

Evidence for the Stepwise Stress Model: *Gambusia holbrooki* and *Daphnia magna* under Acid Mine Drainage and Acidified Reference Water Stress

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The Stepwise Stress Model (SSM) states that a cascade of regulative behavioral responses with different intrinsic sensitivities and threshold values offers increased behavioral plasticity and thus a wider range of tolerance for environmental changes or pollutant exposures. We tested the SSM with a widely introduced fish *Gambusia holbrooki* (Girard) (Pisces, Poeciliidae) and the standard laboratory test species *Daphnia magna* Straus (Crustacea, Daphniidae). The stress was simulated by short-term exposure to acid mine drainage (AMD) and to acidified reference water (ACID). Recording of behavioral responses with the multispecies freshwater biomonitor (MFB) generated continuous time-dependent dose–response data that were modeled in three-dimensional (3D) surface plots. Both the pH-dependent mortalities and the strong linear correlations between pH and aqueous metals confirmed the toxicity of the AMD and ACID gradients, respectively, for fish and *Daphnia*, the latter being more sensitive. AMD stress at $\text{pH} \leq 5.5$ amplified circadian rhythmicity in both species, while ACID stress did so only in *G. holbrooki*. A behavioral stepwise stress response was found in both species: *D. magna* decreased locomotion and ventilation (first step) (AMD, ACID), followed by increased ventilation (second step) (AMD). *G. holbrooki* decreased locomotion (first step) (AMD, ACID) and increased ventilation at intermediate pH levels (second step) (AMD). Both species, although from different taxonomic groups and feeding habits, followed the SSM, which might be expanded to a general concept for describing the behavioral responses of aquatic organisms to pollution. Stepwise stress responses might be applied in online biomonitors to provide more sensitive and graduated alarm settings, hence optimizing the “early warning” detection of pollution waves.

Introduction

Exposure time is a crucial mediator of toxicity. However, it is underestimated in risk assessment schemes, which currently rely on the use of fixed end points such as LC_50 's, EC_{50} 's, and NOECs as the basis of deterministic or probabilistic ecological risk assessment (1). Alternatively, new approaches

concentrate on regression model fittings to toxicity data from different end points (1, 2) and consideration of exposure time in acute toxicity (mortality) and bioconcentration kinetics (3–5). The application of the time-to-death concept, described more than 30 years ago (6), has been reluctant (7), even though response surfaces of mortality as a function of toxicant concentration and acute exposure time have successfully been used to predict chronic toxicity of metals in *Daphnia magna* (8). Next to prediction of mortality, 3D concentration–response–time surfaces might predict sublethal end points (e.g., behavior) as well, taking empirical models (e.g., Stepwise Stress Model, see below) as rationale (9). Behavioral parameters add more sensitivity and ecological relevance to standard toxicity testing (10) and represent either reversible transient short-term responses to an external stressor in order to maintain the organism's functions, which are used in online biomonitors, or represent adaptive effects after long-term exposure to stressors (11).

Exposure time is indeed an important parameter for early detection of pollution waves, performed in so-called “Biological Early Warning Systems” (BEWS) or online biomonitors. These fully automated instruments use sensitive nondestructive behavioral or physiological responses of a test species to rapidly detect sublethal pollution pulses of short duration (12). Since the development of quantitative recording systems (e.g., video, IR-light beams, ultrasound, and impedance techniques), computer-aided, automated online data recording and analysis methods based on concentration–response–time analysis, behavioral ecotoxicology, and its application in online biomonitors are expanding (12).

The Stepwise Stress Model (SSM) is a conceptual model, postulating that an organism displays a time-dependent sequence of different regulatory or compensatory behavioral stress responses during exposure to pollutants above their respective thresholds of resistance (12, 13). When, for example, exposed to a pollution pulse, the first response might be avoidance (increased swimming), followed by a second response later on under continued exposure, which might be increased ventilation of the gills. Such a cascade of stress responses was first observed for *Gammarus pulex* (12, 13). Avoidance responses in *G. pulex* have been observed under insecticide exposure (14). We anticipate an ecological advantage behind this strategy of behavioral plasticity, which might result in a wider range of tolerance toward environmental change and pollution stress.

The aim of the study was to validate the SSM with two different species, the standard toxicity test species, *D. magna*, and the fish species *G. holbrooki* with the multispecies freshwater biomonitor under short-term exposure to a whole effluent of acid mine drainage (AMD) and acid reference water (ACID). Once validated, the SSM allows for a new evaluation and understanding of alarms in automated online biomonitors, which are widely used for biomonitoring of surface water toxicity in large European rivers. Species following the SSM may allow for more sensitive stepwise alarm definitions.

Methods

Study and Reference Sites. São Domingos Mine in Portugal (37°39'56" N, 7°28'46" W) is an abandoned cupriferrous pyrite mine still emitting a permanent, seasonally varying flow of AMD (acids, metals, salts). The study zone was at the confluence of the AMD with the stream Mosteirão, a dilution zone, and pH gradient (15–17). Next to the mine, a water reservoir, where the test species *G. holbrooki* occurred was taken to provide reference water for the experiments. Water

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TABLE 1. Concentrations of Metals and Salts in Water of the Acid Mine Drainage in the Laboratory Experiments with *Gambusia holbrooki* and *Daphnia magna*

pH	As ($\mu\text{g/L}$)	Ca (mg/L)	Cd ($\mu\text{g/L}$)	Co ($\mu\text{g/L}$)	Cu (mg/L)	Fe (mg/L)	K (mg/L)	Mg (mg/L)	Mn (mg/L)	Na (mg/L)	Pb (mg/L)	Zn (mg/L)	S (mg/L)	Cl (mg/L)
3.3	19.1	223.1	42.6	317.7	2.1	8.6	10.5	128.9	8.1	98.9	182.0	12.6	482.7	103.0
4.4	36.4	227.2	19.0	147.6	0.7	1.1	10.6	135.2	3.8	105.4	43.2	5.6	410.4	104.7
5.0	7.5	233.5	14.0	76.6	0.5	0.2	10.7	138.3	2.9	108.5	27.9	3.9	418.3	109.0
5.5	23.5	245.6	12.1	64.8	0.2	0.9	11.4	145.4	2.3	113.6	13.3	3.3	410.9	114.4
6.4	5.8	228.2	5.5	27.4	0.1	0.2	11.0	136.8	0.5	105.6	4.7	1.0	422.0	114.2
6.8	20.5	32.1	0.2	0.2	0.02	0.03	7.5	12.9	0.0	37.1	8.0	0.2	28.9	44.3

chemistry data from the field sites in different seasons are given elsewhere (16).

Test Animals. *G. holbrooki* Girard (Pisces, Cyprinodontiformes: Poeciliidae), known as “mosquitofish”, is a small viviparous key predator with an intermediate position in the food web, feeding mostly on copepods, cladocerans, rotifers, and insects at the surface (18). It has been proposed for monitoring pollution exposure, bioavailability, and biological effects (19). The fish, native from the coastal region of the Eastern United States, has been widely introduced through mosquito control programs all over the world. On the Iberian peninsula, *G. holbrooki* plays an important role as predator in agro-ecosystems such as rice fields and has invaded the lowest stream sections, wetlands, and coastal lagoons (18). *D. magna* is the largest herbivorous cladoceran in the Northern Hemisphere and inhabits eutrophic small ponds, permanent or temporary (20). Daphnids are widely used in toxicity tests for quantifying the toxicity of single (21) or multiple (22) substances, especially metals (23), estimating NOEL and MATC values (24). Being one of the test organisms in the OECD guidelines (e.g., refs 25 and 26), *Daphnia* spp. is used in toxicity tests based on immobilization and more recently also on swimming activity, a parameter highly relevant for online toxicity monitoring (12). We performed our experiments with the *D. magna* clone BEAK issued from the culture at the University of Aveiro.

Multispecies Freshwater Biomonitor. The Multispecies Freshwater Biomonitor (MFB), based on quadropole impedance technology, is an automated biotest and biomonitor system, recording behavioral parameters (time spent on locomotion and ventilation) and survival (time to death) of aquatic invertebrates and vertebrates exposed individually in flow-through test chambers (12, 13, 27, 28).

Experimental Setup. Short-term toxicity bioassays (48 h) with water from the natural pH/metal gradient (pH 6.4, 5.5, 5.0, 4.4, 3.3; replicates: 3) of the AMD as well as reference water acidified with HNO_3 (*G. holbrooki*: reservoir; *D. magna*: ASTM (American Society for Testing and Materials)) were performed in a climate room (20 °C, 16:8 h photoperiod, illumination with two neonlights of 30 W). Unfiltered water was recirculated through artificial streams (polypropylene: 40 cm \times 16.5 cm \times 15 cm, 4 L), leading to a renewal time of 4 h. In each stream-channel 6 cylindrical test chambers (length 4 cm \times depth 2 cm) were placed, containing each one individual (either fish (length ca. 2.0 cm) or daphnid in separate experiments (length ca. 0.25 cm)). Moreover, 15 free swimming organisms of the respective species were placed in the channels for daily visual control of survival according to the LC_{50} approach.

Chemical Analyses. The following elements were analyzed as total concentrations in the water from each treatment: With inductively coupled plasma mass spectrometry (ICP-MS), we determined low concentrations (below 30 ppb) of Cd, Co, Cu, Zn, Mn, Pb, and As. With inductively coupled plasma atomic emission spectrometry (ICP-AES), we determined higher concentrations of Fe, Na, Mg, S, Ca, and As. Chlorine was analyzed with ion chromatography (Table 1).

Modeling of Relationships in Water Chemistry Parameters. Forward stepwise multiple linear regression analyses were used to describe relationships between (i) metals in water versus pH and (ii) mortality probits versus aqueous metals. Moreover, binary linear regression analysis was used to describe the relationship between mortality probits and pH in the AMD and ACID exposures for both species.

Survival Modeling. LC_{50} was assessed by regression of mortality percentages transformed to probits (29) versus pH of the whole AMD effluent and the acidified reference water ACID. The continuous survival data generated by the MFB were used for time-to-death (TTD) analysis. LT_{50} 's were calculated using the log-logistic survivor function, and statistical comparisons were made with a χ^2 test for censored TTD data.

In a conservative approach, we applied partial linear 3D bivariate models with two explanatory factors (time, pH) and one response variable (mortality) instead of bivariate non-linear regression models, such as correlated Gaussian, mixture, and product functions (30). We anticipate the two explanatory factors to be linear within specified ranges. The approach based on 3D concentration–response–time surfaces (3D CRT surfaces: concentration = pH in AMD or ACID; response = mortality) was applied for mortality probits according to models described in the literature (8, 29, 31). Two basic models are described for quantal data (31), Model A, corresponding to a linear multiple regression:

$$\text{mortality expressed in probits } P = \alpha + \beta C + \gamma T \quad (1)$$

where the derivate of P with respect to C (concentration) is constant at each acute exposure time T (parallelism), and model B, including an interaction term $C \times T$:

$$P = \alpha + \beta C + \gamma T + \delta [C \times T] \quad (2)$$

which allows for a change in slope as exposure time varies. C and T can be included in the models as actual values or logarithms, T also as reciprocal (the latter case has been used by Barata et al. (8)), all leading to different forms of the same basic models. The parameters α , β , γ , and δ were estimated by an iterative convergence quasi-Newton process with as loss function, (observed – predicted)². *D. magna* exposed to ACID followed model A with $C \times T = 0$. In the case $C \times T > 0$, we used model B. The obtained equations allow for an extrapolation of toxic responses (mortality) in function of time for the pH range of this study, hence facilitating predictions of LOEC and chronic toxicity.

Behavioral Modeling. Even though the above-mentioned model (eq 2) has originally been developed for quantal data, the same models were used for modeling the continuous behavioral data because principally a function is independent of the type of effect distribution that is under consideration (2). Different types of behavior of the test species were assigned to specific signal frequency ranges by simultaneous visual observation and MFB recording in the real-time modus: *G. holbrooki*, 0–0.5 Hz: tail and body movements (“swimming”); and 2.5–3.0 Hz: regular monofrequent fast undulations of opercula and mouth (“ventilation”). *D. magna*

TABLE 2. Relationships ($p < 0.05$) between Mortality Probits ($0 < P < 10$) of *D. magna* and *G. holbrooki* Exposed to AMD or ACID and Aqueous pH, Metals (2D relationships, $P < 0.001$), and Additional Exposure Time (3D CRT surface models, see Figure 1)

species	exposure	regression equation	r^2
2D Relationships			
<i>D. magna</i>	AMD	$P = 0.53 + 3.06 \log[\text{Zn}] + 2.80 \log[\text{As}] - 2.6 \log[\text{Fe}]$	0.87
	AMD	$P = 15.24 - 1.73 \text{ pH}$	0.76
	ACID	$P = 14.93 - 2.01 \text{ pH}$	0.69
<i>G. holbrooki</i>	AMD	$P = 13.11 + 8.3 \log[\text{Cu}] - 3.9 \log[\text{Cd}] + 3.9 \log[\text{Zn}] - 1.9 \log[\text{Fe}] - 0.7 \log[\text{Co}]$	0.85
	AMD	$P = 13.88 - 1.82 \text{ pH}$	0.57
	ACID	$P = 11.61 - 1.58 \text{ pH}$	0.60
3D CRT Surface Models			
<i>D. magna</i>	AMD	$P = 10.6 - 1.48 \text{ pH} + 0.08T - 0.001 \text{ pH} \times T$	0.78
	ACID	$P = 14.93 - 2.01 \text{ pH}$	0.69
<i>G. holbrooki</i>	AMD	$P = 15.93 - 2.20 \text{ pH} + 0.04T - 0.003 \text{ pH} \times T$	0.79
	ACID	$P = 2.22 - 0.05 \text{ pH} + 0.21T - 0.03 \text{ pH} \times T$	0.53

showed at 0–0.5 Hz movements with the second antennae, defined as “swimming” and at 1–1.5 Hz “ventilation” with the pleopods. Behavioral data were normalized to reference data (pH 6.8) for each data point throughout the whole exposure period ($f(x) = x/\text{ref} \times 100$). This normalization flattened out the normal circadian rhythm, while any circadian variation left in the curves could be interpreted as amplification of the rhythmicity. 3D CRT surface plots (concentration = pH; response = behavior) were fit to least squares for graphical representation. The modeling was complicated by the underlying circadian rhythmicity. To mitigate this effect, we divided the whole plane into several response planes with different slopes, distinguished in dependence to exposure time or pH.

These segments could be linearized into planes with different slopes and intercepts along the pH axis or the time axis. The assumption for this procedure is that a change in the slope of the plane indicates a change in response (e.g., a slope of 0 indicates no response in either the time or the concentration axes, i.e., resistance, whereas a slope different from 0 indicates a response). The degree of slope along the pH axis indicates the sensitivity of the organism while along the time axis it indicates the rapidity of response. The intercept indicates the intensity of the response. Threshold values for the changes in slopes and intercepts could be derived from the surface plots and served as input values for the SSM. Some 3D CRT surfaces could not be linearized due to complex interaction effects between pH and exposure time on the circadian behavior of the test species. In one case, a complex early warning ventilation response in *Gambusia* created a polynomial function in both axes (pH and time), at best described by the quadratic function:

$$B = \alpha + \beta C + \gamma T + \delta [C \times T] + \theta C^2 + \sigma T^2 \quad (3)$$

Results

Metal Concentrations in the AMD Water. Most metal levels in the water were elevated as compared to background values for freshwater (Table 1) (16) and at pH 3.3 and 4.4 above the recommended threshold values for fish life (e.g., Cd: up to 40 times; Cu and Zn: ca. 100 times; Pb: 1000 times) (32). In the laboratory experiments with *D. magna* and *G. holbrooki*, metal concentrations of Cd, Co, Cu, Fe, Zn, and to a lesser extent Pb in the water increased with decreasing pH levels, whereas levels of As and Mn as well as concentrations of the group I and II metals were not pH-dependent (Table 1). A forward stepwise multiple linear regression analysis between pH and metal levels in the water from all experiments retained Zn, Cd, and Fe ($r^2 = 0.99$, $p < 0.05$, $\text{pH} = 6.75 - 0.76[\text{Zn}] + 0.09[\text{Cd}] + 0.34[\text{Fe}]$).

Survival: Relationship to pH and Metals. A forward multiple regression analysis revealed the following relationships between mortality probits (P), pH, and aqueous metals (Table 2). The mortality of *D. magna* in AMD was statistically mostly related to pH, Zn, Fe, and As, while that of *G. holbrooki* was mostly related to pH, Zn, Fe, Cd, Cu, and Co. The aqueous concentrations of Zn had the highest predictive capacity on mortality (probit) of *D. magna*, with $r^2 = 0.81$. The same analysis performed for *G. holbrooki* revealed Cu (aqueous) to be the best predictor of mortality ($r^2 = 0.75$). For both species, the regressions between probit and pH in AMD were not significantly different from the regressions in ACID. This indicates pH as most important determinant for mortality in both ACID and AMD.

Survival: Fixed End Point Analysis. The LC_{50} 's (Table 3) of animals exposed in the MFB chambers did not differ from those of the free-living animals within the respective experiments, hence showing that the captivity did not cause additional mortalities. Although both species had lower LC_{50} 's in the ACID experiment as compared to the AMD experiment, the difference was more pronounced for *G. holbrooki*, being clearly more sensitive to AMD than to ACID. *D. magna* proved more sensitive to both AMD and ACID than *G. holbrooki*.

The LT_{50} 's for both species in the AMD experiment were pH-dependent (Table 3), (*D. magna*: $X^2 = 50.6$; *G. holbrooki*: $X^2 = 22.97$, $p < 0.0001$). The LT_{50} analysis shows the rapidity of the responses. For example in AMD at pH 5.0, *G. holbrooki* reached 50% mortality within 10 h as compared to *D. magna* within >20 h. In terms of TTD, *D. magna* appeared to be slightly more sensitive to ACID stress (pH 3.3 and 4.4) and less sensitive to AMD stress (pH 4.4 and 5.0) than *G. holbrooki*.

Survival: 3D CRT Surface Models. Whereas the LT_{50} analysis gives response times for a pre-set and fixed number of animals (50%), 3D CRT allows for continuous analysis of the response time kinetics of all organisms. *D. magna* exposed to ACID showed with decreasing pH a steep linear pH-dependent increase in mortality independent of exposure time, hence indicating immediate strong toxicity. Under AMD exposure, exposure time caused additional increases in mortality, indicating increasing toxicity of metals and low pH as a function of time (Figure 1A, Table 3). *G. holbrooki* exposed to ACID showed lower mortalities as compared to AMD exposure at all pH values (see intercepts, Figure 1B, Table 3). During the first 12 h, a steep pH-dependent increase of mortality (ACID, AMD) was followed by a second increase on the second day of exposure in ACID at $\text{pH} \leq 4$. Whereas ACID stress caused mortality at $\text{pH} \leq 5$, AMD-toxicity increased linearly in the range of pH 7–3.3.

Normal Behavior and Circadian Rhythms. The behavioral signals of both species when exposed to water of their

TABLE 3. LC₅₀ Values (±95% CI) (pH, Probit Method), LT₅₀ Values (h, Logit Method) for *Gambusia holbrooki* and *Daphnia magna* Exposed to Acid Mine Drainage (AMD) and ACID in the Laboratory during 48 h^a

		<i>Gambusia holbrooki</i>			<i>Daphnia magna</i>		
		LC ₅₀ (95% CI) (pH)	r ²	p	LC ₅₀ (95% CI) (pH)	r ²	p
AMD	chamber	5.8 (-)			5.9		
	free	5.4 (4.8–5.9)	0.58	0.004	6.1 (5.8–6.4)	0.88	0.0005
	ch + fr	5.5 (5.2–5.9)	0.65	0.00002	6.0 (5.8–6.2)	0.68	0.0009
ACID	chamber	3.9 (-)			4.9 (4.5–5.1)	0.86	0.008
	free	4.3 (4.0–4.6)	0.89	0.0002	5.1 (4.2–5.6)	0.69	0.003
	ch + fr	4.2 (3.9–4.4)	0.89	0.003	5.0 (4.5–5.3)	0.62	0.00003

		<i>Gambusia holbrooki</i>			<i>Daphnia magna</i>		
		LT ₅₀ (95% CI) (h)	r ²	p	LT ₅₀ (95% CI) (h)	r ²	p
AMD	3.3	2.55 (2.4–2.7)	0.97	0	0		
	4.4	7.6 (7.2–8.0)	0.96	0	27.0 (26.0–28.3)	0.96	0
	5.0	9.4 (7.5–10.8)	0.86	0.0003	23.6 (21.0–26.3)	0.93	0.00002
	5.5	48			34.0 (30.7–38.4)	0.91	0.0007
	6.4	> 48			48		
ACID	3.3	14 (-)		ns	0		
	4.4	48			2.5 (-)		ns
	5.0				48		
	5.5	> 48			> 48		
	6.4	> 48			> 48		

^a LC₅₀ was calculated for free living animals (free), animals in the MFB chambers (chamber), and both pooled (ch + fr). LT₅₀ was calculated for animals from time-to-death data generated by the multispecies freshwater biomonitor. The r² and p values refer to the regressions between mortality probits and pH values and logits and time, respectively.

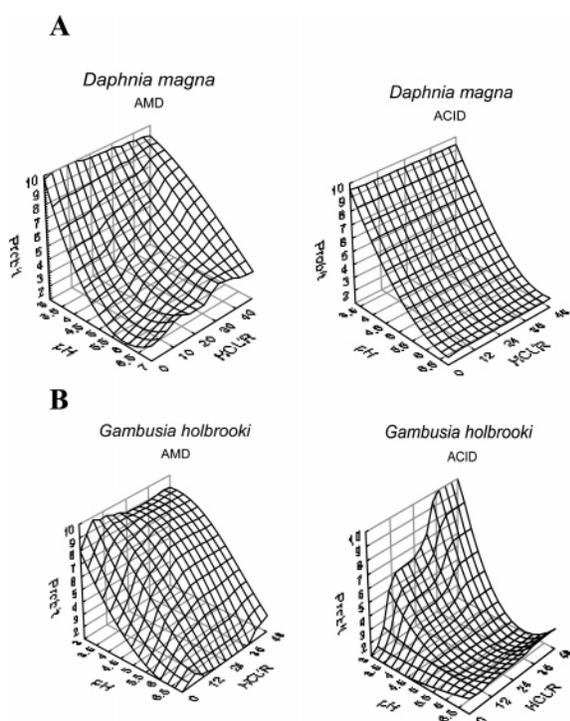


FIGURE 1. 3D CRT surface patterns of mortality (probits) as a function of pH and time of (A) *Daphnia magna* and (B) *Gambusia holbrooki*, when exposed during 48 h to acid mine drainage (AMD) from the São Domingos mine, Portugal, or ACID.

respective original habitat (*G. holbrooki*) or culture (*D. magna*) showed their normal behavior with inter-individual variability (*D. magna*: SE, ca. 10%; *G. holbrooki*: SE, ca. 20% of the means). During the night (Figure 2), *D. magna* showed increased swimming combined with decreased ventilation when exposed to control ASTM water. The fish *G. holbrooki* showed decreased swimming and increased ventilation at the end of the night (Figure 2), in antiphase with the rhythm of the zooplanktonic *D. magna*.

Behavior under Stress: 3D CRT Surface Models.

Under AMD stress an overall pH-dependent decrease in swimming and ventilation of *D. magna* (first pH step) was found between pH 7 and pH 5.5 (Table 5, Figure 3). From pH 5.5 on, the decrease of swimming continued with a steeper slope (second pH step), however, with amplified circadian rhythm. A similar but weaker pattern was found for ventilation. Toward the end of exposure, ventilation behavior increased again at low pH (second time step). To ACID exposure, *D. magna* reacted with a steep linear pH-dependent decrease in both swimming and ventilation (Figure 3) (one pH step, Table 5), which remained stable over time (Table 4). Both behavior types showed synchronous response surfaces and similar models. Whereas the circadian activity pattern in swimming and ventilation of *D. magna* was not affected under ACID stress, it was clearly amplified under AMD stress at pH ≤ 5.5.

When exposed to AMD, *G. holbrooki* showed a pH (pH step 1)- and time-dependent (first time step) decrease in swimming, followed by a second increase in swimming after 36 h (second time step) (Figure 4, Tables 4 and 5). Ventilation behavior showed a clear immediate increase (0–6 h) at intermediate pH (5–5.5) (pH and time step 1), followed by a pH- and time-dependent decrease (step 2) (Figure 4, Tables 4 and 5). Behavior remained influenced by amplified circadian rhythmicity. When exposed to ACID, *G. holbrooki* showed a pH-dependent decrease (pH 7–5.5: first pH step) in swimming and ventilation. Below pH 5.5 circadian rhythmicity was enhanced in both swimming and ventilation. The response planes under ACID exposure showed strong pH dependency and, to the contrary of *D. magna*, influences or amplification of the circadian rhythm, especially at pH ≤ 5.5.

Discussion

Water Chemistry. Most metal levels in the water revealed a clear negative linear relationship with pH. Increasing acidification has been reported to (i) increase dissolution of metal ions from metal salts, (ii) increase desorption, and (iii) decrease complexation at pH below 5 (33). Zn, Cd, and Fe were retained as most significantly pH-dependent in the multiple regression model, whereas the pH dependency of

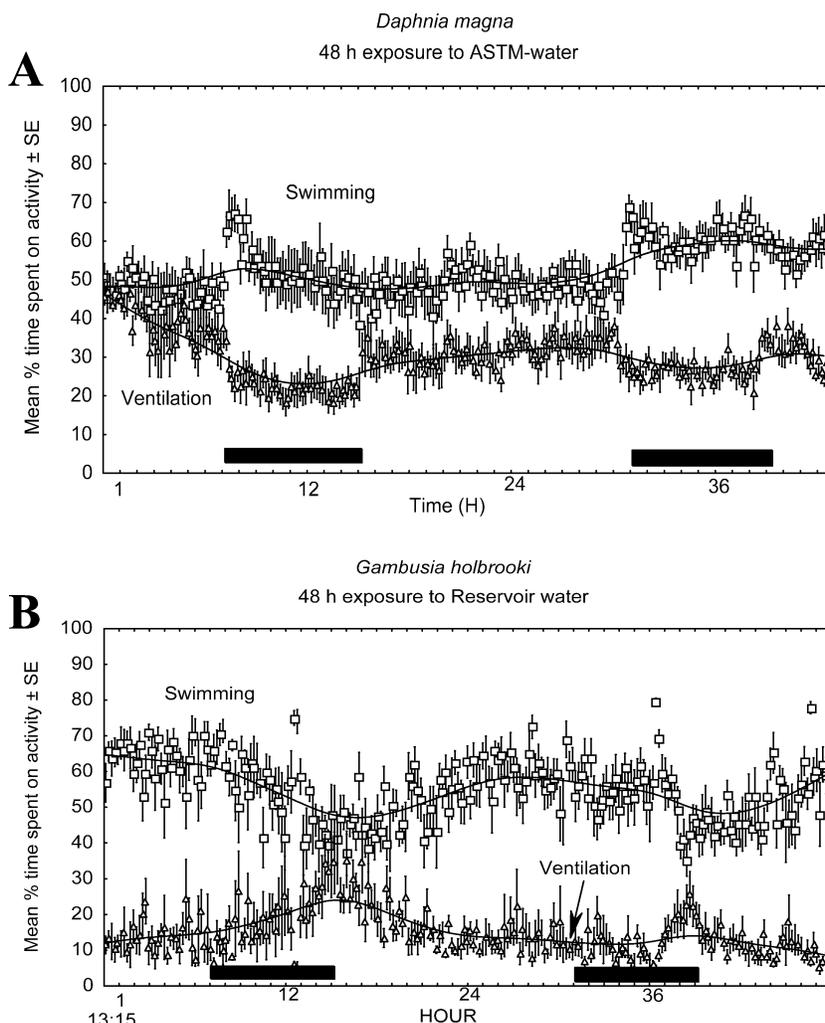


FIGURE 2. Mean activity (% of time \pm SE) recorded by the multispecies freshwater biomonitor of (A) *Daphnia magna* and (B) *Gambusia holbrooki* when exposed during 48 h to the water of their origin (control). Dark bars represent night periods in the climate room.

TABLE 4. Stepwise Stress Model as a Function of Exposure Time^a

species stressor behavior	time steps in SSM	time-thresholds & range (h)	regression equation/remarks	r^2
<i>D. magna</i>				
AMD				
swimming ^b	1		amplification of circadian rhythm (nonlinear)	
ventilation ^b	1	0–12	amplification of circadian rhythm (nonlinear)	
	2	35–48	$B = -70.04 + 24.51 \text{ pH} + 0.07T$	0.74
ACID				
swimming	no time steps			
ventilation	no time steps			
<i>G. holbrooki</i>				
AMD				
swimming	1	0–12	$B = -34.78 + 24.29 \text{ pH} - 2.61T$	0.61
	2	36–48	$B = -1692.76 + 271.37 \text{ pH} + 40.93T - 6.20 \text{ pH} \times T$	0.73
ventilation ^c	1 + 2	0–12	$B = -1043.62 + 1.69T + 493.3 \text{ pH} + 5.51 \text{ pH} \times T - 3.16T^2 - 49.85 \text{ pH}^2$	0.56
ACID				
swimming ^b	1	0–12	$B = 6.79 + 11.83 \text{ pH} + 3.31T - 0.25 \text{ pH} \times T$	0.33
ventilation ^b	2		amplification of circadian rhythm (nonlinear)	

^a 3D CRT surfaces for the different types of behavior normalized to reference of *D. magna* and *G. holbrooki* exposed to AMD or ACID during 48 h. B = behavior; T = exposure time. The modeling ($p < 0.05$) covers the whole pH range (3.3–6.8). Each step is defined as a significant change of linear slope. ^b Amplified circadian rhythm at $\text{pH} \leq 5.5$. ^c Polynomial along both axes of, respectively, pH and time, representing increase (step 1 during first 6 h) and decrease (step 2 during 7–12 h).

other metals seemed to be weakened and counteracted by the presence of other metals. In another study (34), a correlation analysis of metal levels in water and sediment with (i) pH and (ii) survival of *D. magna* revealed that

especially Fe, followed by Mn and Zn levels in the sediment were the most predictive toxicity indicators, supporting our results for *D. magna* (aqueous concentrations of Zn correlated best with mortality, with $r^2 = 0.81$).

TABLE 5. Stepwise Stress Model as a Function of pH^a

species stressor behavior	pH steps in SSM	pH thresholds & range	regression equation/remarks	r ²
<i>D. magna</i>				
AMD				
swimming ^b	1	7–5.5	$B = 141.80 - 6.17 \text{ pH} - 3.84T + 0.58 \text{ pH} \times T$	0.41
	2	5.5–3	circadian rhythm, but clear decrease	
ventilation ^b	1	7–5.5	$B = -88.37 + 27.87 \text{ pH} - 0.22T$	0.77
	2	5.5–3	circadian rhythm, increase after 36 h	
ACID				
swimming	1	7–3	$B = -95.2 + 30.52 \text{ pH} - 0.80T + 0.09 \text{ pH} \times T$	0.76
ventilation	1	7–3	$B = -104.57 + 31.88 \text{ pH} - 0.4T + 0.04 \text{ pH} \times T$	0.77
<i>G. holbrooki</i>				
AMD				
swimming	1	7–3	15–35 h: $B = 49.11 + 12.88 \text{ pH} - 7.46T + 0.95 \text{ pH} \times T$	0.55
ventilation ^c	1 + 2	7–5.5–3	$B = -1043.62 + 1.69T + 493.3 \text{ pH} + 5.51 \text{ pH} \times T - 3.16T^2 - 49.85 \text{ pH}^2$	0.56
ACID				
swimming ^b	1	7–5.5	$B = 99.41 - 1.44 \text{ pH} - 3.33T + 0.54 \text{ pH} \times T$	0.16
	2	5.5–3	circadian rhythm	
ventilation ^b	1	7–5.5	not significant	0.08
	2	5.5–3	circadian rhythm	

^a 3D CRT surfaces for the different types of behaviour normalized to reference of *D. magna* and *G. holbrooki* exposed to AMD or ACID during 48 h. *B* = behavior; *T* = exposure time. If not specified, the modeling ($p < 0.05$) covers the whole time range (0–48 h). Each step is defined as a significant change of linear slope. ^b Amplified circadian rhythm at pH ≤ 5.5. ^c Polynomial along both axes of, respectively, pH and time, representing increase (step 1 at pH ≥ 5.5) and decrease (step 2 at pH ≤ 5.5) within a short period of 12 h.

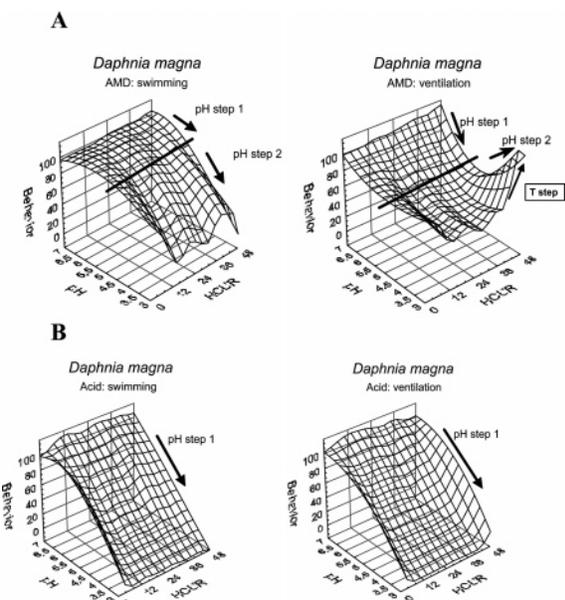


FIGURE 3. 3D CRT surface patterns of different types of behaviour measured by the multispecies freshwater biomonitor (mean time % spent on selected frequencies of movement, $N = 8-12$ animals, normalized to the control) of *Daphnia magna*, when exposed to (A) acid mine drainage (AMD) from São Domingos mine, Portugal, or (B) ACID. Behaviour (Y-axis) = % time activity.

Survival Modeling. *D. magna* has been reported to be among the most sensitive aquatic species regarding organic contaminants and metals (20). In the present study, traditional LC₅₀ analysis proved *D. magna* to be more sensitive to both AMD and ACID stress than *G. holbrooki*. The calculation of the index of relative sensitivities ($S = \log(\text{LC}_{50} 48 \text{ h}_{D. magna} / \text{LC}_{50} 48 \text{ h}_{\text{test-species}})$) (35) reveals for *G. holbrooki*: $S = 0.03$ (AMD) and $S = 0.07$ (ACID). Using this index, lower relative sensitivity as compared to *D. magna* resulted also for *Choroterpes picteti* (36) and *Chironomus* sp. (17), equal sensitivity for the crustacean *Atyaephyra desmaresti* (16), additional test species that have been exposed to the same AMD and ACID stress in similar experiments. In a similar study (34), *D. magna* exposed to AMD with a pH of 3.3–3.7

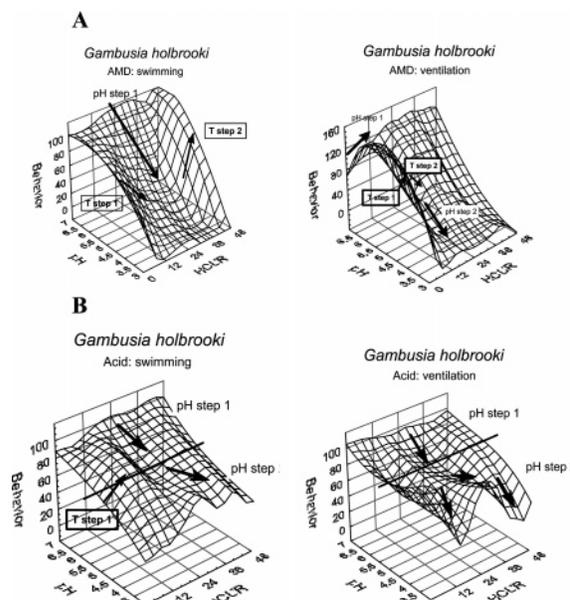


FIGURE 4. 3D CRT surface patterns of different types of behaviour measured by the multispecies freshwater biomonitor (mean time % spent on selected frequencies of movement, $N = 8-12$ animals, normalized to the control) of *Gambusia holbrooki*, when exposed during 48 h to (A) acid mine drainage (AMD) from São Domingos mine, Portugal, or (B) ACID. Behaviour (Y-axis) = % time activity.

from a mine in the United States showed 100% mortality after 48 h of exposure, water column toxicity being most strongly correlated with pH. In our study, the higher sensitivity of *D. magna* concerning the LC₅₀'s might be due to the fact that it originated from a laboratory clone culture, whereas the fish were collected from a genetically variable field population and probably adapted to fluctuating environmental factors inclusive pH and metal levels. Ecological risk assessment based on LC₅₀'s of *D. magna* might overestimate the risk for ecosystem health and integrity (37). In situ survival tests with *D. magna* as compared to local zooplankton in São Domingos Mine at pH 3.5 (24 h) revealed, however, similar sensitivities of both local and cultured crustaceans

(15). *G. holbrooki* seemed more affected by combined metal and acid stress as compared to acid-only stress, which might be attributed to interactions such as substitution of H^+ ions by metal ions at the receptors of the gills, primary sites of metal bioaccumulation and toxicity during acute exposure (38–40).

The calculated fixed 48-h LC_{50} end points fit well into the 3D CRT graphs for both species exposed to AMD and ACID. The 3D CRT surfaces of both species showed different patterns in both time and pH axes, which could not be deduced just from 48-h LC_{50} calculations. These different patterns express the kinetics of toxicity as a function of time, something completely neglected by the classical 48-h LC_{50} approach. The calculated LT_{50} 's within each pH level give an idea about the rapidity at which half of the population dies due to pollution stress. However, these values represent data points in just one plane (at $probit = 5$), in the 3D CRT, hence neglecting useful information provided by the other probit values. Lee et al. (41, 42) reported in a similar approach how the time course of PAH toxicity in *Hyalella azteca* was determined not only by the bioconcentration kinetics but also by the cumulative toxicity with increasing exposure time (circadian(time) = "toxicity time course" (41, 42)). Therefore, TTD or hazard models must be developed as complement to toxicokinetic models. The equations fitted to the present 3D CRT models would predict that after an exposure of, for example, 2 weeks both species would reach 100% mortality at $pH < 6.4$ in AMD or ACID. In literature, good agreement has been reported when using concentration–response–time surfaces to predict chronic metal toxicity for *D. magna* from 72-h short-term exposures (8).

Behavior. Behavioral parameters mirror sublethal toxicity, thus behavioral responses might be faster and more sensitive toxicity parameters than mortality (43). The MFB proved to be a reliable tool for recording different behavioral responses caused by AMD and ACID stress, and the electrical alternating current of 100 kHz did not affect the behavior of neither *G. holbrooki* nor *D. magna*, which is supported by other studies on fish (44, 45) and crustaceans (Kirkpatrick, Queen's University Belfast, personal communication).

D. magna showed a steep pH-dependent decrease of similar slopes both for swimming and ventilation when exposed to ACID, which, according to the definition mentioned in Weltje (46), indicates a high pH-sensitivity but low phenotypic behavioral plasticity for clones and vegetatively reproducing species. Decreased locomotory activity of a shrimp was shown to be related to higher maintenance costs resulting in higher metabolic rates in other nonmuscular tissues (47). Exposure to AMD resulted in less steep response surfaces than to ACID, indicating less sensitivity and toxicity, probably due to antagonistic effects of H^+ and Me^{2+} ions at the sites of toxic action.

G. holbrooki exposed to ACID responded directly with increased swimming, indicating an avoidance response. This kind of locomotory escape behavior has been observed in other animal groups exposed to other pollution types as well (e.g., *Gammarus pulex* exposed to insecticides (14) or to copper (28)), resulting in increased drift. Behavioral avoidance–attractance responses were found in one-third of 75 tested chemicals in fish, another third being not avoided, and one-third producing variable or inconsistent reactions (48). Overall, there was a pH-dependent decrease in swimming and ventilation, which in ACID below $pH 5.5$ was affected by the amplification of the circadian rhythm of *G. holbrooki*. Under AMD stress strong pH-dependent effects masked circadian effects.

As the gills in fish are the main targets for metals and acid stress (38–40), ventilation behavior seemed to be an appropriate parameter in this study, showing direct and dramatic changes in relation to pH and exposure time.

Increased ventilation can be interpreted as a trial to "remove" the toxins, often in combination with increased mucous production, from the respiratory surfaces by increasing the water flow over the gills. Ventilation and cough rates have been reported to be sensitive behavioral/physiological indicators of metal exposures in fish (10). Gill ventilation frequency of larval and adult rainbow trout decreased significantly during acute short-term exposure to a complex metal mixture, which could be linked to haematological and respiratory biomarkers. Moreover, fish were found capable to sense and avoid the metal mixtures (38). Heart rate of brown trout doubled and ventilation rate increased significantly under exposure of 0.28–0.45 mg/L Al at $pH 4.7$ (39). In another study with the MFB (44), the stickleback decreased swimming and increased ventilation under ammonia stress. Upon exposure to the herbicides atrazine or diuron, the goldfish displayed burst swimming reactions and changes in grouping behavior and surfacing activity (49).

The slopes of the response surfaces of a behavioral trait to the environmental gradient, in our study the AMD gradient, were species-specific and different for the types of behaviors observed in fish and daphnid. This reflects different sensitivities and response thresholds, linked to different costs and benefits of performing specific behaviors as stress regulator. The observed differences of behavioral responses between ACID and AMD stress might be attributed to (i) different sites and modes of action of the different pollution types; (ii) different morphometry and surface-size relationships of the target organs, such as the gills; or (iii) different intrinsic behavioral plasticity for the two stress responses. The high sensitivity of *D. magna* clone combined with low behavioral plasticity, as shown by the steep equi-clinal linear response surface planes for survival, swimming and ventilation, combined with the compliance to the SSM only for AMD exposure seems to support the hypothesis that behavioral plasticity may be (i) related to genetical variation in a population and (ii) higher in opportunistic, ecologically successful and expansive species compared to clones.

Circadian Rhythmicity. Amplification of endogenous circadian rhythm under toxicant stress (*D. magna*: AMD; *G. holbrooki*: AMD and ACID) might be a new potential behavioral stress parameter. Circadian rhythm of *Daphnia* was not affected by ACID. However, at AMD $pH < 5.5$ the circadian rhythm of *D. magna* was amplified for both swimming and ventilation. Apparently, toxic action of heavy metals in acid environment stimulated physiological mechanisms for endogenous rhythmicity. In a field scenario it might have ecological consequences of, for example, changed predation pressure. Using the MFB, Gerhardt et al. (50) could clearly measure a negative phototactic swimming behavior of *D. magna*, a well-known anti-predation strategy among zooplankton (51), aquatic insects (e.g., ref 52), and other crustaceans (47, 53, 54). Moreover, Gerhardt et al. (50) demonstrated for *D. magna* that this endogenous rhythm continued, even under manipulated photoperiods. Both ACID and AMD could amplify circadian rhythms in swimming and ventilation behavior of fish. Heavy metals can amplify circadian hormonal peaks, both in invertebrates (55) and in vertebrates (56). However, as reported by Handy (57), some essential metals such as copper may have a reducing effect on swimming speed upon fish as well as an abating effect on circadian rhythmicity. The latter is related to endocrine disrupting action of copper on melatonin and serotonin physiology. Such loss of rhythmicity has also been observed in the fish *Anabas testudineus* exposed to the insecticide lindane (58) or to the mayfly *Choroterpes picteti* when exposed to acid water but not so when exposed to AMD (36). In humans and rats, altered circadian clock, due to exposure to light at night, suppresses melatonin, being normally produced at the end of dark phases (59). Also, pollutants

known as “nerve poisons” such as lead and other acetylcholinesterase inhibitors are known to alter daily activity patterns (43).

Stepwise Stress Model and Online Biomonitoring. Behavioral responses occurred directly in both fish and crustacean and followed the concept outlined in the SSM, supporting previous findings for crustaceans and fish exposed to dilutions of complex effluents (45). Hence they might be used in BEWS for automated biomonitoring with a graduated alarm system using swimming and ventilation as parameters (12, 13, 45). Concerning exposure time (LT₅₀ and 3D CRT), *G. holbrooki* died faster than *D. magna* when exposed to AMD (steeper increases in mortality during the first 12 h of exposure). This is especially important for BEWS and automated biomonitoring, which, next to behavioral changes, also integrate mortality in their alarm settings and where a pollution pulse has to be detected as soon as possible. Both behavioral plasticity and rapid response patterns of *G. holbrooki*, the latter being interpreted as early warning, illustrate some advantages using this local species as compared to the standard test organism *D. magna*. As stated by Cairns already in 1992 (60), multispecies tests in ecotoxicology are more ecologically reliable in predicting responses at higher biological levels. This strategy should also be adopted in online biomonitoring (i.e., replacing single species biomonitoring by multispecies biomonitoring).

However, circadian activity rhythms might complicate the behavioral concentration–response–time patterns, causing increased noise in the alarm system (13), as seen in the present study: on one hand, behavior under stress has to be compared with a control containing a day–night cycle, and on the other hand, this cycle can even be altered in the exposure treatments as compared to the control. As circadian rhythmicity is a widespread phenomenon among aquatic invertebrates and vertebrates, also in those species used in online biomonitoring all over Europe, future basic research is urgently needed in order (i) to define changes in circadian rhythmicity as new behavioral test parameter and (ii) to develop models for prediction and evaluation of this parameter as confounding factor in early warning systems for pollution detection.

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