, were separately exposed to 9 different cadmium concentrations for 5 days to simulate a short-term pulse of xenobiotics in an aquatic environment, followed by a recovery period of 15 days. In terms of mortality, females were much more sensitive to cadmium than males; 4.28 ± 2.45 μg Cd^{2+}/l resulted in strong effects on the rate of survival of females but not males. In both sexes, mortality occurred predominantly within the first 5 days of the recovery period. At the cellular level, cadmium induced an hsc/hsp70 response. The lower Cd^{2+} concentrations we used led to an induction of stress proteins while higher Cd^{2+} concentrations resulted in a proportionately reduced hsc/hsp70 response, most likely due to pathological damage. Surviving individuals retained their capacity to induce stress protein production in the recovery period, even if the stress protein response system was overwhelmed by cadmium during the exposure period.

Introduction

The metal cadmium is a class B metal (Nieboer & Richardson 1980) with no known biological function (Hiatt & Huff 1975). In low concentrations, it is common all over the world, and mostly associated with zinc, lead or phosphate. The worldwide production of cadmium is between 12,000 and 15,000 tonnes/year (Marquardt & Schäfer 1997). Cadmium has been used in alloyins, for corrosion protection, as a stabilizer in synthetic materials and batteries, and is still in use in some countries as a component of pigments. The aquatic environment, including vertebrate and invertebrate biota, may be contaminated by cadmium through various human-related sources. When accidents occur in the mining industry, waste-water and sediments containing cadmium are often released in a shock-wave from landfills and secondary containments. Strongly contaminated waterways can result from such accidents, even though the concentration of cadmium decreases gradually with distance from the source. Cadmium can occur in surface waters, and its toxicity then depends on the chemical milieu in which it is found (e.g., Sunda *et al.* 1978; Coombs 1979; Graney *et al.* 1984; Muskó *et al.* 1990). After an accident, the effective retention period at a site contaminated by a single pulse lasts several hours to a few days only. After the pulse, Cd^{2+} residues are frequently hard to measure in the water or in the sediment. Often, no significant toxic potential remains for invertebrates after the initial shock wave. However, organisms that do not die immediately from acute contamination often die several days after exposure (Taylor 1983). The consequences and possible recovery periods for organisms exposed to such metal pulses

therefore need to be determined across long periods of time.

Considerable information on LC_{50} values for Cd^{2+} to different freshwater amphipod species has been published (Rehwoldt *et al.* 1973; Thorp & Lake 1974; Abel & Gardner 1986; Martin & Holdich 1986) but much less information on accumulation strategies after short term contamination or physiological consequences of such a pulse shock (Stuhlbacher & Maltby 1992; Borgmann *et al.* 1993; Stephenson & Turner 1993; Ritterhoff *et al.* 1996). The purpose of this study, therefore, was to assess the consequences of pulse contamination in a model freshwater invertebrate. We chose the amphipod, *Gammarus fossarum* Koch, 1835, a species common to European waters, and focused (i) on mortality following a cadmium pulse. Furthermore, we (ii) monitored the impact of the pulse on the integrity of the stress response system using hsc/hsp70, a predominent stress protein known to be induced by cadmium. We applied the combination of an acute toxicity test with a physiological stress parameter within both a shock phase of severe contamination and a subsequent period under control conditions. We aimed to simulate the situation after a mining accident, with a view to elucidating the temporal aspects of toxic action of a single pulse and the kinetics and degree of subsequent physiological recovery in a model aquatic invertebrate.

Materials and methods

Test animals

The experiments were performed using sexually mature specimens of the freshwater amphipod *Gammarus fossarum* Koch, 1835 in the precopula phase. The source of the animals was the headwaters of the Steinlach, close to the stream's source, located in southern Germany (9°07′33″ W, 48°22′57″ N). Four hundered pairs were randomly collected by hand and the sexes seperated with caution in the laboratory. The total body length (distance between the base of the first antenna to the distal end of the telson) of females was 7 ± 2 mm and of males was 12 ± 2 mm. Animals of each sex were kept in 10 separate 5 litre glass tanks (40 gammarids per tank) filled with the original stream water (photoperiod: 14 h light: 10 h dark) at 7.5 °C for 2 days before the experiments commenced. Animals were fed *ad libitum* during the entire experiment with partially decomposed alder leaves (*Alnus glutinosa*) from the original habitat. To avoid possible disturbance by water aeration (with an air stone) and possible cannibalism, the aquaria were supplied with small pieces of polyethylene gauze as substrate (Borgmann *et al.* 1993).

Experimental design

Nine different experimental Cd^{2+} concentrations were prepared from stream water using a $CdCl_2$ stock solution of 1 g Cd^{2+}/l. Since premliminary studies had shown that, when high quantities of cadmium were introduced, they were from the aqueous phase (probably by absorption to the leaf litter material, substrate, or the tank wall), the following volumes of stock solution were added to aquaria (3.5 l) (Table 1). For each treatment there were two aquaria, one housing females and one males. After 72 h, the cadmium and control solutions in the tanks were replaced by a fresh cadmium solution in order to maintain a constant Cd^{2+} concentration. The cadmium concentrations in the aquerous phase of the experiments A-J following (i) initial contamination and (ii) solution replaced after 72 h were measured by atomic absorption sectrometry (Perkin Elmer AAnalyst 300) and are listed in Table 1. The exposure to cadmium was terminated after 125 h at day 6; subsequently all gammarids were kept in uncontaminated, original stream water, provided with polyethylene gauze and food. At day 10 and 15, the water was changed and all experiments terminated at day 20. During each water change, water samples were analysed for temperature (electronic thermometer integrated in WTW OXI 340), conductivity (electronic conductivity meter WTW LF 330), and pH (electronic pH meter WTW pH 330). In addition, water samples were analyzed for carbonate hardness and total hardness (titration total hardness test and titration carbonate hardness test, all Aquamerck, Darmstadt, Germany).

To study the time course of Cd^{2+} effects on stress protein levels, specimens were randomly sampled (for each exposure and control) from the survivors at day 5, 10, 15 and 20 and frozen immediately in liquid nitrogen; for replicate numbers, see Table 2. Dead animals were removed and survivors counted every 24 h. Survival rates were calculated considering removal of individuals for the stress protein analyses.

Stress protein analysis

The frozen specimens were individually homogenized with an Ultra-Turrax tissue homogenizer for

Table 1. Cadmium concentrations [μg/l] applied in the study: initial Cd^{2+} concentrations (nominal) and real Cd^{2+} concentrations after 48 h, 96 h and after the first recovery period (216 h) in the aqueous phase of the experiments A-J. Data are means $\pm$ standard deviations.

Experiment	Cd^{2+} stock solution volume [μL]	Cd^{2+} concentration initial (nominal)	Cd^{2+} concentration after 48 h	Cd^{2+} concentration after 96 h	after 216 h
A	control	0	0.15 ± 0.07	0.10 ± 0.00	0.87 ± 0.07
B	27	8	3.37 ± 2.88	4.28 ± 2.45	4.08 ± 4.33
C	55	16	5.15 ± 0.07	14.64 ± 6.27	0.46 ± 0.07
D	109	31	16.83 ± 13.42	18.67 ± 3.61	0.97 ± 0.50
E	218	62	0.31 ± 0.14	12.29 ± 3.82	1.02 ± 0.58
F	437	125	1.07 ± 0.07	38.61 ± 18.97	3.01 ± 3.82
G	875	250	3.32 ± 0.22	49.22 ± 10.31	1.07 ± 0.50
H	1750	500	11.58 ± 7.57	93.43 ± 61.31	1.17 ± 1.37
I	3500	1000	11.53 ± 0.58	62.12 ± 18.03	1.02 ± 0.00
J	7000	2000	30.50 ± 3.61	102.82 ± 104.87	0.92 ± 0.29

Table 2. Replicate numbers of females and males used for the stress protein analysis.

Male replicate number [n]	A	B	C	D	E	F	G	H	I	J
day 5	10	11	10	9	10	10	9	10	10	10
day 10	10	8	9	9	9	8	7	9	7	7
day 15	9	10	9	8	10	9	–	–	–	–
day 20	8	10	10	7	11	10	12	–	–	–
Female replicate number [n]	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
day 5	10	10	10	11	10	10	10	10	10	10
day 10	9	7	7	9	7	7	5	1	5	9
day 15	9	5	6	–	–	4	–	–	–	–
day 20	10	6	6	6	8	7	2	–	–	–

5 s in a buffered extraction solution (10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris HCL, 5% SDS, 2% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), pH 7.4, the volume of which was adjusted to the specimen's weight) and the homogenate subsequently centrifuged (10 min, 20000 *g* at 4 °C). The total protein concentration in each supernatant was determined by a protein assay based on the method of Bradford (1976). Constant protein weights of the supernatant (40 μg) were separated by SDS-PAGE (12% acrylamide-bisacrylamide) for 20 min at 80 V and 120 min at 120 V, transferred to a nitrocellulose filter, and the filter blocked for 2 h with 50% horse serum in Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris pH, 7.5). After removal from the solution, the nitrocellulose filter was incubated with a primary antibody against hsc/hsp70 (mouse anti-human, Dianova, Germany, broadly cross-reactive among different invertebrate taxa with all members of the hsc/hsp70 family except for grps) at room temperature overnight. Subsequently, the filter was washed for 5 min with TBS. The secondary antibody (peroxidase-conjugated goat anti-mouse IgG, Dianova, Germany) was added for 2 h at room temperature. After repeated washing for 5 min in TBS, the antibody cross reaction was visualized by 4-chloro(1)naphthol. The grey value intensity of the hsc/hsp70 bands in the immunoblots were quantified by a densitometric image analysis (Herolab E.A.S.Y., Germany). For this system, a standard curve relating the different weights of supernatant hsc/hsp70 and signal density was established by Schill *et al.* (2002). Each gel contained a prestained protein ladder (Bench-

mark, GibcoGRL, Life Technologies, Gaithersburg, MD, USA) and a *G. fossarum* hsp70 standard to minimize methological variability. Additionally, gels were stained with Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL, USA) after the transfer procedure to ensure that complete protein transfer was accomplished.

Statistical significance of differences was tested using the Mann-Whitney U-test.

Results

Water quality parameters

The water quality parameters for each tank and every water change were measured and no significant changes were observed across the course of the experiment. The pH averaged 8.52 ± 0.08 (average ± SD for all experiments); the average value for the conductivity was 467 ± 58 μS/cm and the average temperature was 7.63 ± 0.21 °C. The water had a total hardness of 213.6 mg $CaCO_3$/l and carbonate hardness of 249.2 mg $CaCO_3$/l and was influenced by the bedrock of the water source. Due to the pH, conductivity and total/carbonate water hardness, the water source (Steinlach) can be characterized as a carbonate stream water. The oxygen supply was not measured because we ensured strong artificial ventilation. In addition to a lack of possible anthropogenic contamination of our water source, it can be considered as rather unpolluted due to its location close to the stream's source and the lack of any sewage plant or other industry above the source.

Survival rates

The relationship between exposure/recovery period and the rate of survival of *G. fossarum* is given in Figure 1. The female and male control groups showed a very high rate of survival during the course of the experiment. At Cd^{2+} concentrations from experiment B up to F, there was no mortality in either sex at day 5 (Table 3). A strong decrease in survival, even within the first 5 days, occurred in the female gammarids exposed to concentrations higher than in experiment G. In male animals, the first effects on the rate of survival occured at a concentration from experiment H at day 5, and increased, in contrast to the females, only slightly with increasing Cd^{2+} concentrations. The rate of survival in females started to decrease rapidly after the first 5 days of the experiment, even though the cadmium stressor was removed after day 5. Up to experiment G, the survival curve of males was similar to that of the control group except for onset D which resulted in an unexpectedly high Cd^{2+} concentration in the water after initial contamination. In experiment H, however, the rate of survival after 20 days was about one third that of the control group. At higher concentrations, it was not possible to record a rate of survival for day 20 since survivors had all been used for hsc/hsp70 analysis. In general, females were more sensitive to Cd^{2+} than males, except at the highest concentrations given. Considering temporal aspects, the steepest drop in the rate of survival was between day 5 and day 15, after the stressor had been removed.

Stress protein (hsc/hsp70) reactions

For methodological reasons, data on hsc/hsp70 could only be generated for surviving specimens. Animals taken at day 5, at the end of Cd^{2+} exposure, showed a trend toward decreasing stress protein (hsc/hsp70) levels with increasing, and probably pathological, Cd^{2+} concentrations (Figures 2a, 3a) in both sexes. Only in females did the lowest Cd^{2+} concentration (measured at day 4) result in a tendentially elevated hsc/hsp70 mean, compared to the control. After a 'recovery' period of 5 days (at day 10), the maximum hsc/hsp70 means of surviving males and females was recorded for onset G, but animals which had been exposed to higher Cd^{2+} concentrations still exhibited a rather low stress protein level (Figures 2b, 3b). These levels measured at day 10 for onset G animals were the highest recorded across the entire experiment. Amphipods which recovered for 10 days in cadmium-free water showed similar hsc/hsp70 levels independent of the preceding Cd^{2+} treatment, and both sexes were similar in this regard (Figures 2c, 3c). Amphipods sampled at day 20 had similarly low hsc/hsp70 levels (Figures 2d, 3d). All hsc/hsp70 levels recorded for treatment groups at days 15 and 20 were not significantly different from the control gammarids.

Discussion

In this study, laboratory tests were used to assess the mortality and the stress protein (hsc/hsp70) response of *G. fossarum* exposed to a short-term pulse of contamination comprising different concentrations of cadmium. The adverse effect of the cadmium pulse was sufficient to result in an ongoing mortality, even

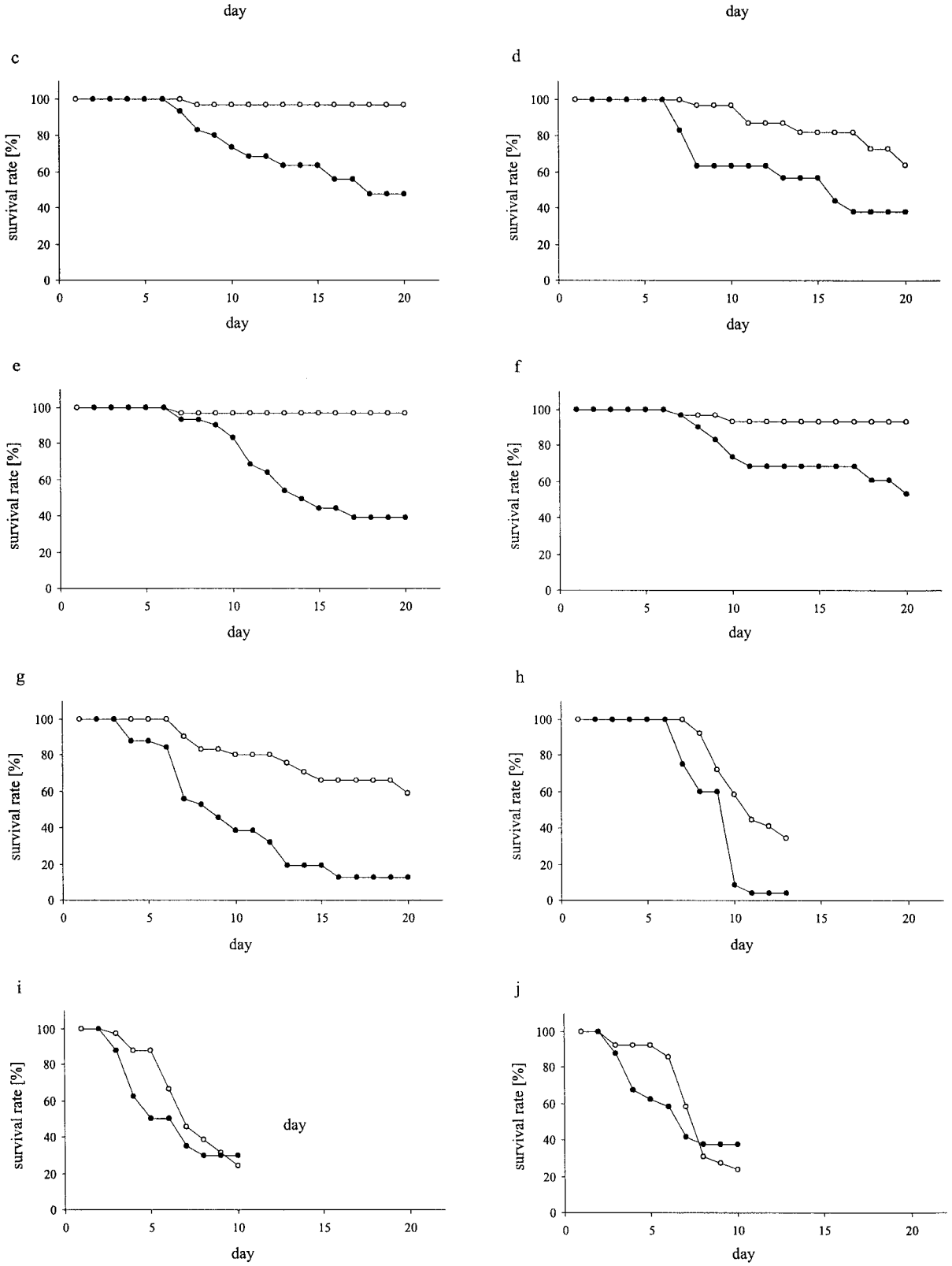

Fig. 1. Suvival rate [%] of female (black dots) and male (circles) *G. fossarum* in experiments A (a, control), B (b), C (c), D (d), E (e), F (f), G (g), H (h), I (i) and J (j).

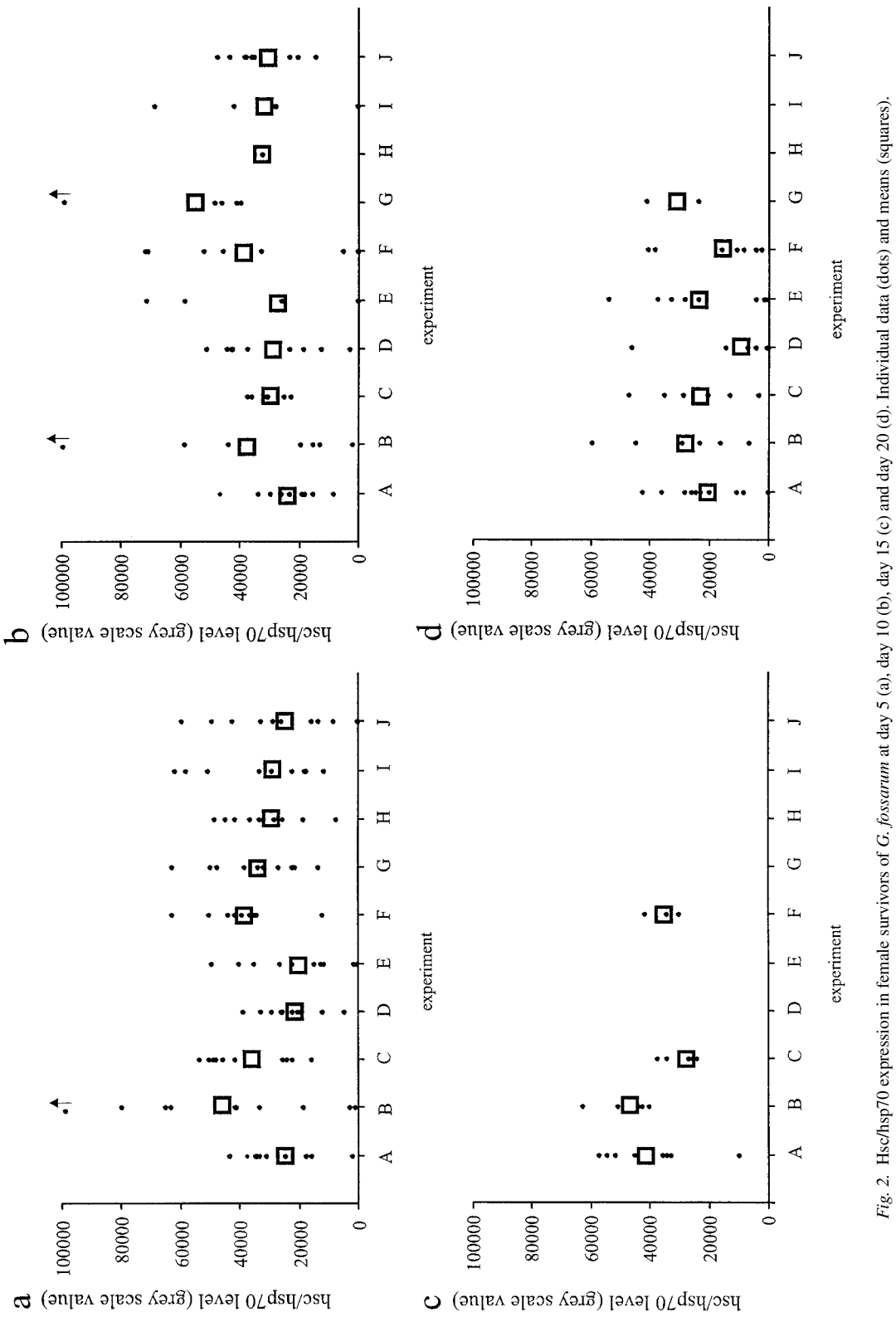

Fig. 2. Hsc/hsp70 expression in female survivors of *G. fossarum* at day 5 (a), day 10 (b), day 15 (c) and day 20 (d). Individual data (dots) and means (squares).

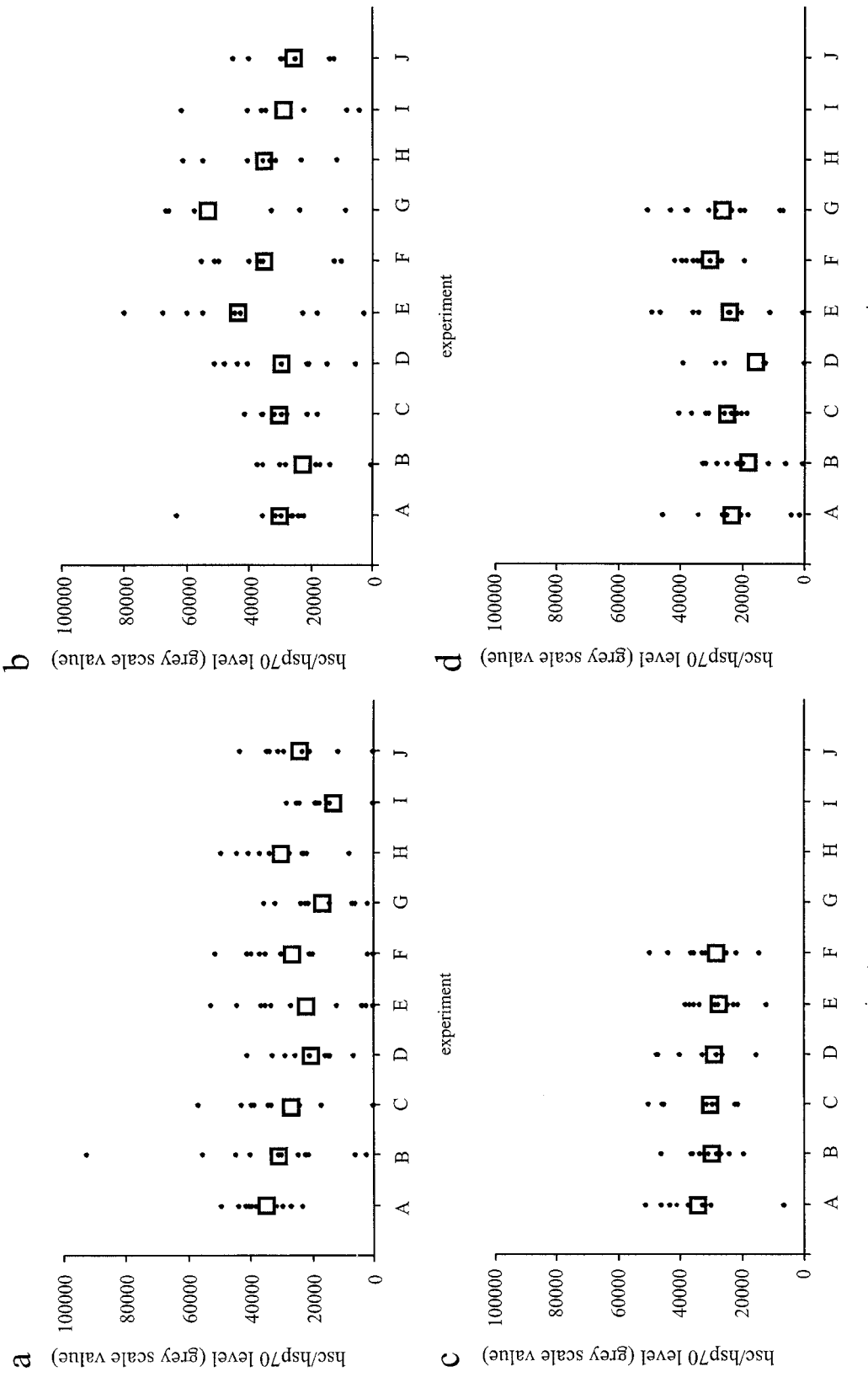

Fig. 3. Hsc/hsp70 expression in male survivors of *G. fossarum* at day 5 (a), day 10 (b), day 15 (c) and day 20 (d). Individual data (dots) and means (squares).

Table 3. Rate of survival [%] of female and male *G. fossarum* at day 5 (a), day 10 (b), day 15 (c) and day 20 (d).

Male survival rate [%]	A	B	C	D	E	F	G	H	I	J
day 5	100.00	100.00	100.00	100.00	100.00	100.00	100.00	92.50	87.50	92.50
day 10	100.00	93.33	96.67	96.67	96.67	93.33	80.00	34.26	24.50	23.98
day 15	100.00	93.33	96.67	82.17	96.67	93.33	65.88	n. d.	n. d.	n. d.
day 20	90.00	93.33	96.67	63.91	96.67	93.33	58.56	n. d.	n. d.	n. d.
Female survival rate [%]	A	B	C	D	E	F	G	H	I	J
day 5	100.00	100.00	100.00	100.00	100.00	100.00	87.50	60.00	50.00	62.50
day 10	90.00	76.67	73.33	63.33	83.33	73.33	38.50	4.29	30.00	37.50
day 15	90.00	62.29	63.56	57.00	44.12	68.44	19.25	n. d.	n. d.	n. d.
day 20	90.00	46.72	47.67	38.00	39.22	53.23	12.83	n. d.	n. d.	n. d.

n. d.: not determined

in the phase of zero stress when the cadmium was removed. In particular, female gammarids showed a drastically increased rate of mortality in comparison to males during cadmium exposure and the 5 days following exposure. Naylor *et al.* (1990) found that juveniles of *G. pulex* were the life stages most sensitive to acidity and zinc. Larger males and brooding females were least sensitive. The tolerance of brooding females surprized Naylor *et al.* (1990) because reproduction should already impose a stress that might be expected to weaken tolerance to toxins. However, they suggested that the high resistance could, at least in part, be due to the fact that females do not moult when brooding, and moulting has been shown to increase susceptibiliy to some toxins (McCahon & Pascoe, 1988). For this short-term pulse and regeneration experiment, female and male gammarids in the precopula phase were used. In general, this phase of the reproductive period may be highly relevant for studies using crustaceans as bioindicators. The disruption of precopula pairs is frequently used as a toxicological endpoint and offers the opportunity to use reproductive traits as indicators of environmental quality or integrity (Rinderhagen *et al.* 2000). Adult females of *G. fossarum* are thought to moult approximately six to ten times, usually releasing a brood at each moult. The numer of moults in adult males is not known (Pöckel 1992). Investigations of Pöckel (1993) showed the reproduction sequence of *G. fossarum* at 10 °C: precopula phase – moulting of the female – fertilisation of the eggs – development and post-hatch time (44 days). After 44 days, the reproductive period started anew. It is possible that, in our case, the females we used were still stressed and weakened through their preceding reproductive effort or their energy allocation into egg development. But moulting and an increased susceptibiliy to toxins could not have been the reason for the higher mortality in our study since animals were used during the precopula phase before female moult.

Direct uptake from the water phase is the dominant pathway of Cd^{2+} ingestion in *Asellus aquaticus* (van Hattum *et al.* 1993) and, supposedly, is equally important in *G. fossarum*. Many investigations (e.g., Maltby 1995) observed that ventilation rates in gammarids are approximately twice that of asellids. This may result in higher rates of metal transfer across the gill membrane and a faster rate of toxic metal bioaccumulation in gammarids over asellids. Assuming ingestion of food to be a potentially important pathway of Cd^{2+} uptake, gammarids as well as isopods (Eckwert & Köhler 1997) should be sensitive enough to detect the unpalatability of contaminated food and stop feeding. *G. fossarum* is able to survive periods of starvation of about three weeks (Hervant *et al.* 1997), but was shown to respond to food deprivation with a marked and transitory hyperactivity. In natural conditions, this probably corresponds to an attempt to move to a better habitat at the beginning of the period of starvation. Such a transitory hyperactivity, however, may also lead to increased metabolic rate and result in a higher metal uptake via the gills.

The effect of Cd^{2+} on aquatic organisms in general depends on both its biological availability and the physiological status of the organism. Cd^{2+} occurs in ionic form at a low pH (Hem 1972) and in compounds in an alkaline medium (Coombs 1979).

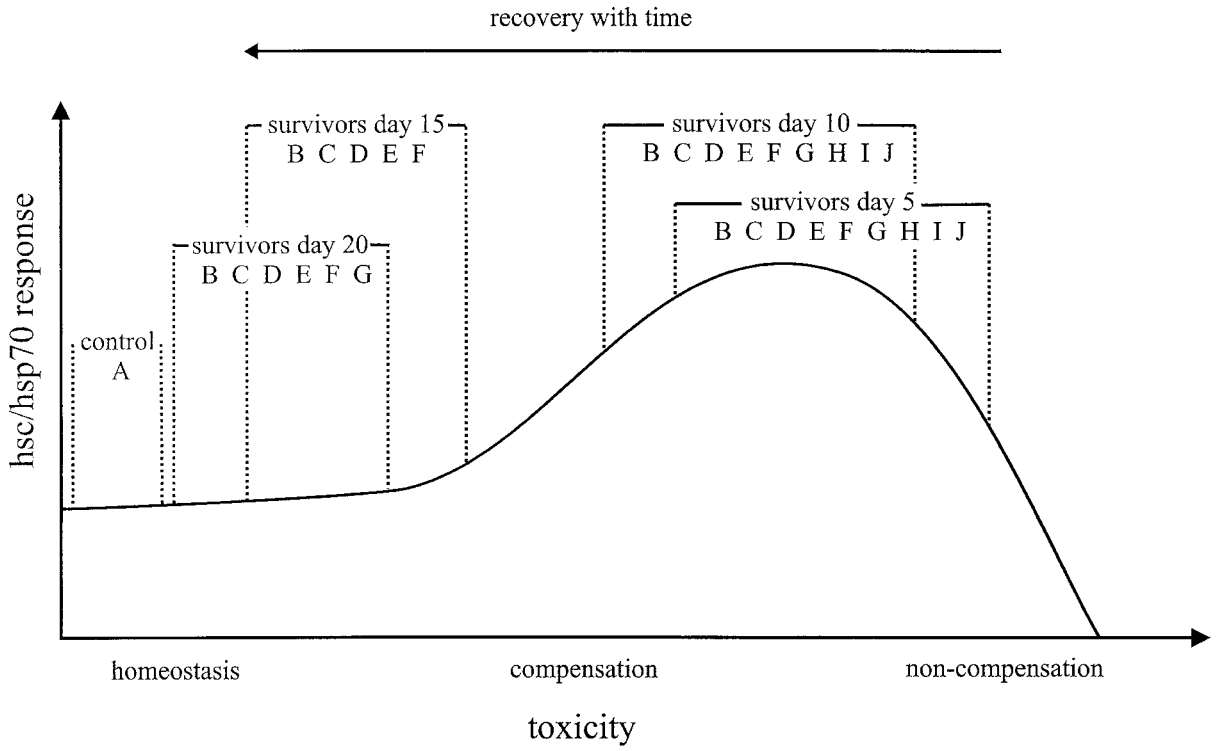

Fig. 4. Hsc/hsp 70 induction kinetics according to Eckwert *et al.* (1997) and interpretation of the data recorded for surviving specimens in this study. Explanation in the text.

At rather low Cd^{2+} concentrations (10–50 μg/l), a more acid medium (pH 4.5) was described to be more toxic than a less acidic one (pH 6.0) (Muskó *et al.* 1990). In contrast, at higher Cd^{2+} concentrations, the situation was the opposite. Our animals were very sensitive to Cd^{2+} at pH 8.5, especially during the first days of exposure. Increased mucus secretion on the surface of the respiratory organ at low pH can be responsible for reduction of the intensity of respiration and metabolism and, consequently, for the reduction of metal toxicity under acidic conditions (Whitley & Sikora 1970). Furthermore, water temperature seems to be crucial for acute toxicity. In acute toxicity tests, Muskó *et al.* (1990) determined LC_{50} values of 6.2 μg Cd^{2+}/l (96 h), 15 μg Cd^{2+}/l (48 h) and 82 μg Cd^{2+}/l (24 h) for *G. fossarum* at a temperature of 16 °C, a pH of 8.5 and water hardness of 173 mg $CaCO_3$/l. The pH measured in our experiment averaged 8.52 ± 0.08 and, consequently, was disadvantageous for survival in a cadmium solution. But it seemed that the much lower temperature we used assisted the survival of the gammarids for a longer time in spite of their exposure to higher cadmium concentrations than those given by Muskó *et al.* (1990).

In general, *G. fossarum* has proved to be more sensitive to changes in its environment than other gammarids, both in laboratory experiments (Brehm & Meijering 1982) and in field studies (Meijering & Pieper 1982). The sensitivity of *G. fossarum* is reflected also in the hsc/hsp70 stress response being overwhelmed by most of the tested Cd^{2+} concentrations, already after 5 days of exposre. Despite the high variability we observed among individuals, the results for hsc/hsp70 levels followed clear trends. An obvious reason for inter-individual difference in stress response is genetic variation between individuals; other factors may be of phenotypic character, such as health status and age.

In most organisms, hsp70 is among the most prominent proteins induced by heat, and early experiments have shown a close relation between the induction of these proteins and the induction of tolerance (e.g., Li *et al.* 1982). This classical 'heat shock response' has important roles to play in the everyday life of crustaceans exposed to variable temperature (McLennan & Miller 1990). In recent years,

there has been a growing interest in tracing contaminant effects using the hsp70 level in crustaceans such as isopods (Eckwert & Köhler 1997; Köhler *et al.* 2000; Arts *et al.* 2002) and amphipods (RO Schill & H-R Köhler, unpublished). Eckwert *et al.* (1997) found that, in response to increasing metal concentrations, the hsp70 level in the woodlouse, *Oniscus asellus*, increased to a 'maximum' of induction and declined at high metal concentrations, which was most probably due to a pathological impact on the protein synthesis machinery. This effect has been reported for other taxa, from nematodes (Guven *et al.* 1994) to vertebrates (Köhler *et al.* 2001), and seems to be a ubiquitous phenomenon when stress effects become too severe. According to Eckwert *et al.* (1997), three phases of the response curve, corresponding to homeostasis (unstressed), compensation (stressed but actively responding with stress protein induction) and non-compensation (severely stressed, increasingly pathologically damaged) can be distinguished. Figure 4 displays the hsp70 response curve on the basis of the findings of Eckwert *et al.* (1997) and Köhler *et al.* (2001). The level of stress proteins we recorded for *G. fossarum* at days 5, 10, 15 and 20 can be given the following interpretation:

Concomitant with the high mortality following Cd^{2+} exposure, the low hsc/hsp70 levels in response to the higher cadmium concentrations at day 5 most likely reflect a stress response system which is overwhelmed and, consequently, the data should be interpreted as belonging to the 'non-compensation phase' of the hsp70 kinetic curve. This interpretation is strengthened by the fact that, after 5 days of exposure, the lowest Cd^{2+} concentrations resulted in a rather high level (stress response, still 'intact') whereas higher Cd^{2+} concentrations lowered the stress response in both sexes of *G. fossarum*. The maximum hsc/hsp70 response at day 10 (after 5 days of recovery) was now found amongst animals exposed to a higher Cd^{2+} concentration. According to the model of Eckwert *et al.* (1997), this could be explained by a re-constitution of the stress response in those gammarids which had survived and recovered from high Cd^{2+} concentrations, and an attempt towards homeostasis in those animals which had been exposed to low Cd^{2+} levels only. Surviving *G. fossarum* analysed at day 15 or 20 showed a low hsc/hsp70 level similar to the control animals. This effect is most likely to be interpreted as a further stabilisation of the stress response system (homeostasis) and, furthermore, reflects the observation of a cessation in mortality from day 15 onwards.

Even though mortality was high (particularly in the females) during the first days after the Cd^{2+} 'shock-wave', and even though the capacity of the survivors to respond to stress was shown to be overwhelmed by the exposure, survivors were able to recover from the Cd^{2+} pulse within 10 days. In the field, such survivors are suspected to re-establish new gammarid populations after a mining accident.

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References

Abel PD, Gardner SM. 1986 Comparisons of median survival times and median lethal exposure time for *Gammarus pulex* exposed to cadmium, permethrin and cyanide. *Water Res* **20**, 579–582.

Arts MSJ, Schill RO, Knigge T, Eckwert H, Kammenga JE, Köhler H-R. 2002 Stress proteins (hsp70, hsp60) induced in isopods and nematods by field exposure to a gradient near Avonmouth, UK. *Ecotoxicology* (in press).

Borgmann U, Norwood WP, Clarke C. 1993 Accumulation, regulation and toxicity of copper, zinc, lead and mercury in *Hyalella azteca. Hydrobiologia* **259**, 79–89.

Bradford MM. 1976 A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt Biochem* **72**, 248–254.

Coombs TL. 1979 Cadmium in aquatic organisms. In: Webb M, ed. *The Chemistry, Biochemistry and Biology of Cadmium.* North-Holland, New York: Elsevier; 93–139.

Eckwert H, Köhler H-R. 1997 The indicative value of the hsp70 stress response as a marker for metal effects in *Oniscus asellus* (Isopoda) field populations: variability between populations from metal-polluted and uncontaminated sites. *Appl Soil Ecol* **6**, 275–282.

Eckwert H, Alberti G, Köhler H-R. 1997 The induction of stress proteins (hsp) in *Oniscus asellus* (Isopoda) as a molecular marker of mutiple heavy metal exposure: I. Principles and toxicological assessment. *Ecotoxicology* **6**, 249–262.

Graney RL Jr, Cherry DS, Cairns J Jr. 1984 The influence of substrate, pH, diet and temperature upon cadmium accumulation in the asiatic clam (*Corbicula fluminea*) in laboratory artificial streams. *Water Res* **18**, 833–842.

Guven K, Duce JA, de Pomerai DI. 1994 Evaluation of a stress-inducible transgenic nematode strain for rapid aquatic toxicity testing. *Aquat Toxicol* **29**, 119–137.

Hem JD. 1972 Chemistry and occurrence of cadmium and zinc in surfacewater and groundwater. *Water Resour Res* **8**, 661–679.

Hervant F, Mathieu J, Barré H, Simon K, Pinon C. 1997 Comparative study on the behavioral, ventilatory, and respiratory response

of hypogean and epigean crustaceans to long-term starvation and subsequent feeding. *Comp Biochem Physiol* **118 A**, 1277–1283.

Hiatt V, Huff JE. 1975 The environmental impact of cadmium: an overview. *Internl J Environ Studies* **7**, 277–285.

Köhler H-R, Eckwert H. 1997 The induction of stress proteins (hsp) in *Oniscus asellus* (Isopoda) as a molecular marker of multiple heavy metal exposure. II: Joint toxicity and transfer to field situations. *Ecotoxicology* **6**, 263–274.

Köhler H-R, Zanger M, Eckwert H, Einfeldt I. 2000 Selection favours low hsp70 levels in chronically metal-stressed soil arthropods. *J Evol Biol* **13**, 569–582.

Köhler H-R, Bartussek C, Eckwert H, Farian K, Gränzer S, Knigge T, Kunz N. 2001 The hepatic stress protein (hsp70) response to interacting abiotic parameters in fish exposed to various levels of pollution. *J Aquat Ecosyst Stress Recov* **8**, 241–260.

Li GC, Werb Z. 1982 Correlation between synthesis of heat shock proteins and developement of thermotolerance in Chinese hamster fibroblasts. *Proc Natl Acad Sci USA* **79**, 3218–3222.

Maltby L. 1995 Sensitivity of crustaceans *Gammarus pulex* (l.) and *Asellus aquaticus* (L.) to short term exposure to hypoxia and unionized ammonia: observations and possible mechanisms. *Water Res* **29**, 781–788.

Marquardt H, Schäfer SG. 1997 Lehrbuch der Toxikologie, Heidelberg, Berlin: Spektrum Akademischer Verlag.

Martin TR, Holdich DM. 1986 The acute lethal toxicity of heavy metals to peracarid crustaceans (with particular references to freshwater asellids and gammarids). *Water Res* **20**, 1137–1147.

McCahon CP, Pascoe D. 1988 Cadmium toxicity to the freshwater amphipod *Gammarus pulex* (L.) during the moult cycle. *Freshwat Biol* **19**, 197–203.

McLennan AG, Miller D. 1990 A biological role for the heat shock response in crustaceans. *J Therm Biol* **15**, 61–66.

Muskó IB, Meinel W, Krause R, Barlas M. 1990 The impact of Cd and different pH on the amphipod *Gammarus fossarum* Koch (Crustacea: Amphipoda). *Comp Biochem Physiol* **96C**, 11–16.

Naylor N, Pindar L, Calow P. 1990 Inter- and intraspecific variation in sensitivity to toxins; The effects of acidity and zinc on the freshwater crustaceans *Asellus aquaticus* (L.) and *Gammarus pulex* (L.). *Water Res* **24**, 757–762.

Nieboer E, Richardson DHS. 1980 The replacement of the nondescript term 'heavy metals' by a biologically and chemically significant classification of metal ions. *Environ Pollut* **1B**, 3–26.

Pöckel M. 1992 Effects of temperature, age and body size on moulting and growth in the freshwater amphipods *Gammarus fossarum* and *G. roeseli. Freshwat Biol* **27**, 211–225.

Pöckel M. 1993 Beiträge zur Ökologie des Bachflohkrebses (*Gammarus fossarum*) und Flussflohkrebses (*Gammarus roeseli*). *Natur und Museum* **123**, 114–124.

Rehwoldt R, Laski L, Shaw C, Wirkowski E. 1973 The acute toxicity of some heavy metal ions towards benthic organisms. *Bull Environ Contam Toxicol* **10**, 291–294.

Rinderhagen M, Ritterhoff J, Zauke G-P. 2000 Biomonitoring of Polluted Waters – Reviews on Actual Topics. *Environ Res Forum* **9**, 161–194.

Ritterhoff J, Zauke G-P, Dallinger R. 1996 Calibration of the estuarine amphipods, *Gammarus zaddachi* Sexton (1912), as biomonitors: toxicokinetics of cadmium and possible role of inducible metal-binding proteins in Cd detoxification. *Aquat Toxicol* **34**, 351–369.

Schill RO, Gayle PMH, Köhler H-R. 2002 Daily stress protein (hsp70) cycle in chitons (*Acanthopleura granulata* Gmelin, 1791) which inhabit the rocky intertidal shoreline in a tropical ecosystem. *Comp Biochem Physiol* **131C**, 253–258.

Stephenson M, Turner MA. 1993 A field study of cadmium dynamics in *Hyalella azteca* (Crustacea. Amphipoda). *Water Air Soil Poll* **68**, 341–361.

Stuhlbacher A, Maltby L. 1992 Cadmium Resistance in *Gammarus pulex* (L.). *Arch Environ Contam Toxicol* **22**, 319–324.

Sunda WG, Engel DW, Thuotte RM. 1978 Effect of chemical speciation on toxicity of cadmium to grass shrimp, *Palaemonetes pugio*: Importance of free cadmium ion. *Environ Sci Techn* **12**, 409–413.

Taylor D. 1983 The significance of the accumulation of cadmium by aquatic organisms. *Ecotoxicol Environ Saf* **7**, 33–42.

Thorp VJ, Lake PS. 1974 Toxicity bioassays of cadmium on selected freshwater invertebrates and the interaction of cadmium and zinc on freshwater shrimp, *Paratya tasmaniensis* Rick. *Aust J Mar Freshwater Res* **25**, 97–104.

van Hattum B, Korthals G, van Straalen NM, Govers HAJ, Joosse ENG. 1993 Accumulation patterns of trace metals in freshwater isopods in sediment bioassays – influence of substrate characterisitcs, temperature and pH. *Water Res* **27**, 669–684.

Whitley LS, Sikora RA. 1970 the effects of three common pollutants on the respiration rate of tubificid worms. *J Wat Poll Control Fed* **42**, 57–66.