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EFFECTS OF ACID MINE DRAINAGE ON LARVAL *CHIRONOMUS* (DIPTERA, CHIRONOMIDAE) MEASURED WITH THE MULTISPECIES FRESHWATER BIOMONITOR®

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Abstract—The abandoned São Domingos mine (Portugal) offers a pH and metal gradient of acid mine drainage (AMD), and is an ideal model for ecotoxicological studies. Short-term 24-h bioassays with water from the AMD (pH 3.3, 4.4, and 5.5, and control) were performed with fourth instars of *Chironomus* in the laboratory and in situ (AMD at pH 5.5) in artificial flow-through channels. This was compared to reference water acidified to the respective pH values (acid only). Behavioral stress responses were monitored online with the Multispecies Freshwater Biomonitor® (MFB). The exposure to AMD and acid only was in the sublethal range (mortality 0–20%). The use of MFB chambers did not affect survival. Stress behavior of *Chironomus* consisted mainly of decreased locomotory activity in AMD and increased activity in acid-only tests, indicating that the metals in the AMD played a role as stress factor. Field exposure in the AMD mixing zone (pH 5.5) generated similar activity as in the corresponding laboratory exposure.

Keywords—Acid mine drainage *Chironomus* Behavior Online biomonitoring

INTRODUCTION

Pollution spills from mining retention basins in the Guadiamar River (1998, Spain) [1] and Tisza River (2000, Rumania) [2] had dramatic consequences. A large area at the border with Cota Doñana National Parc, an internationally protected wetland, was polluted with heavy metals and a massive fish kill occurred along the Tisza and the Danube rivers. Although this kind of accidental pollution is relatively rare, its sporadic occurrence will continue to pose a considerable threat, not only to the health of freshwater ecosystems, but also to nature conservation, fisheries, human health, and local rural economies. Therefore, it is essential to better understand the biological effects such pollution causes at all levels of the food chain of the affected water bodies, and to develop fast and cost-effective biotests for early warning, early diagnosis, and screening.

Heavy metals and water acidity play a major role in such spills, and the São Domingos mine in southern Portugal was chosen as a model site to study effects of acid mine drainage (AMD) on several aquatic organisms in an attempt to develop fast and cost-effective biotests (Cost Effective Tools for Ecological Risk Assessment project). Online biomonitoring represents a continuous and automated biotest, the aim of which is to detect early warning signals from organisms reacting to stress provoked by a passing wave or pulse of a pollutant or a pollutant mixture. The assumption is that the behavior of organisms will change rapidly and sensitively because of toxic substances and that the detection of early warning signals will be ecologically more relevant, faster, and cheaper than chemical detection of a restricted selection of chemicals. During the last 20 years, online biomonitoring was developed for a variety of organisms, such as bacteria, algae, fish, mollusks, and crustaceans [3]. A variety of biological responses, ranging from biochemical and physiological responses to simple movements and complex behavior can be measured online by means of automatic detectors [3]. Because of the key position larvae of members of the family Chironomidae have among the invertebrates in freshwater ecosystems, they are receiving increasing attention in ecotoxicological studies, especially concerning the benthic, abundant, and pollution-tolerant *Chironomus* [4–6]. The nonbiting midges of the genus *Chironomus* commonly are used in whole effluent toxicity testing [7]. A study by Gerhardt and Janssens de Bisthoven [8] showed that the behavior of *Chironomus* measured with the Multispecies Freshwater Biomonitor® (MFB; Limco International, Ibbenbüren, Germany) could indicate polluted water. However, other than a few case studies [8,9] that showed the potential of using the behavioral responses of *Chironomus* as early warning signals, the use of *Chironomus* in online biomonitoring needs to be further investigated, calibrated, and validated.

In the present study, behavioral change was investigated to indicate sublethal responses and effects of AMD pollution. Behavior was chosen because of its nondestructive nature, in opposition to other biomarkers, its fast response time and sublethal sensitivity, and the possibility for online automated recording with the MFB [3]. The MFB can be installed at risk sites for continuous monitoring of water quality [3,10]. The aim in the present study was to determine in 24-h acute exposures at which AMD level the midge larvae start showing behavioral stress, whether such stress reactions can be used in situ in the MFB, whether the behavioral responses occur in the sublethal range, and to test whether changes in metal bioaccumulation occur within 24 h of exposure to AMD.

MATERIALS AND METHODS

Study site

The São Domingos mine is situated in the watershed of the Chança River (tributary of the Guadiana River) at the border

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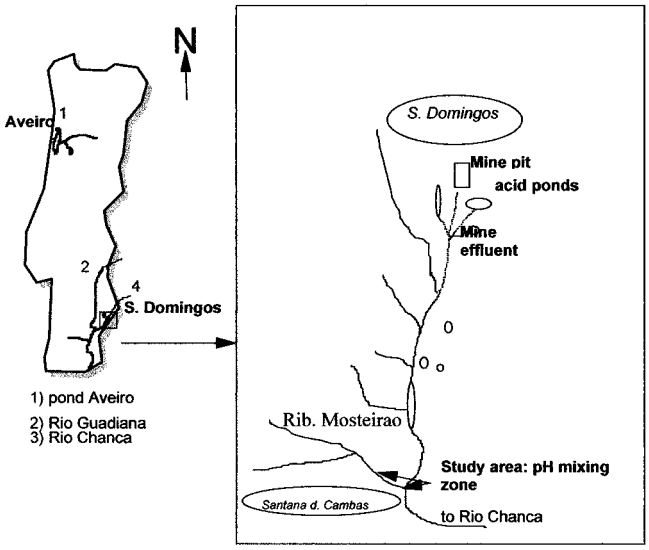

Fig. 1. Map of study area (Portugal).

with Spain in Alentejo, southern Portugal (Fig. 1). The climate is semiarid. The mine is of the cupriferrous pyrite type and has been abandoned for more than 30 years. Over the years, shafts and retention basins were filled with water and a continuous stream of AMD with a pH of 2.3 now flows down the main excavated valley toward the Chança River. Along its course, the AMD receives a few tributaries with pH 6 to 7, creating small mixing zones. By the time it reaches an artificial reservoir on the Chança River, the water pH may have climbed to 5 in rainy periods. However, the pH may remain very low in dry periods. Therefore, it is appropriate to describe this system as a dynamic pH gradient that is dependent on pluviometry. At the confluence of the Mosteirao stream (southern Portugal) with the AMD main channel, a dynamic pH gradient between 6.4 and 2.3 exists over less than 200 m and provided test water for the laboratory exposures and the AMD pH 5.5 site for the in situ experiment. The pond from which the larvae originated, situated on the university campus in Aveiro (Portugal), provided control water for the laboratory exposures. The sites from the AMD gradient were named according to their pH value.

Test animals

We used fourth instars of members of the genus *Chironomus*, sampled in a concrete basin (called a pond in the text) on the campus of Aveiro University, because of its proximity to the laboratory. Chemical data are presented in Table 1. For the field experiment, the larvae were transported in cool boxes to São Domingos. Another study (L. Janssens de Bisthoven, unpublished data) confirmed the presence of *Chironomus* spp. in the AMD, in small densities, so that the use of *Chironomus* spp. could be considered as ecologically relevant for the mine area. We collected larvae in the field because the AMD was anticipated to be highly toxic and we expected field-collected larvae to be more resistant than the usual cultured larval *Chironomus riparius*. Moreover, the whole experiment was expected to be ecologically more relevant if conducted with field-collected larvae rather than cultured larvae. After identification with larval morphological characters according to the key of Webb and Scholl [11], the species obtained could be assigned to three species groups (group allocation of Lindeberg and Wiederholm [12]): *Chironomus* group *thummi* (10%), *C.* group *plumosus* (18%), and *C.* group *anthracinus* (72%), which were equally distributed over all experimental conditions.

Experimental design

AMD experiment. The short-term (24-h) exposure was performed with fourth instars of *Chironomus* with water from the natural pH gradient in the São Domingos mine (pH 5.5, 4.4, and 3.3) and reference water (pond) (chemical data are given in Table 1) and without addition of food. The laboratory exposures were carried out in a climate room at 20°C, a 16:8 h day:night cycle, and illumination of each channel with two 30-W neon lights. The pH, conductivity (330 WTW pH electrode, VWR International, Frankfurt, Germany), oxygen, and temperature were recorded daily. Water temperature was between 17.0 and 18.3°C. The pH values measured did not vary much from the nominal pH values (standard deviation [SD] = 0.02–0.17) and showed no drift. Oxygen concentration remained above 7 mg/L. The setup was composed of a series of triplicate water-filled 5-L polyethylene buckets, from which water was pumped via a multichannel pump (maximal speed of 18 ml/min, renewal time of water approximately 4 h; Watson/Marlow 2058, Fallmouth, UK) to a series of artificial flow-through channels (polypropylene of 40-cm length, 16.5-cm width, and 15-cm depth, and a volume of 4 L). The overflow of each channel was directed back to the respective buckets, thus creating a closed circuit. Each bucket, aerated by an air stone, contained a polyethylene box with 10 larvae. These larvae were used to visually record survival after the end of

Table 1. Mean metal content of the water (μg/L, mg/L) and larval *Chironomus* (μg/g dry wt) after 24 h exposure to acid mine drainage (pH 5.5, pH 4.4, and pH 3.3) and the control treatment (pond)

	As	Ca	Cd	Cl	Co	Cu	Fe	K	Mg	Mn	Na	Pb	S	Zn
							Water							
Pond	5.50	49.10	0.26	53.15	0.80	23.82	189.24	20.08	10.88	60.8	49.93	9.55	13.01	83
pH 5.5	23.50	245.61	12.10	114.38	64.80	172.00	929.6	11.44	145.36	2.32	113.61	13.3	410.96	3,310
pH 4.4	36.37	227.21	19.00	104.68	147.58	701.00	1,129.7	10.59	135.24	3.77	105.44	43.2	410.43	5,646
pH 3.3	19.06	223.09	42.57	103.01	371.67	2,114.00	8,604.2	10.46	128.95	8.10	98.89	182.0	482.75	12,594
							Larvae							
Pond	7.01	—	0.13	—	0.55	54.69	2,054.64	—	—	70.73	—	60.77	—	285.66
pH 5.5	82.18	—	0.41	—	6.55	120.45	5,055.33	—	—	502.69	—	72.00	—	260.10
pH 4.4	142.25	—	0.16	—	3.07	51.63	6,702.45	—	—	204.99	—	42.76	—	125.48
pH 3.3	60.88	—	0.28	—	1.99	29.31	2,655.15	—	—	95.65	—	11.34	—	151.56

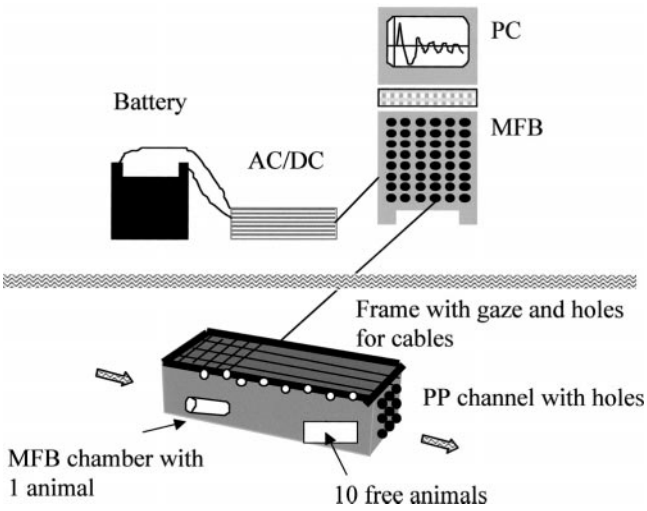

Fig. 2. Experimental setup for the in situ experiments. PC = personal computer; MFB = Multispecies Freshwater Biomonitor; AC/DC = analogue–digital converter; PP = polypropylene.

the 24-h exposure. These polyethylene boxes (10 × 10 × 5 cm) had windows on all sides, covered with 100-μm-mesh-size nylon nets, except in the bottom. Each flow-through channel contained four chambers connected to the MFB (see below), each containing one individual larva, to record online the behavior and time to death during the 24-h exposure. A few larvae escaped through the nettings from the boxes and the MFB chambers. Therefore, mortalities were calculated on the basis of the number of surviving nonescaped and escaped (still in the same water, but swimming in the bucket or the artificial channel) larvae.

Acid experiment. In a second set of experiments, midges were exposed in duplicate channels during 24 h to pond water (chemical data are given in Table 1), and to pond water that was acidified to pH 3.3, 4.4, and 5.5 with HNO_3, to test whether the observed behavioral reactions in the first experiment were influenced by metal mixture toxicity plus acidity or rather by acidity alone. No pH drift occurred. All experimental conditions were as in the AMD experiment. Escape was prevented by gluing finer nettings to the various containers.

Field experiment. The same artificial channels as used in the laboratory were placed in duplicate in the pH gradient of the AMD mixing zone, which at the time of the experiment had a pH of 5.5 (Fig. 1). To allow for a continuous flow-through, nine 9-mm-diameter holes were bored at both short sides and covered with 1-mm-mesh nylon nets to prevent intrusion of outside animals (Fig. 2). The channels were topped by a wooden frame that spanned a net to prevent disturbance from above. In the wooden frame, 8-mm-diameter holes were bored to pass the cables of the MFB chambers. The channels were placed parallel to the current and fixed to poles. Each channel contained a box with 10 free-living individuals and four MFB chambers each containing one individual. At the start of the exposure (after a 1-h acclimation time), and after 14 h, the chambers were connected to the MFB (and a laptop computer), which was powered by a battery. The animals' behavior was then recorded in 4-min traces. A catastropic flood event caused by a sudden hail storm stopped the experiment after 18 h.

Metal analysis

Metals and other elements in the water and from a duplicate of two to three surviving larvae per treatment (only the AMD experiment) were analyzed with inductively coupled plasma–mass spectrometry for low concentrations (Cd, Co, Cu, Zn, Mn, Pb, and As) and with inductively coupled plasma–atomic emission spectrometry for higher concentrations (Fe, Na, Mg, S, Ca, and high As values). Chlorine was determined with ion chromatography. Replicate analyses agreed very well with each other. The larval samples were analyzed after one digestion in 500 μl of 30% HNO_3 at 80°C overnight, a second digestion in 100 μl of H_2O_2 at 80°C, and then addition of 100 μl of HNO_3 and 900 μl of ultradistilled water. No difference in metal content in the water was found at the start and at the end of the experiments.

Multispecies Freshwater Biomonitor

The MFB allows for repeated recording (4-min traces every 10 min) of the behavior of up to 96 test chambers, each containing one animal. The animal moves freely between two pairs of steel plates that serve as electrodes. Movements of the animal provoke typical signals by impedance conversion [13]. Data analysis is based on a discrete fast Fourier transformation, resulting in a histogram of the behavioral frequencies that occurred in the original behavioral signal. Different behaviors can be assigned to different frequencies. The low frequencies (0.5–2.5 Hz) were summarized in band 1; the higher frequencies (3.0–8) were summarized in band 2. Band 1 expresses the percent of time the animals spend on locomotory and other slow activity and is referred to in the study simply as locomotion. Band 2 expresses the percent of time the animals spend on faster movements, such as undulating with the body in a regular monofrequent pattern (ventilation). For additional details, see Gerhardt [3,14] and Gerhardt and Janssens de Bisthoven [8]. The time spent on different frequencies can be plotted as a function of exposure time. These long-term graphs are performed by the MFB software. For detailed manual statistical analysis, transformations, curve fitting, and layout, the long-term data were processed in Statistica® (StatSoft, Tulsa, OK, USA).

Statistics

Normality (chi-square goodness of fit) and homogenity of variance (Levene's test) were tested on the data before analysis of covariance. Mortalities (after transformation of the frequencies to arcsine($x^{1/2}$) of the free-living animals were compared between the pH conditions by one-way analysis of variance. To test the eventual influence of the MFB chamber on larval survival, mortalities in the MFB chambers (visual recording of surviving animals at the end of the exposure) were compared with a chi-square test among each other and with the means of the mortalities of the free-living animals. Furthermore, mortalities were transformed to probits and put in linear regression with pH and metal values to determine the pH (always in association with highly correlated metals) or some selected metals at which 50% of the larvae would die. In the case of mortality $< 50\%$, extrapolation of the regression line provided the median lethal concentration (LC50). From the continuous MFB data, cumulative survival curves (in probit) in function of natural logaritm (ln) (time) were constructed to calculate the time after which 20% of the organisms have died (LT20). After the MFB data were transferred via Excel®

(Microsoft, Redmond, WA, USA) to Statistica, graphs of means for band 1 (summarizing slower locomotory movements) were constructed and fitted to least squares. Because the faster ventilatory movements represented on average <6% of the activity time, they were not graphically represented. For every time block of 6 h, the band 1 and band 2 data were compared by analysis of covariance (covariate time) after arcsine($x^{1/2}$) transformation between the different experimental conditions. Degrees of freedom were provided by the number of 4-min recordings × number of larvae. Bioaccumulation data were compared after ln(x + 1) transformation by one-way multivariate analysis of variance between the different AMD conditions and correlated by Spearman rank tests with aqueous concentrations and with the pH values of the respective sites. Linear relationships were explored by forward stepwise linear regressions.

RESULTS

Metal concentrations

Water. The chemical characteristics of the water are given in Table 1. The metals Cd, Co, Cu, Fe, and Zn, as well as S, increased with decreasing pH (Spearman rank test , $r < -0.73$, $p < 0.04$).

Metals in larvae. Chironomus showed increasing As content from high to low pH (Spearman rank test, $r = -0.82$, $p < 0.04$). Of all the metals (Table 1), only As in water and larvae correlated positively (Spearman rank test, $r = 0.83$, $p < 0.04$). The larvae exposed in the pond water contained significantly less As than those exposed to pH 4.4 and pH 5.5 (analysis of variance, $F = 5.5$, $df = 6, 13$, $p = 0.005$). The larvae exposed to pond water contained less Co ($F = 30.7$, $df = 6, 13$, $p < 0.0001$) and Mn ($F = 10.04$, $p < 0.0001$) than the larvae exposed to pH 5.5. Surprisingly, the larvae exposed to pH 3.3 contained significantly less Cd, Co, Cu, Mn, and Pb than larvae from pH 5.5 ($F > 3.8$, $df = 6, 13$, $p < 0.02$).

Mortalities

No statistical difference existed between the mortalities of the larvae in the MFB chambers and free-living larvae ($p > 0.05$). Mortality of free-living larvae and MFB larvae in the AMD exposure ranged between 5 and 20% and was not different among the treatments. Within the acid experiment, mortality ranged between 0 and 15%. The control (pond) mortality was 5%. The mortality of *Chironomus* generated in the acid water exposure of pH 3.3 (5%) was lower than in the pH 3.3 AMD water exposure (11%) ($F = 8.97$, $df = 1, 5$, $p = 0.03$). In the field AMD (pH 5.5), mortality of *Chironomus* was higher (20%) than in the laboratory exposures (4.7%; $p < 0.05$). The overall low mortalities and the absence of clear differences among the treatments indicates that the experimental exposures were in the sublethal range for fourth instars of *Chironomus*.

The regression equation between probits and pH for the laboratory AMD experiment was $y = 5.91 - 0.53$ (pH) ($r^2 = 0.47$, $p < 0.05$). For the acid experiment, the equation was not significant. The LC50 (pH + metals) for laboratory AMD was not reached and therefore was estimated by extrapolation at << pH 3.3 (+ associated metals). The LC50s for selected metals were estimated as Cu, 3,000 μg/L, Fe, 17,000 μg/L, Pb, 290 μg/L, Zn, 18,000 μg/L, Mn, 11,000 μg/L.

A forward stepwise multiple regression analysis on aqueous Cu, Fe, Pb, and Zn revealed a mortality–metal dependency with the following equation: mortality probit = $2.38 + 6.10^{-6}$ (Zn) $- 6.10^{-4}$ (Fe) $+ 3.10^{-3}$ (Cu), with intercept and the slope of Fe being significant ($r^2 = 0.58$, $p < 0.05$). When Mn was added in the analysis, the slope of Cu became significant as well. A similar analysis between body burdens and mortality probits revealed bivariate linear regressions between the single metals Cd, Cu, Pb, and Zn ($r^2 > 0.3$, $p < 0.05$) and a multiple regression, mortality probit = 2.5 + 0.0003 Zn − 0.005 Co ($r^2 = 0.4$, $p < 0.03$).

Only two treatments had enough mortality data for LT20 determination. In the acid at pH 3.3, 20% of the larvae died after 1 h and in the AMD at pH 4.4, 20% of the larvae died after 22 h.

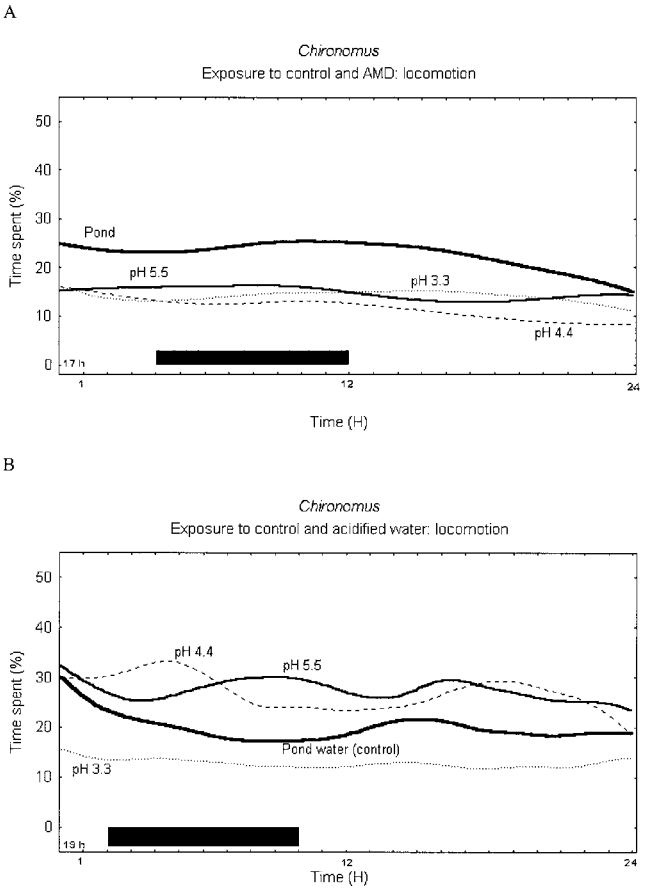

Fig. 3. Mean percentage of time spent on locomotory activity of *Chironomus* larvae (n = 5–15 larvae) to (**A**) acid mine drainage (AMD) water and control pond and to (**B**) acid stress and control pond during 24 h. Dark bar = night. Least square fit of the means. The variance is explained in the text. Online data provided by the Multispecies Freshwater Biomonitor.

Behavior

The normal behavior of *Chironomus*, observed in pond water, consisted of periods of locomotion and periods of ventilation. The percentage of time spent on locomotion ranged on average between 15 and 28 ± 15% SD (Fig. 3) and was less than 6 ± 15% SD for ventilation.

In the acid-only experiment, no significant difference in activity time could be discerned between day and night. The activity in pH 3.3 remained lower than in the other conditions during the course of the exposure ($p < 0.05$), showing morbidity. The mean activity time ranged between 12 (pH 3.3) and 27 (pH 5.5) ± 15% (SD) (Fig. 3).

In the AMD exposure, no consistent difference in time spent on locomotion could be discerned between day and night. The locomotion activity, which was similar in pH 3.3, 4.4, and 5.5 (~15%), was the highest in the pond water (~20%; $p < 0.05$). The mean percentage of activity time ranged between 11 (pH 4.4) and 15 (pH 5.5) ±15% (SD).

One-hour field exposure of larvae to AMD at pH 5.5 generated higher activity (25.6%, standard error [SE] 2.9) than in the laboratory (13.3%, SE 1.6; $F = 21.9$, $df = 1,17$, $p < 0.001$). However, after 14 h, the difference had disappeared (respectively, 15.8%, SE 3.7, and 9.6%, SE 4.9).

Activity in acid only was generally higher than in AMD treatments ($p < 0.05$).

DISCUSSION

Tolerance of larvae of the Chironomini *to acidity*

Larvae of *Chironomus* and other species from the tribe Chironomini are more tolerant to acid-metal stress than are other Chironomidae [15], and therefore are suitable candidates for online biomonitoring in AMD. This tolerance is largely attributed to the presence of hemoglobin in the hemolymph, which is alkaline (pH 7.2–7.7 [16]) and has a buffering capacity [17]. Jernelöv et al. [17] demonstrated that acid-adapted larval *Chironomus* contain even more hemoglobin with higher buffering capacity than nonadapted larvae. The adapted larvae could survive 7 d in pH 3.5. The exposures to AMD confirmed this tolerance with a calculated LC50 < AMD at pH 3.3.

Accumulation of metals in larvae as a function of pH

Larvae are documented to accumulate more metals and other elements when water acidity is higher. In a study on acidified lakes, this was the case for Al, Mn (highest effect), and Zn; however, it did not hold true for Cu, Fe, S, and P [15]. Krantzberg and Stokes [18] mentioned Al, Cd, Cu, Fe, Mn, Ni, and Pb to be higher in chironomids originating from pH 4.4 to 5.5 than at pH levels higher than 5.8. On the other hand, larvae from metal-contaminated lakes are able to regulate accumulation of Cu, Ni, and Mn [19], and Zn and Cu are essential regulated metals in *Chironomus* [20]. After 14 d of exposure to a mixture of metals in a synthetic sediment, Harrahy and Clements [21] found the highest bioaccumulation rates for Cd and the lowest rates for Pb, with Cu and Zn having intermediate values. Uptake was strongly correlated with exposure time. Examination of our chemistry data suggests that 24 h is too short of a period to allow for a significant uptake of metals by the larvae, except for arsenic. This could be explained by the fact that As, and to a lesser extend Pb, are bound externally on the body surface and to food particles present in the gut, in contrast to Cd, Cu, and Zn, which are bound in the body tissues [22]. The lack of metal accumulation is in sharp contrast to the results of Timmermans et al. [20], who obtained bioaccumulation of Cd, Pb, Cu, and Zn within 24 h; however, their results were obtained in lake water of pH 7.6. Low pH and high metal concentrations apparently play antagonistic roles in the bioaccumulation of metals from AMD. This is confirmed by findings of Campbell and Stokes [23], who predicted a decrease of metal uptake with increasing H^+ competition for binding sites. In the present study, lower body burdens of metals were found at pH 3.3. Indirectly, physiological effects of low pH also could change the bioaccumulation.

Mortality and behavior

Although the larvae did not show much pH dependency of metal burden, examination of the mortality and behavior data indicated sublethal toxic stress. The stress may be partly due to the acidity, especially at pH 3.3. During most of the exposure to pH 4.4 and pH 5.5, larvae exposed to AMD showed significantly lower activity than larvae exposed to acidified pond water of similar pH. This higher activity in the acid water could be interpreted as an escape response to acid stress, whereas the higher morbidity in the AMD may be a response to stress by metal mixture plus acidity. The estimated 24-h LC50 for Cu of 3,000 μg/L is two times the 96-h median effective concentration (where the effect is immobilization) found by Gauss et al. [24] for hard water, which seems plausible, given the different experimental conditions (mixture toxicity vs single metal). Moreover, our Cu data fit (after extrapolation) to the single-species 48-h LC50 of 1.2 mg/L [25] and of 0.74 mg/L [26]. For Zn, the LC50 corresponded roughly with results of Khangerot and Ray [27]. However, for Pb, our LC50 was ±180 times lower than data from the same study [27]. The use of fourth instars of *Chironomus* to test the toxicity of whole effluents underestimates the toxicity for the viability of the species, as suggested by Gauss et al. [24], who found a much higher sensitivity to Cu in first instars. However, the small size of first instars makes it difficult to sample them in the field and makes them sensitive to transport and handling stress. Hence, at this stage, first instars are not practical for routine biomonitoring. Moreover, currently most ecotoxicological data for *Chironomus* involve the fourth instar, allowing for comparison. An acute 24-h biotest obviously will provide responses that are only relevant when extrapolated to short-term pulse events.

The use of field-collected larvae

Chironomus is a genus that has a difficult taxonomy [11,28]. Because we experienced incongruencies when using the key of Webb and Scholl [11], we preferred to refer to the genus name only. The use of a species consortium of *Chironomus* concurs with the reality [29,30]. In field studies, *Chironomus* often remains unidentified or is assigned to a species group. For online biomonitoring tests, collection of larvae from the field as test organisms may be more cost effective than the long-term maintenance of a laboratory culture, provided a relatively unpolluted field site with sufficient larvae is available. We expect field-collected larvae to be less prone to artifacts due to varying environmental conditions other than pollution. The choice between cultured larvae and field-collected larvae remains a point of debate, depending on the scientific goals, the financial possibilities, and weighed against the level of sensitivity wanted.

Automated biomonitoring and behavior

The good swimming and movement ability of *Chironomus* has been used in several studies to detect changes in water pollution [9,31–33]. In all these studies, the main response was a decreased activity, eventually with an increased ventilation, irrespective of the type of pollution (organics or metals). Another study demonstrated behavioral responses of *Chironomus* in polluted river water, measured online with the MFB [8]. This study not only demonstrated that *Chironomus* had different levels of activity (e.g., passivity, escape behavior, and ventilation) as a function of differently polluted sites, but also depending

on other sublethal effects such as morphological deformities and depleted developmental rate. Simultaneously to our experiments, local mayflies (*Choroterpes picteti*) also were evaluated with the MFB in the São Domingos mine and showed similarly lower metal body burdens at pH 3.3 than at higher pH of AMD, similar mortalities, and a day–night activity. (A. Gerhardt, unpublished data.)

In the present study, we tested natural AMD in the laboratory, followed by a field test for pH 5.5. In contrast to more classical approaches where laboratory tests with artificially spiked water are compared with field assays [7], our approach can be qualified as whole-effluent toxicity testing under controlled conditions. Stuijfzand et al. [34] demonstrated the protective role (against metals) of humic acids present in the water in situ on larval *Chironomus*. That shows that field validation of laboratory tests remains crucial. Our preliminary field study illustrates the possibility of doing online biomonitoring with the MFB, when using larvae of *Chironomus*. The laboratory experiment was capable of differentiating pH 3.3 (plus associated metals) from the less affected sites. We expect a finer differentiation of the sites with increasing time of exposure. Further in situ validation with water-only and solid-phase conditions are required.

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