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Effects of Acid Mine Drainage and Acidity on the Activity of *Choroterpes picteti* (Ephemeroptera: Leptophlebiidae)

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Abstract. Survival and behavior of the mayfly *Choroterpes picteti* (Leptophlebiidae) exposed to acid mine drainage (AMD: pH 3.3–6.4) and a reservoir polluted with arsenic (pH 6.8) from São Domingos mine (Portugal) were studied in laboratory and *in situ* bioassays (48 h) with the Multispecies Freshwater Biomonitor, and compared with water from a reference river and acidified reference water (acid only). Metal body-burdens showed a negative pH dependency for Mn and As, a positive one for Pb, and for Zn, Cu, Co, and Cd a decrease at pH < 4.4. Generally, survival decreased with decreasing pH. The 48-h LC_{50} (pH) for AMD and for acid only were similar (pH 4.8–4.9); however, the LT_{20} (h) at pH 3.3 revealed AMD to be less toxic than acid only. *C. picteti* show diurnal rhythm with increased locomotor activity in the night. The circadian rhythm was weakened by acid exposure, but less so by AMD exposure. Compared to reference river water, ventilation was stimulated at pH < 6.0 in acid only and in reservoir water. Locomotion was stimulated at pH 5 in acid only and reservoir; however, it was reduced in all other treatments, when compared to reference river water. Under acid-only exposure, both locomotion and ventilation were significantly higher compared to AMD exposure at the corresponding pH values. The laboratory results were field validated.

Accidental spills of acid water and metals from ongoing mining activities and drainage from abandoned mining areas are of concern for the integrity of freshwater ecosystems, fisheries, human health, and local rural economies (Feasby and Jones 1994). The most obvious effects of such pollution are reduced abundance and biodiversity of the aquatic fauna, because of extinction of sensitive species (*e.g.*, Burton *et al.* 1982; Singer 1982; Soucek *et al.* 2000). Toxicity is related to (1) pH alone, (2) increased bioavailability of metals at low pH, and (3) precipitation of metal hydroxides, these factors being difficult to separate (Campbell and Stokes 1985). Replacement of sensitive species by tolerant ones may compensate for biodiversity loss at low pH, combined with shifts in the food web structure towards more predators and changes of activity patterns in individuals (Gerhardt *et al.* 2004).

The use of behavioral parameters as indicators of sublethal toxicity was initially suggested by Warner *et al.* (1966) listing three advantages, namely, that behavioral change (1) is the final integrated result of biochemical and physiological processes and so a more comprehensive measure than a single biochemical or physiological parameter, (2) is a sensitive indicator of sublethal toxicity, and 3) can be measured without harming the animal. Little (1990) concluded that behavioral observations provide a unique toxicological perspective because they link biochemical causes of pollutants with ecological consequences on the population and community levels. Behavior can be quantified and automated with modern technology, such as, for example, light beam technique (Aagaard *et al.* 1991), time-lapse photography and video-filming (Vanderploeg and Pfaffenhöfer 1985; Atchison *et al.* 1987), and, as in the present study, with impedance conversion technique (*e.g.*, Gerhardt 1999a; Heinis *et al.* 1990).

To keep close to ecological reality, we chose the locally resident mayfly *Choroterpes picteti*, belonging to the Leptophlebiidae, a mayfly family found in high abundance in streams and lakes worldwide (Elliott *et al.* 1988), and therefore relevant for this kind of investigation. Their large size, long life cycle, and their detritivorous feeding habits may be arguments for their use as biotest species (Johnson *et al.* 1993; Janssens de Bisthoven and Gerhardt 2003). Their tolerance towards acidity and metals and their occurrence in mining areas (Engblom and Lingdell 1984; Gerhardt 1993) make them ecologically relevant biotest species for sublethal effect studies in freshwater ecosystems affected by mines. Exposure to single metals has been shown to induce behavioral changes in mayflies (Gerhardt 1994; Gerhardt and Palmer 1998). These changes were associated with an increased bioaccumulation of the concerned metals. However, the combined action of acidity and a mixture of heavy metals may be different from the action of acidity alone or single metals at selected pHs. For example, iron will precipitate to Fe(III)hydroxide at pH > 3.8, creating

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an orange crust where only some chironomids thrive (Koryak *et al.* 1972), as observed in the study site (Gerhardt *et al.* 2004; Janssens de Bisthoven *et al.* 2005). Also, at low pH, metals tend to desorb from insects (Krantzberg and Stokes, 1988), and toxicity of divalent metal ions is known to decrease with decreasing pH because of competition of the hydrogen ion with metal ions at binding sites (Campbell and Stokes 1985; Gerhardt 1993).

The purpose of the present study was to test the following hypotheses for the mayfly *C. picteti*: (1) short-term toxicity (survival) of acid mine drainage (AMD) differs from that of acid only at corresponding pH values, and (2) *C. picteti* elicits a quantifiable behavioral effect outside the normal range of variability when exposed to AMD or acid only.

Materials and Methods

Study Sites

The experiments were conducted in a whole effluent toxicity design (Chapman 2000) with water from (1) the River Vascão (reference); (2) the Mosteirão stream, polluted by acid mine drainage from an abandoned cupriferrous pyrite mine; and (3) a nearby water reservoir, all situated within a 50-km radius from the village of "Minas do São Domingos," SE Portugal [37° 39′ 56″ N, 7° 28′ 46″ W] (Pereira *et al.* 1999). The River Vascão is characterized by slightly alkaline pH (pH 7.0–7.8), a conductivity of 630 $\mu S\ cm^{-1}$, and the absence of any metal or other anthropogenic pollution. Here *C. picteti* was found in great abundance and was collected for the experiments. The polluted Mosteirão stream receives AMD from the mine, creating a dynamic pH/metal gradient between pH 6.4 and 2.3 with a conductivity of 1332 $\mu S\ cm^{-1}$. The reservoir has approximately neutral pH and a conductivity of 352 $\mu S\ cm^{-1}$ and is polluted by arsenic.

Multispecies Freshwater Biomonitor

The Multispecies Freshwater Biomonitor (MFB) is an automated biotest system based on the quadropole impedance conversion technique (Gerhardt *et al.* 1998; Gerhardt 1999a). The test organisms were placed individually in flow-through acrylglass test tubes, sealed on both ends with polyamide rings with nylon netting (mesh size: 500 μm) in order to allow for water exchange. Two pairs of stainless steel electrode pairs were attached to the chamber walls, one generating a high-frequency (100 kHz) alternating current, the other non-current carrying pair serving as sensor of impedance changes, caused by the test organisms' movements in the chamber. By observing individuals placed in an MFB chamber under a binocular microscope and simultaneously recording the activity signals in real time by the MFB, different types of behavior, such as locomotion (low frequency and high amplitudes: 0–2.5 Hz) and ventilation (high frequency and low amplitudes: >2.5 Hz) have been assigned and are supported by previous studies (Gerhardt and Palmer 1998). The occurrence of signal frequencies in percentage of the total record (4 min) was calculated by the MFB software via a discrete Fast Fourier Transformation Analysis.

Experimental Design

Three 48-h experiments were performed: (1) a laboratory exposure to reference water from River Vascão, a reservoir (both habitats for *C. picteti*) as well as to AMD-polluted water of the Mosteirão stream at different pHs; (2) a laboratory exposure to acidified reference water from the River Vascão; and (3) an *in situ* exposure in the reservoir and in the AMD-polluted Mosteirão stream. A few days prior to the experiments, nymphs of *C. picteti* were collected in the River Vascão by gentle manual rinsing of cobbles in water filled buckets and transported to the laboratory in cooling boxes. In the laboratory the animals were allowed to acclimate to the conditions for at least 2 days prior to the experiments. No food was added to the short-term experiments.

Short-term bioassays (48 h) with water from the above-mentioned locations (reservoir: pH 6.8; AMD: pH 5.0, pH 4.4, and pH 3.3; reference river water) were performed in triplicate in artificial flow-through polypropylene channels of 40-cm length, 16.5-cm width, and 15-cm depth and a volume of 4 L. Water from 5 L polyethylene buckets, aerated with air stones, was pumped via a multichannel pump (Watson/Marlow 2058) through each artificial stream at a maximal speed of 18 ml/min and back to the buckets. Oxygen saturation remained more than 80% in all treatments. The renewal time of water in the stream was 4 h. The whole set-up was placed in a climate room at 18°C (SD = 0.5), 16 h day/8 h night diurnal cycle and illumination per channel with two 30-W neon lights. pH, oxygen, conductivity, and temperature were monitored daily. Water and animal samples were taken at the end of the experiments for chemical analysis (see below). Thirteen MFB chambers with one nymph each were distributed among the three channels per pH treatment (4+4+5). Moreover, each of the triplicate channels per pH treatment contained 10 free-living nymphs, thus exposed to the same conditions (water, neither substrate nor food) as the ones captive in the MFB chambers. The average length of the nymphs was 6 mm (SD = 1.3, N = 125). The measured pH followed the nominal pH values without drift and with minor variations (SD = 0.02–0.2) during 48 h of exposure. Survival of the free-living nymphs was counted after 48 h. Survival and behavior of the nymphs exposed in the MFB chambers were recorded automatically every 10 min for periods of 4 min throughout the 48-h exposure.

In a second set of experiments, mayflies (length: mean = 8 mm, SD = 1.4, N = 124) were exposed in duplicate artificial stream channels in the climate room exactly as described above during 48 h to unpolluted reference water (River Vascão) and to Vascão water that was acidified to pH = 3.3, 4.4, and 5.0 with HNO_3, in order to test whether the observed behavioral reactions in the first experiment were due to acid exposure in combination with a metal mixture or to acid exposure alone.

In the *in situ* test, the same artificial streams were placed in duplicates in the pH/metal gradient in the São Domingos mine (pH 5.0) and the reservoir (pH 6.8). The water temperature fluctuated between 16 and 24°C during the day, and the water level was higher because of rainfall than during sampling for the laboratory experiment. To allow for a continuous flow, nine holes (diameter 9 mm) were bored at both ends of the channels, which were sealed with nylon nets (mesh size: 500 μm), glued with hot glue to prevent escape of the animals. The channels were topped by a wooden frame that spanned a net (mesh size: 500 μm). In the wooden frame, holes (9-mm diameter) were bored to pass the cables of the MFB-chambers (Janssens de Bisthoven *et al.* 2004). The channels were exposed in the current and fixed to poles on the stream bottom. Each channel contained 10 free-living individuals and 4 MFB chambers with one individual in each. After 1-h acclimation, and after an additional 8, 22, and 48 h of exposure, the chambers were plugged to the MFB without moving them in the water. The MFB was connected to a laptop with power supply from a battery and the mayflies' behavior was then recorded (a trace of 4 min). At the end of the experiments, survival of the free-moving animals was assessed manually and samples of mayflies were taken for metal analysis.

Table 1. Chemical characteristics (μg/l) of the water used in the experiments with *Choroterpes picteti*

	As	Ca[a]	Cd	Cl[a]	Co	Cu	Fe	K[a]	Mg[a]	Mn[a]	Na[a]	Pb	S[a]	Zn[a]
Laboratory experiment (mean [± SD], N = 2)														
Vas.	3.2	25.4	0.1	109.0	0.3	15.2	36.1	3.9	42.9	14.7	82.1	5.5	16.7	0.056
	(0.3)	(0.5)	(0.001)	(4.0)	(0.0)	(1.2)	(4.6)	(0.1)	(0.1)	(3.8)	(0.6)	(1.2)	(0.1)	(0.002)
Res.	15.6	24.7	0.1	24.7	0.2	15.0	27.9	6.1	10.1	17.3	28.2	16.8	21.6	0.144
	(0.1)	(0.5)	(0.001)	(0.3)	(0.0)	(1.0)	(5.2)	(0.07)	(0.1)	(4.2)	(0.4)	(1.6)	(0.08)	(0.002)
pH 5.0	1.3	71.4	11.0	44.1	71.8	965.0	77.0	30.0	37.5	1.9	44.9	129.0	110.4	2.45
	(0.1)	(2.2)	(0.5)	(5.5)	(0.5)	(5.0)	(18.0)	(0.2)	(0.5)	(0.01)	(0.02)	(9.0)	(1.0)	(0.03)
pH 4.4	1.7	72.3	16.8	39.5	99.0	1429.0	111.5	3.4	36.8	2.7	41.8	269.0	128.1	3.28
	(0.01)	(1.0)	(0.3)	(5.5)	(1.2)	(1.0)	(38.0)	(0.1)	(0.6)	(0.2)	(0.1)	(42.5)	(1.1)	(0.5)
pH 3.3	2.5	69.1	24.5	29.5	143.0	2203.0	2170.1	3.7	35.8	3.8	37.4	307.0	151.6	4.90
	(0.5)	(1.3)	(0.4)	(0.5)	(1.0)	(38.0)	(78.0)	(0.3)	(1.2)	(0.2)	(1.2)	(25.0)	(2.5)	(0.1)
Field experiment														
Res.	15.3	24.6	0.05	29.0	1.5	43.0	1.6	3.6	9.5	5.3	25.8	16.9	18.4	30.2
	(0.4)	(2.1)	(0.05)	(3.5)	(1.1)	(25.0)	(1.0)	(0.9)	(1.2)	(2.7)	(2.0)	(12.4)	(1.9)	(3.2)
pH 5.0	5.5	30.9	0.5	39.5	17.9	49.1	52.0	3.3	16.3	0.9	22.3	34.2	28.6	51.0
	(3.8)	(0.0)	(0.4)	(14.5)	(3.1)	(22.2)	(21.4)	(2.4)	(13.3)	(0.2)	(17.5)	(30.4)	(26.2)	(4.0)

[a] mg/L
Res.= Reservoir, Vas.= River Vascão, sites in the AMD pollution gradient are named according to their pH.

Chemical Analyses

Water samples were analyzed for Cd, Co, Cu, Zn, Mn, Pb, and As with ICP-MS (detection limit: <0.01 μg/L) and for Fe, Na, Mg, S, Ca, and high values of As with ICP-AES (detection limit: <10 μg/L). Chlorine was determined with ion chromatography (detection limit: 0.01 mg/L). Animal samples from each treatment and all experiments were taken in triplicates and were kept frozen (–18°C) until subsequent determination of metal body burdens with ICP-MS and ICP-AES after wet digestion in 500 μL 30% HNO_3 at 80°C overnight, followed by digestion in 100 μL H_2O_2 at 80°C and solution of the remnants in 100 μL HNO_3 and 900 μL ultradistilled water. Quality control of metal analysis was performed using destruction blanks and reference materials (IAEA shrimp MA-A-3/TM and IAEA simulated freshwater W4 (Timmermans and Walker 1989).

Statistics

Multiple (stepwise forward) and bivariate linear regressions were calculated for the sites in São Domingos Mine (pollution gradient) in order to show statistically relevant relationships between (1) metals and pH values in the water, (2) pH values in the water and mayfly metal burdens, and (3) metal values in the water and organisms. After testing the assumptions for analysis of variance (ANOVA), one-way ANOVA was used to determine significant differences of metal levels in water or mayflies between the different treatments.

Survival data were analyzed by linear regression of probit-transformed mortality data plotted against pH after 48 h (LC_{50}) (Weber 1986). Mortalities of animals exposed freely compared to animals exposed in MFB chambers were tested with chi-square tests. From the continuous survival data, generated by the MFB, we also calculated LT_{20} values according to a log-logistic regression model (Dixon and Newman 1991).

Test for normality of the behavioral raw data (chi-square goodness of fit) and homogeneity of variances (Levene's test) were performed and raw data were transformed to arcsine ($x^{0.5}$) prior to analysis of covariance on the 48-h data. ANOVAs for activities in day and night blocks throughout the experiment allowed for analysis of diurnal rhythms.

Results

Metal Concentrations in the Water

In São Domingos mine metal levels (Table 1) were dependent on pH of the sites (water: pH = 6.5 – 0.47 Zn – 0.48 Cu + 0.1 As + 0.01 Mn, $r^2 = 0.99$, $p = 0.006$). Metal levels increased with decreasing pH values ($F(6,7) = 45–6231$, $p = 0.01–0.0001$). Linear regressions of single metals against pH values revealed significant bivariate relationships between most elements and pH ($r^2 > 0.86$, $p < 0.03$): Cd, Co, Cu, Pb, Zn, and S, from which some were disaffirmed in the multiple regressions. All metals in the reservoir (except for As) were significantly lower than in the AMD gradient ($F(6,7) = 45–6231$, $p = 0.01–0.0001$) and all metals in the River Vascão were significantly lower compared to the AMD sites. The reservoir contained significantly more arsenic than all other sites ($F(6,7) = 515$, $p = 0.001$). In the field experiment at pH 5.0, all metals except Fe were significantly lower than those in the laboratory experiment, which we link to rainfall and variations in discharge ($F(1,2) = 27–1950$, $p = 0.02–0.0005$).

Metal Body Burdens

Generally, body burdens of essential metals were orders of magnitude higher than those of toxic metals, and were related to pH values (Figure 1, body burdens: pH = 4.33 + 0.27 Cu + 0.68 Pb – 0.55 As + 0.25 Fe, $r^2 = 0.99$, $p = 0.03$). At low pH (pH 3.3 and 4.4) metal burdens were lower than at higher pH levels for Cu ($F(1,7) = 49.8$, $p = 0.0002$), and Zn ($F(1,7) = 4.43$, $p = 0.07$). Pb body burdens at pH 3.3 were significantly lower than in other pH treatments ($F(6,13) = 3.9$, $p = 0.02$). There was no clear dependency of metal body burdens on metal concentrations in the water, except for mayflies exposed at pH 3.3, where the highest metal concentrations in the water corresponded to the lowest metal body burdens (Figure 1). Metal burdens of mayflies exposed to River Vascão water

Table 2. LT_{20} (logit regression model: y = logit mortality and x = time) for *Choroterpes picteti* exposed during 48 h to AMD, acid-only water, Reservoir (Res.) and River Vascão (Vas.) water.

Site/pH	Regression	LT_{20} (h)	CI: 95%	r^2	p
AMD					
Vas.	No mortality				
Res.	$y = 2.67 - 0.82$ x	20	17.3 – 21.4	0.42	0.0001
5.0	$y = 3.10 - 1.49$ x	10.0	8.7 – 11.4	0.63	0.0001
4.4	$y = 7.32 - 2.29$ x	13.5	13.0 – 14.0	0.79	0.0001
3.3	$y = 8.99 - 3.64$ x	3.5	3.0 – 4.1	0.77	0.0001
ACID-only					
5.0	$y = 3.1 - 1.4$ x	13.0	12.0 – 14.0	0.45	0.0001
4.4	$y = 3.1 - 1.5$ x	10.0	9.0 – 10.5	0.66	0.0001
3.3	$y = 0.04 - 1.45$ x	0.25	0.0 – 1.0	0.37	0.0001

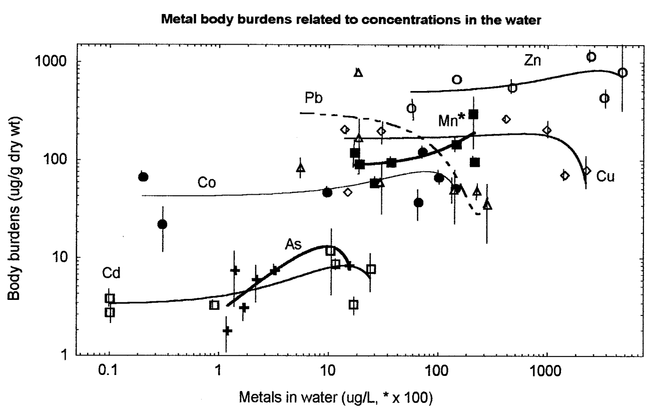


Fig. 1. Metal body burdens (means, SE) in nymphs of *Choroterpes picteti* after 48 h exposure in the laboratory to water from the AMD gradient and the reference. Curve fitting with least-squares method. Points from left to right side represent metal burdens at sites with decreasing pH value.

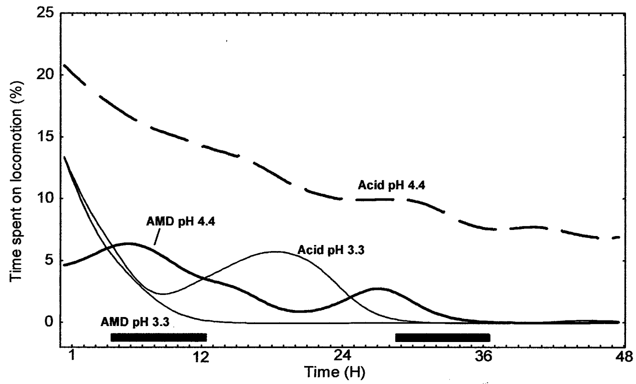
(reference) were significantly lower than in all other treatments for Cu, Fe, and Mn ($F(9, 40) = 3.0$, $p < 0.005$). Metal body burdens of mayflies exposed *in situ* were not different from those exposed in the laboratory, except for Fe, being higher *in situ* (F (1,2)= 29, $p = 0.03$).

Survival

In the laboratory and *in situ* experiments with AMD, *C. picteti* survived equally well when exposed free in the artificial streams compared to those in the test chambers ($p > 0.05$). Mortality within 48 h of exposure ranged from 0% (River Vascão) to over 8% (reservoir) to 100% (pH 3.3 in AMD and in acid only). In the field exposure, survival was similar compared to survival in the laboratory experiment ($p > 0.05$). The 48-h LC_{50} (pH) (AMD) was reached at pH 4.8 (95% CI = 4.7–5.1, $r^2 = 0.78$, $p = 0.0003$). The mortality in the *in situ* exposure at pH 5.0 was situated exactly on the probit regression line fitted for the laboratory data of corresponding pH. Survival of *C. picteti* under acid-only exposure was significantly pH-dependent and decreased at low pH values ($F(5,6) = 11.4$, $p = 0.005$). The 48-h LC_{50} (pH) (acid only) was reached at pH 4.9 (95% CI: 4.7–5.0, r^2 = 0.71, $p = 0.0001$). The LT_{20} values for the AMD laboratory experiment decreased with decreasing pH (Table 2). In water from the reservoir some *C. picteti* died, probably because of the high As concentrations. In general, toxicity of AMD and acid only started to be apparent at pH values of pH 5.0 and below. The LT_{20} values showed that toxicity of AMD or acid only can be detected with *C. picteti* already within 15 h of exposure, hence faster than with the fixed-time 48-h LC_{50} analysis.

Behavior

A typical behavioral record (4 min) of *C. picteti* exposed in water of their origin (River Vascão) was characterized by long periods of inactivity (up to 80% of a record) alternated with periods of locomotory activity (up to 25 % of a record) and ventilation of the gills (up to 5% of a record). The variation in behavior among the mayflies was low (locomotion: SE ± 5, ventilation SE 0.5), very similar to *Adenophlebia auriculata* (Gerhardt and Palmer 1998). *C. picteti* showed a diurnal activity pattern with elevated locomotion at night combined with decreased ventilation (Figure 2), in the reservoir, River Vascão, at pH 5.0 and pH 4.4 (Res.: $F(1, 329) = 30.4$, $p = 0.0001$; Vascão: $F(1, 208) = 8.3$, $p < 0.005$; pH 4.4: $F(1, 355) = 25.9$, $p = 0.0001$ and especially at pH 5.0: $F(1, 416) = 190.6$, $p = 0.0001$). At pH 3.3, the animals died too quickly to prove diurnal activities in AMD or acid water. The phenomenon of diurnal activity patterns was less expressed under acid exposure alone in the laboratory experiments. Mayflies exposed to water from the reservoir were significantly more active than in the AMD gradient and water from River Vascão ($F(6, 2297) = 93.4$, $p < 0.001$). In all treatments, locomotor activity of mayflies slightly decreased with exposure time. Generally, mayflies were more active in acid water than in AMD water (pH 4.4: $F(1, 1584) = 331$, $p = 0.001$; pH 5.0: F (1, 788) = 84.8, $p = 0.001$) (Figure 2). At pH 3.3, some mayflies were still alive and active, whereas in AMD at pH 3.3 they had already died (F (1, 458) = 57.6, $p = 0.001$). Generally the animals spent only a little time on gill ventilation (less than 5% of the behavior record) (Figure 3), and ventilation in the water from the reservoir was significantly higher than in the River Vascão and other treatments (F(6, 11873) = 95, $p = 0.0001$). In the acid-only treatments, ventilation was significantly higher than in the AMD treatments, especially at pH 4.4 ($F(1, 4171) = 297$, $p = 0.0001$) (Figure 3). A summary of mean activity levels of the mayflies from the whole exposure time shows the differ-



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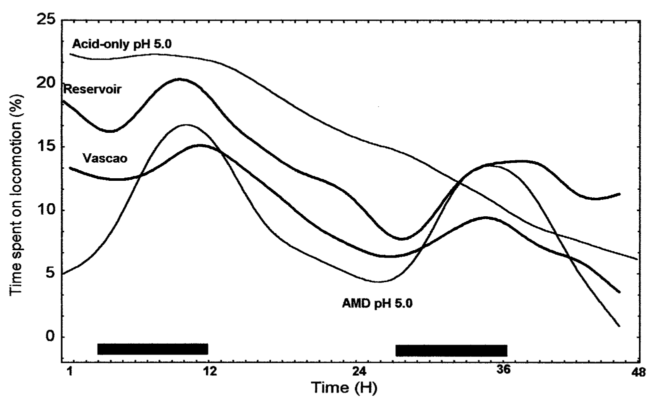

Fig. 2. Locomotory activity of *Choroterpes picteti* in the different AMD and acid-only conditions in laboratory exposures (48 h). Least-square fit of the raw data (N = 12–16, means presented, SD bars omitted for clarity, SD: 2–5%). Dark bars = night. (A) pH 3.3 and 4.4 and (B) Vascão (= reference), reservoir and pH 5.0.

ences in the response pattern of *C. picteti* to acid-only and AMD exposure as a function of pH (Figure 4). A steep pH-dependent relationship of decreasing locomotory activity with decreasing pH was only found in the acid-only treatments.

The *in situ* validation showed significantly higher locomotory activity of *C. picteti* at pH 5.0, especially at the beginning, and in the reservoir, compared to the respective laboratory experiment, especially during the first 8 h of exposure ($F(2,23) = 11.3$, $p < 0.001$) (Figure 5). Ventilation was higher in all field exposures compared to laboratory experiments ($F(1, 81) = 62.27$, $p = 0.0001$).

Discussion

Water Chemistry

The River Vascão showed low metal concentrations in the range of natural background values (Jørgensen *et al.* 1991; Kelly 1991). This confirms the choice of the River Vascão as a good reference site. The reservoir was contaminated by As, probably because of a combination of storm water and atmospheric transport (Gerhardt *et al.* 2004), which could be the reason why *C. picteti* only occurred there in low numbers. It has been documented that the acid mine drainage of São Domingos mine is characterized by a temporal and spatial variability in pH and concentrations of metals and salts, which is due to seasonal changes in rainfall and discharge, causing extreme conditions, from desiccating pools to sporadic flash floods (Gerhardt *et al.* 2004; Janssens de Bisthoven *et al.* 2005). In the AMD gradient, aqueous metal concentrations increased linearly with decreasing pH values for most metals. Increased acidification has been proven to increase dissolution of metal ions from metal salts, *e.g.*, for Cu (pH < 6), Pb (pH < 6), or Al (pH < 5) (Campbell and Stokes 1985; Gerhardt 1993).

Metal Body Burdens

Mayflies exposed to reference water from River Vascão had significantly lower metal body burdens, as compared to animals exposed to AMD water. It indicates that mayflies exposed to AMD increased their metal body burden for essential and toxic metals during 48 h, either externally adsorbed and/or internally taken up. Lead body burdens of the mayflies were positively related to pH in the water, whereas other metals (Zn, Cu, Co, and Cd) showed a decrease only at low pH (pH 3.3 and pH 4.4). Mn and As, on the other hand, increased gradually with decreasing pH. Krantzberg and Stokes (1988) mentioned similar pH dependencies for body burdens of Al, Cd, Cu, Fe, Mn, Ni, and Pb in chironomids. The reasons for decreased metal body burdens at pH < 4.4 might be the following: (1) in spite of high extent of dissolution and bioavailability of metals at low pH, competition with other mono- or bivalent ions at the membrane receptors might counteract increased metal uptake above a saturation level, which might already be reached at a pH around 5 (Campbell and Stokes 1985; Wren and Stephenson 1991; Gerhardt 1993). (2) Metal body burdens during 48-h exposure might have consisted mostly of externally adsorbed metals, which desorbed from the organisms surface at extremely low pH (pH 3.3–4.4) (Krantzberg and Stokes 1988).

Survival

Survival of mayflies exposed in MFB chambers did not differ from mayflies freely exposed in the artificial streams. This reveals that survival of mayflies was not negatively affected by the limited space in the test chambers and the high-frequency alternating current in the MFB chambers, as confirmed earlier for fish (Craig and Laming 2004), amphipods (Kirkpatrick, University of Belfast, personal communication), and chironomids (Janssens de Bisthoven *et al.* 2004). Survival of *C. picteti* in the laboratory experiments was also similar to that in the field experiments. As far as survival is concerned, the MFB proved reliable for short-term toxicity bioassays in the laboratory as well as *in situ.* Because survival data can be recorded automatically on a continuous basis, the MFB data can generate more reliable LT values than when survival would be ascertained sporadically on a visual basis.

Mayflies can be found down to pH 4.5 (Kelly 1991), which supports our experimental results. Survival of mayflies exposed to AMD or acidified water decreased significantly

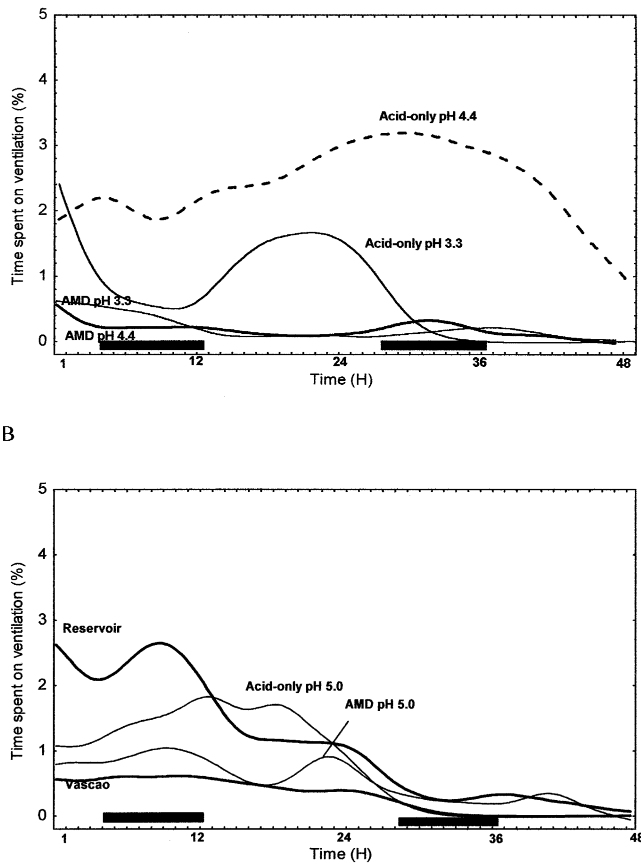

Fig. 3. Ventilation activity of *Choroterpes picteti* in the different AMD and acid-only conditions in laboratory exposures (48 h). Least-square fit of the raw data (N = 12–16, means presented, SD bars omitted for clarity, SD: 0.2–1%). Dark bars = night. (A) pH 3.3 and 4.4 and (B) Vascão (= reference), reservoir and pH 5.0.

with decreasing pH values. Although the 48-h LC_{50} (pH) for acid-exposed and AMD-exposed individuals was similar (pH 4.8–4.9), the LT_{20} for pH 4.4 and 3.3 was reached earlier in acid-only water compared to AMD. Organic matter, a possible confounding factor, was similar in both experiments (Gerhardt, unpublished data). Both the River Vascão (acid-only experiment) and Mosteirão stream (AMD experiment) belong to the same catchment. Furthermore, the observed differences between effects of AMD and acid only cannot solely be attributed to the type of acidity (H_2SO_4 in AMD experiment *versus* HNO_3 in the acid-only experiment) itself: we expect the difference in effects between both types of acids at comparable pH values to be several orders of magnitude smaller than the difference caused by the number of metals in the AMD and their respective concentrations.

The 48-h LC_{50} (pH) for acid stress has been reported to be pH 4.0 for the mayflies *Baetis* sp. and *Leptophlebia marginata* (Økland and Økland 1986), thus indicating *C. picteti* to be somewhat more sensitive than those species. During short-term exposure (96–192 h) to low pH, *Leptophlebia cupida* showed loss of Na^+ and Cl^- at pH 3.5 and 30% mortality, but not at pH 4.5 in the range of Ca^{2+} and K^+ regulation with 100% survival (Rowe *et al.* 1989). Fluxes of Na^+ showed a higher net loss in mayfly nymphs at pH 4 compared to pH 5 and 6, with the

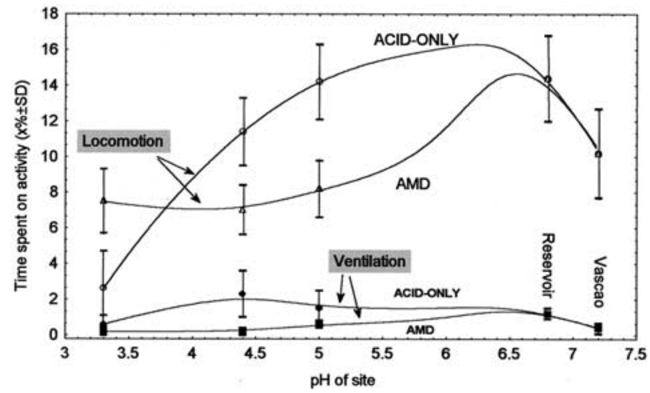

Fig. 4. Mean activity levels of *Choroterpes picteti* at different AMD- and acid-only treatments.

presence of the metal Al reducing this effect (Herrmann *et al.* 1993). Under acid exposure, insects are generally better to maintain internal Na^+ and Cl^- balances than crustaceans, thus supporting the acid tolerance of insects (Havas 1981).

Toxicity of divalent metal ions is known to decrease with decreasing pH because of competition of the hydrogen ions with metal ions at receptor sites (Campbell and Stokes 1985; Gerhardt 1993). A similar toxicity-mediating effect was found for the mayfly *Ephemera danica* exposed to pH 5 and Al (Herrmann *et al.* 1993). Earlier short-term toxicity studies (LC_{50}) for *Leptophlebia marginata* showed increased acute toxicity of Cd, Pb, and Fe at pH 4.5–5.0 compared to pH 7 (Gerhardt 1992b; Gerhardt 1994). However, the metal concentrations were much higher than in the present study, except for the Fe values, being close to the previously reported LC_{50}s for Leptophlebiidae.

Moreover, chronic exposure to Fe during 30 days at pH 4.5 and 7.0 revealed less activity and lower survival of the mayfly *Leptophlebia marginata* at pH 4.5 at concentration levels similar to our present study (Gerhardt 1992a). This indicates that Fe levels in the AMD might have seriously contributed to sublethal behavioral effects as well as mortality in *C. picteti*. The Pb values in the present experiments ranged between 0.01 (pH 5.5) to 0.3 (pH 3.3) mg/L, much below the 96-h LC_{50} (>5 mg/L at pH 7 and 1.09 mg/L at pH 4.5) found for *Leptophlebia marginata* (Gerhardt 1994). Copper concentrations in the AMD water ranged between 0.97 mg/L (pH 5.0) and 22 mg/L (pH 3.3) and were up to 1000 times greater than natural background levels (Jørgensen *et al.* 1991). At pH ±7 the 48-h LC_{50} for the leptophlebiid mayfly *Adenophlebia auriculata* was 0.79 mg/L Cu (Gerhardt and Palmer 1998). Values for As in the AMD were below the 48-h LC_{50} for daphnids, chironomids, and fish (Jørgensen *et al.* 1991). The LC_{50} range (48–96 h) for Cd in aquatic species is between 2 μg Cd/L to 4.6 g Cd/L (Hoekstra *et al.* 1994); therefore it is in the toxic range of Cd levels in the AMD in the mine. However, for the mayfly *Atalophlebia* sp., 840 μg Cd/L was reported (Wang 1987), which might indicate tolerance of leptophlebiid mayflies including *C. picteti* to Cd. We remain careful in the interpretation of mixture toxicity effects because investigations with (1) more than binary metal mixtures in equitoxic mixtures and (2) metals in realistic mixtures are rare, (3) interaction models become complicated the more metals are added, and (4) the

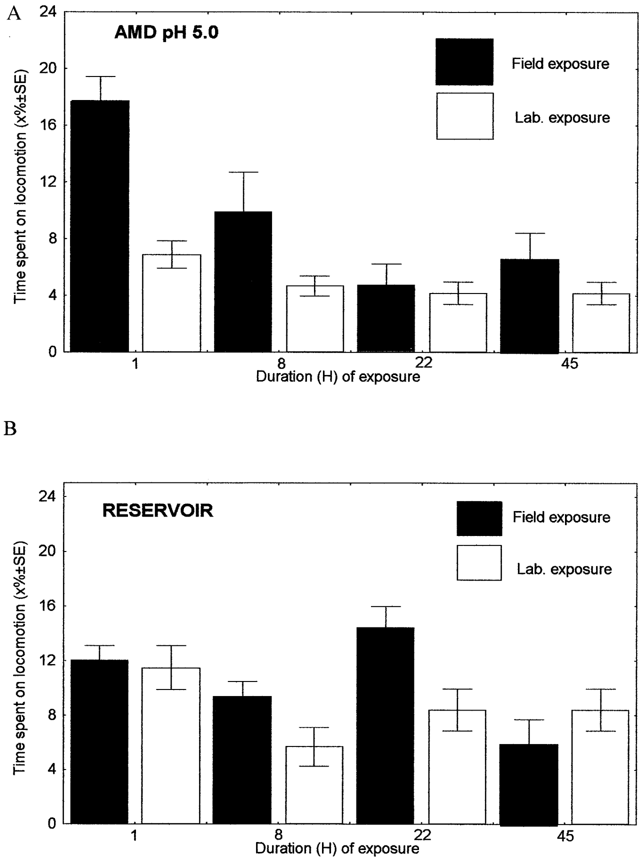

Fig. 5. Comparison of activity of *Choroterpes picteti* exposed to AMD (pH 5.5) and the reservoir (pH 6.8) in the laboratory and *in situ.* (A) pH 5.0 and (B) reservoir.

biotic ligand model (Santore *et al.* 2001) was not applied here because of shortcomings in the extent of application to different test species, metals in mixtures, and extreme pH values.

Behavior

Compared to pelagic fish (Gerhardt *et al.* 2002) and planktonic species, for example, *Daphnia* sp. (Gerhardt *et al.* 2003), the mayfly *Choroterpes picteti* was a rather inactive species, typical for benthic organisms, such as *Gammarus pulex* (Gerhardt 1995). In the MFB, locomotory activities of benthic mayflies and chironomids represented around 25% of the recording time (Gerhardt and Palmer 1998; Janssens de Bisthoven *et al.* 2004). *C. picteti* showed a diurnal activity pattern with increased locomotory activity and decreased ventilation during the night. Diurnal activity patterns have often been observed in invertebrates, for example, with *Hydropsyche angustipennis* (Gerhardt 1996), *Gammarus pulex* (Gerhardt *et al.* 1998; Gerhardt and Quindt 2000), Ephemeroptera (Elliott *et al.* 1988; Huhta *et al.* 2000), or the plecopteran *Dinocras cephalotes* (Gerhardt 2000).

In the reservoir, ventilation and locomotory activity were significantly higher than in the AMD gradient and the River Vascão, which might be because the water contained the highest arsenic values, but low values for other metals. The exposure to AMD resulted in pH-dependent behavioral changes of *C. picteti*, consisting of (1) increased diurnal activity variations at pH 5 compared to all other treatments, and (2) decreased locomotion at low pH (pH 3.3 and 4.4). Under acid-only exposure, both locomotion and ventilation were significantly higher compared to AMD exposure at the corresponding pH values and the reference water. Under acid-only exposure, diurnal rhythms were weakened. At pH 4.4 in acidified water, ventilation of *C. picteti* was highest, a pH value that corresponds to the natural minimum pH, where mayflies occur (Kelly 1991; Gerhardt 1993).

Increased locomotion can be interpreted as escape trials and could lead to increased nocturnal drift in nature (Elliott *et al.* 1988). Mayflies exposed to short-term (8 h) acid pulses down to pH 4.6 or 5.2 responded with increased drift (Kratz *et al.* 1994). Under extreme chemical stress, mayflies might also increase day activity and drift, thus explaining the higher general activities and the weakened diurnal rhythms in the acid-only treatments in our experiment. Increased gill ventilation can be interpreted as a trial to remove toxic substances from the gills by increasing the water flow.

The "Toxicity Bioassay Continuum" (Gerhardt 1999b) is a concept explaining the gradient from laboratory bioassays to field bioassays, that is, increasing ecological realism for different factors such as experimental design, test species, test medium, scale of containers, duration, and end points. Therefore, we performed our laboratory tests with whole effluent water from a natural AMD gradient, and we used the same experimental design as in the *in situ* test. Survival of mayflies in the laboratory experiments was similar to that in the field experiments. Good agreement of standard laboratory tests and *in situ* tests of 48 h with daphnids exposed in flow-through chambers (mesh size 50 μm) in the São Domingos mine were previously found at highly contaminated sites; however, at intermediately polluted sites, *in situ* toxicity (mortality) was higher than in the laboratory tests (Pereira *et al.* 1999). Especially at the beginning of the experiments, activities of the field-exposed mayflies were higher compared to those in the laboratory experiments, which might have been because of differences in handling stress (transport of organisms to the study site) and differences in water temperature (laboratory: 18°C and field: 16–24°C) and its diurnal fluctuations.

In conclusion, within the pH-metal gradient of the AMD of São Domingos, exposure of the ephemeropteran *C. picteti* during 48 h at a pH of 4.8 provoked 50% mortality, similar to mortality in acid-only exposure at the same pH. Whereas 48-h LC_{50} (pH) could not differentiate between AMD and acid-only toxicity, LT_{20} (at pH 3.3) did so. Behavioral responses indicated (1) arsenic pollution in the reservoir, and (2) AMD pollution and acid pollution at $\leq$ 48-LC_{50} (pH) values. Behavioral response times are starting already within the first hours.

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References

Aagaard A, Andersen BB, Depledge MH (1991) Simultaneous monitoring of physiological and behavioural activity in marine organisms using non-invasive, computer aided techniques. Mar Ecol Progr Ser 73:177–182

Atchison GJ, Henry MG, Sandheinrich MB (1987) Effects of metals on fish behaviour: a review. Environ Biol Fishes 18:11–25

Burton TM, Stanford RM, Allan JW (1982) The effects of acidification on stream ecosystems. In: D'Itri FM (ed), Acid precipitation. Effects on ecological systems. Ann Arbor Science Publishers, Michigan, pp 209–235

Campbell PGC, Stokes PM (1985) Acidification and toxicity of metals to aquatic biota. Can J Fish Aquat Sci 42:2034–2049

Chapman PM (2000) Whole effluent toxicity testing-usefulness, level of protection and risk assessment. Environ Toxicol Chem 19:3–13

Craig S, Laming P (2004) Behaviour of the three-spined stickleback *Gasterosteus aculeatus* (Gasterosteidae, Teleostei) in the Multispecies Freshwater Biomonitor: a validation of automated recordings at three levels of ammonia pollution. Water Res 38:2144–2154

Dixon PM, Newman MC (1991) Analyzing toxicity data using statistical models for time-to-death: an introduction. In: Newman MC, McIntosh AW (eds), Metal ecotoxicology. Concepts and applications. Lewis Publishers, Chelsea, Michigan, pp 207–241

Elliott JM, Humpesch UH, Macan TT (1988) Larvae of the British Ephemeroptera: A key with ecological notes 49. Freswater Biological Asociation, Ambleside, UK, 145

Engblom E, Lingdell PE (1984) The mapping of short-term acidification with the help of biological pH-indicators. Rep Inst Freshw Res Drottningholm 61:28–33

Feasby G, Jones RK (1994) Report of results of a Workshop on Mine Reclamation, Toronto, Ontario March 10–11, 1994. Workshop hosted by the IGWG-Industry Task Force on Mine Reclamation

Gerhardt A (1992a) Subacute effects of iron (Fe) on *Leptophlebia marginata* (L.) (Insecta: Ephemeroptera). Freshwater Biol 27:79–84

Gerhardt A (1992b) Acute toxicity of Cd in stream invertebrates in relation to pH and test design. Hydrobiologia 239:93–100

Gerhardt A (1993) Impact of heavy metals on stream invertebrates with special emphasis on acid conditions. Water Air Soil Pollution 66:289–314

Gerhardt A (1994) Short term toxicity of iron (Fe) and lead (Pb) to the mayfly *Leptophlebia marginata* (L.) (Insecta) in relation to freshwater acidification. Hydrobiologia 284:57–168

Gerhardt A (1995) Joint and single toxicity of Cd and Fe related to uptake to the mayfly *Leptophlebia marginata* (L.) (Insecta). Hydrobiologia 306:229–240

Gerhardt A (1996) Behavioural early warning responses to polluted water. Performance of *Gammarus pulex* L. (Crustacea) and *Hydropsyche angustipennis* (Curtis) (Insecta) to a complex industrial effluent. Environm Sci Pollut Res 3:63–70

Gerhardt A (1999a) Recent trends in automated biomonitoring for water quality control. In: Gerhardt A (ed), Biomonitoring of polluted water. Reviews on actual topics. Environmental Research Forum Vol 9. TransTecPublications, Zürich, Switzerland, pp 95–118

Gerhardt A (1999b) Biomonitoring for the 21st century. In: Gerhardt A (ed), Biomonitoring of polluted water. Reviews on actual topics. Environmental Research Forum Vol 9. TransTecPublications, Zürich, Switzerland, pp 1–12

Gerhardt A (2000) A new multispecies freshwater biomonitor for ecological relevant control of surface waters. In: Butterworth F, Gunatilaka A, Gonsebatt ME (eds), Biomonitors and biomarkers as indicators of environmental change: Vol. 2. Kluwer-Plenum Press, pp 301–317

Gerhardt A, Palmer C (1998) Copper tolerances of *Adenophlebia auriculata* (Insecta) and *Burnupia stenochorias* (Mollusca) in indoor artificial streams. Sci Total Environ 215:217–229

Gerhardt A, Quindt K (2000) Abwassertoxizität und -überwachung mit den Bachflohkrebsen *Gammarus pulex* (L.) und *Gammarus tigrinus* (Sexton) (Crustacea: Amphipoda). Wasser und Boden 52/10:19–26

Gerhardt A, Carlsson A, Ressemann C, Stich KP (1998) A new automated biomonitoring system for *Gammarus pulex* (L.) (Crustacea): *in situ* test below a copper effluent in South Sweden. Environm Sci Technol 32:150–156

Gerhardt A, Janssens de Bisthoven L, Mo Z, Wang C, Wang Z (2002) Short-term behavioural responses of *Orizias latipes* (Pisces) and *Macrobrachium nipponense* (Crustacea) to municipal and pharmaceutical waste water in Beijing, China. Chemosphere-Section Environ Toxicol Risk Assess 47:35–47

Gerhardt A, Janssens de Bisthoven L, Penders E (2003) Quality control of drinking water from the River Rhine (Netherlands) with the Multispecies Freshwater Biomonitor. Aquat Ecosystem Health Manage 6:159–166

Gerhardt A, Janssens de Bisthoven L, Soares AMVM (2004) Macroinvertebrate response to an acid mine drainage: community metrics combined with automated behavioural toxicity bioassay. Environ Pollution 130:263–274

Havas M (1981) Physiological response of aquatic animals to low pH. In: Singer R. Effects of acidic precipitation on benthos. Proc Symp Benthic Biology, North American Benthological Survey. Hamilton, New York, pp 49–65

Heinis F, Timmermans KR, Swain WR (1990) Short-term sublethal effects of cadmium on the filter feeding chironomid larva *Glyptotendipes pallens* (Meigen) (Diptera). Aquat Toxicol 16:73–86

Herrmann J, Degermann E, Gerhardt A, Johansson C, Lingdell P-E, Muniz IP (1993) Acid-stress effects on stream biology. Ambio 22:298–307

Hoekstra JA, Vaal MA, Notenboom J, Slooff W (1994) Variation in the sensitivity of aquatic species to toxicants. Bull Environm Contam Toxicol 53:98–105

Huhta A, Muotka T, Tikkanen P (2000) Nocturnal drift of mayfly nymphs as post-contact antipredator mechanism. Freshwater Biol 45:33–42

Janssens de Bisthoven L, Gerhardt A (2003) Use of *Adenophlebia auriculata* Eaton 1881 and *Leptophlebia marginata* (L. 1767) nymphs (Leptophlebiidae, Ephemeroptera) in aquatic ecotoxicology. Zool Baetica 13/14:57–69

Janssens de Bisthoven L, Gerhardt A, Soares AMV (2004) Effects of acid mine drainage on larval *Chironomus* (Diptera, Chironomidae) measured with the multispecies freshwater biomonitor. Environm Toxicol Chem 23:1123–1128

Janssens de Bisthoven L, Gerhardt A, Soares AMVM (2005) Chironomidae larvae as bioindication of an acid mine drainage in Portugal. Hydrobiologia 532, in press

Johnson RK, Wiederholm T, Rosenberg DM (1993) Freshwater biomonitoring using individual organisms, populations, and species assemblages of benthic macroinvertebrates. In: Rosenberg DM, Resh VH (eds), Freshwater biomonitoring and benthic macroinvertebrates. Chapman and Hall, New York, pp 159–195

Jørgensen SE, Nielsen SN, Jørgensen LA (1991) Handbook of ecological parameters and ecotoxicology Elsevier, Amsterdam

Kelly M (1991) Mining and the freshwater environment Elsevier, London and New York, 231

Koryak M, Shapiro MA, Sykora JL (1972) Riffle zoobenthos in streams receiving acid mine drainage. Water Res 6:1239–1247

Krantzberg G, Stokes PM (1988) The importance of surface adsorption and pH in metal accumulation by chironomids. Environ Toxicol Chem 7:653–670

Kratz KW, Cooper SD, Melack JM (1994) Effects of single and repeated experimental acid pulses on invertebrates in a high altitude Sierra Nevada stream. Freshwater Biol 32:161–183

Little EE (1990) Behavioural toxicology. Stimulating challenges for a growing discipline. Environm Toxicol Chem 9:1–2

Økland J, Økland KA (1986) The effects of acid deposition on benthic animals in lakes and streams. Experientia 42:471–486

Pereira AMM, Soares AMVM, Gonçalves F, Ribeiro R (1999) Test chambers and test procedures for *in situ* toxicity testing with zooplankton. Environm Toxicol Chem 18:1956–1964

Rowe L, Berrill M, Hollett L, Hall RJ (1989) The effects of short-term laboratory pH depressions on moulting, mortality and major ion concentrations in the mayflies *Stenonema femoratum* and *Leptophlebia cupida*. Hydrobiologia 184:89–97

Santore RC, Di Toro DM, Paquin PR, Allen HE, Meyer JS (2001) Biotic ligand model of the acute toxicity of metals. 2. Application to acute copper toxicity in freshwater fish and *Daphnia*. Environm Toxicol Chemistry 20:2397–2402

Singer R (1982) Effects of acidic precipitation on benthos. In: D'Itri FM (ed), Acid precipitation. Effects on ecological systems. Ann Arbor Science Publishers, Michigan, pp 329–363

Soucek DJ, Cherry D, Currie R, Latimer H, Trent G (2000) Laboratory to field validation in an integrative assessment of an acid mine drainage-impacted watershed. Environ Toxicol Chem 19:1036–1043

Timmermans KR, Walker PA (1989) The fate of trace metals during the metamorphosis of chironomids (Diptera, Chironomidae). Environ Pollut 62:73–85

Vanderploeg HA, Pfaffenhöfer GA (1985) Modes of algal capture by the freshwater copepod *Diaptomus sicilis* and their relation to food-size selection. Limnol Oceanogr 30:871–885

Wang W (1987) Factors affecting metal toxicity to (and accumulation by) aquatic organisms. Overview. Environm Int 13:437–457

Warner RE, Peterson KK, Borgmann L (1966) Behavioural pathology in fish: a quantitative study of sublethal pesticide toxication. J Appl Ecol 3:(suppl)223–247

Weber E (1986) Grundriss der biologischen Statistik. G Fischer Verlag, Jena, pp 534–551

Wren CD, Stephenson GL (1991) The effect of acidification on the accumulation and toxicity of metals to freshwater invertebrates. Environm Pollution 71:205–241