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Phytoassessment of acid mine drainage: *Lemna gibba* bioassay and diatom community structure

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Abstract An integrated multilevel phytoassessment of an acid mine drainage (AMD, pH range 3.3–6.8) in southern Portugal was performed. A 7-day phytotoxicity bioassay with the duckweed *Lemna gibba* (chlorosis, necrosis, growth) was carried out, both in the laboratory and *in situ*, combined with an analysis of the resident epilithic diatom community. The toxicity test was performed with water from the AMD gradient, an unpolluted river control and acidified control water, in order to discriminate potential pH-effects from combined pH- and metal-effects. Diatom communities discriminated well among the sites (alkalophilic species *versus* halobiontic, acidobiontic and acidophilic species), showing inter-site differences to be larger than intra-site seasonal variations. In *L. gibba* exposed to AMD, necrosis and growth inhibition were higher *in situ* compared to the laboratory experiments. *L. gibba* was more sensitive to AMD than to acidified water. Already after 4 days, growth rate inhibition in *L. gibba* proved to be a reliable indicator of AMD-stress. Ecotoxicological thresholds obtained with *L. gibba* corresponded with those obtained previously with animals of intermediate tolerance to AMD. The results were summarised in a multimetric index.

Keywords Phytoassessment · Phytotoxicity · *Lemna* · Diatoms · Acid mine drainage · Acidity · Multimetric index

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Introduction

Phytoassessment (community analysis) and phytotoxicity testing have been underrepresented in aquatic biomonitoring and ecotoxicology, compared to testing and screening with animals (Lewis and Wang 1999). Within the European Water Framework Directive, ratified in 2000, bioassessment should include macrophyte and phytobenthos, especially diatoms (Shaumburg et al. 2005). Among phytoassessment methods, diatoms are widely used as bioindicators of historical changes (e.g. Charles and Smol 1988; Dixit and Smol 1994; Hynynen et al. 2004), trophic state (e.g. Pan et al. 1996; Dasi et al. 1998; Köster and Hübener 2001), and metal pollution (e.g. Ivorra et al. 1999) of aquatic ecosystems. Diatoms are also useful indicators of acidity in streams and lakes (Battarbee et al. 1999) as they occupy niches along the entire pH range. The analysis of diatoms as primary producers should preferably be combined with a suite of monitoring data including physical and chemical measurements as well as analysis of other aquatic communities such as invertebrates (Triest et al. 2001; Lowe and Pan 1996). Verification of field studies can be performed with laboratory bioassays (DeNicola and Stapleton 2002; Schmidt et al. 2002; Gerhardt et al. 2004) on primary or secundary producers. The vascular floating hydrophyte *Lemna gibba* was selected for phytotoxicity tests, since it belongs to the standard species (Stewart et al. 1999), as *Lemna* spp. is explicitly recommended by the OECD (OECD 2002) and in at least five policy acts in the USA (Lewis and Wang 1999). *In situ* application of the *Lemna* assay is relatively rare (e.g. Gardner and Grue 1996, Mkandawire and Dudel 2005, Coors et al. 2006).

The aim of this study was to carry out a phytotoxicity test with the duckweed *Lemna gibba* on an acid mine

drainage (AMD) gradient in southern Portugal, both in the laboratory and *in situ*, combined with an analysis of the resident diatom communities along this pH and metal gradient. This multilevel integrated approach enables a weight-of-evidence assessment of the environmental risk posed by AMD at the autotrophic level, hence at the producer base of food chains. It offers an interesting perspective in that (1) data on the effects of AMD on vascular plants are rare, (2) the combination of testing *L. gibba* and assessing diatom community structure for risk assessment of AMD is a novel approach, and enables a simultaneous analysis at different levels of taxonomy and biological organisation in plants (alga *versus* vascular plant, individual *versus* community), (3) as part of a larger study, the duckweed bioassay can directly be related to previous exposure tests of several animal species to the same AMD (Gerhardt et al. 2005a, b; Janssens de Bisthoven et al. 2004, 2006), and the effects of AMD on diatom communities can be compared with the effects of the same AMD on community responses of macroinvertebrates (Gerhardt et al. 2004) and chironomid larvae (Janssens de Bisthoven et al. 2005). As in Niyogi et al. (2002) for the 'chemical stress index', or Schmidt et al. (2002) and Gerhardt et al. (2004) for AMD and macroinvertebrates, we combine environmental and bioassay parameters into one integrative multimetric index allowing for site ranking in risk assessment.

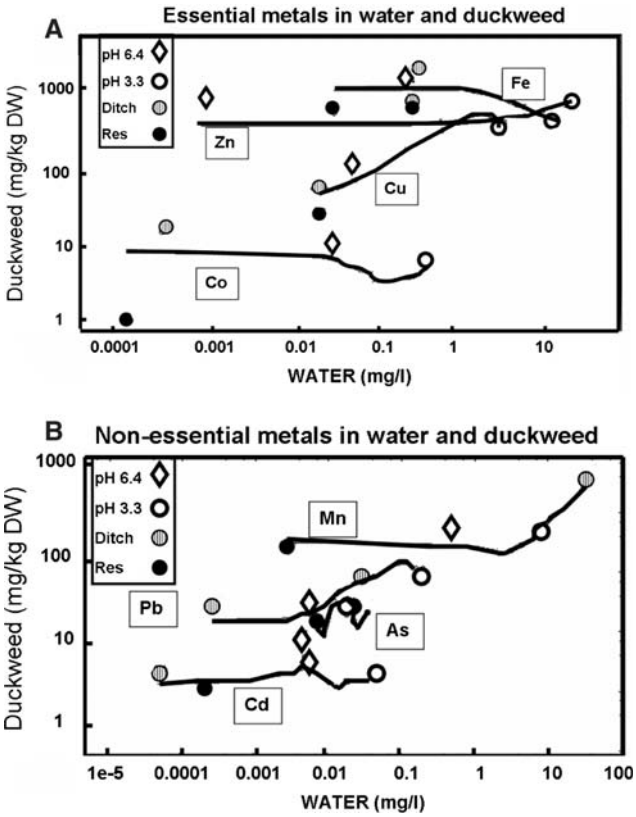

Fig. 1 Essential (**A**) and non-essential (**B**) metals (least squares fit) measured in the water and in the fronds of *Lemna gibba*, after exposure of 7 days to acid mine drainage, with indication of ditch, reservoir (res.), pH 6.4 and pH 3.3. The data obtained in pH 4.4, pH 5.0 and pH 5.5 are situated between pH 3.3 and pH 6.4, but not indicated by symbols for seek of clarity

Materials and methods

Study area and sampling

This work was performed in the years 2000–2001 in the abandoned São Domingos mine (southern Portugal, province Alentejo, 37°38.06 N/07°30.97 E) situated in the south Iberian mining belt, and in the river Vascão, situated south of this mining belt. Drainage systems in the open-cast mine still transport heavy metals in acid effluents, containing, e.g. iron, copper, sulphur, lead and zinc. During the dry estival periods, atmospheric transport of arsenic is also important. Figures of locations are provided in previous publications concerning this large study as listed above.

One study site within the mining area was situated in a reservoir (pH = 6.8 and conductivity = ±350 µS cm^{-1}), containing especially high concentrations of arsenic (Fig. 1, Table 1). The reservoir's outflow runs into the mine's drainage system, upstream of two other sampling sites along the drainage, which were selected for their impact by acid water rich in metals at pH = 3.3 and pH = 4.4 (conductivity = 1,200–1,400 µS cm^{-1}). A lotic control site was selected at river Vascão (37°31′11″ N/07°34′68″ E) in a separate catchment, situated at the edge of Guadiana Nature Reserve in the same ecoregion (circumneutral pH, ca. 630 µS cm^{-1}).

Chemical analysis

Water was collected in 25 l polyethylene tanks in June, September and October for chemical analysis and use for the laboratory bioassays. Metals and other elements in the water and in *Lemna* fronds were analysed either with the multi-element method of induced coupled plasma-mass spectrometry (detection limit ca. 0.1 µg/l) (Cd, Co, Cu, Zn, Mn, Pb, As) or with the single-element method of ICP-atomic emission spectrometry (detection limit ca. 1 µg/l) (Fe, Na, Mg, S, Ca, high As-values). Cl was determined with ion chromatography. Replicate analyses agreed well with each other. Duckweed tissue samples were analysed after a digestion in 500 µl 30% HNO_3 at 80°C overnight, followed by a second digestion in 100 µl H_2O_2 at 80°C and then addition of 100 µl HNO_3 and 900 µl ultra-distillated water (APHA 1992).

Table 1 Heavy metals (μg/l) (A) and chemical composition (mg/l) (B) in the water during laboratory[c] and *in situ*[b] exposure of duckweed to water from reference, ditch and mine (reservoir and AMD), and in the sites analysed for diatom community[a]

Site (season)	Metal (μg/l)							
(A) Heavy metals (μg/l)	As	Cd	Co	Cu	Fe	Mn	Pb	Zn
Reservoir (June)[a]	12.0	0	1.0	20.0	64.0	280.0	6.0	23.0
Reservoir (October)[b,a]	121.2	1.0	2.7	69.6	–	–	29.4	152.0
AMD-pH 3.3 (June)[a]	3.0	25.7	146.0	2,259.0	1,351.8	3,820.0	554.0	5,920.0
River Vascão (June)[a]	1.0	0	0	2.0	36.0	70.0	2.0	15.0
River Vascão (October)[a]	3.2	0.1	0.3	15.2	36.1	147.0	5.5	56.0
Ditch (October)[b]	19.3	0.1	0.4	20.8	216.9	36.6	0.3	69.0
AMD-pH 3.3 (October)[b,a]	15.7	86.2	931.9	2,824.4	7,568.4	42,153.8	168.5	17,186.0
AMD-pH 4.4 (October)[b,a]	50.6	20.0	292.1	2,196.8	4,932.4	16,951.7	184.4	6,462.0

	Element (mg/l)					
(B) Chemical composition (mg/l)	Ca	Cl	K	Mg	Na	S
Reservoir (June)[a]	20.0	16.0	2.9	8.1	19.6	18.4
Reservoir (October)[b,a]	25.5	23.2	5.0	10.2	27.6	12.0
AMD-pH 3.3 (June)[a]	73.8	29.0	4.1	37.5	38.4	164.1
River Vascão (June)[a]	17.1	32.1	1.2	13.6	30.2	7.7
River Vascão (October)[a]	25.4	109.2	3.9	43.0	82.1	16.7
Ditch (October)[b]	90.7	930.5	60.8	102.1	846.5	100.5
AMD-pH 3.3 (October)[b,a]	306.6	62.8	7.0	114.6	62.5	490.8
AMD-pH 4.4 (October)[b,a]	616.3	147.4	16.1	333.7	167.0	1,057.2
Ditch[c](September)	90.7	930.5	60.8	102.1	846.5	100.5
Reservoir[c] (September)	32.1	44.3	7.5	13.0	37.1	28.9
AMD-pH 6.4[c](September)	228.2	114.2	11.0	136.9	105.6	422.0
AMD-pH 5.5[c](September)	245.6	114.4	11.4	145.4	113.6	411.0
AMD-pH 5.0[c](September)	233.5	109.1	10.7	138.3	108.5	418.3
AMD-pH 4.4[c](September)	227.2	104.7	10.6	135.2	105.4	410.4
AMD-pH 3.3[c](September)	223.1	103.0	10.5	129.0	98.9	482.8

The aqueous metal concentrations in the laboratory bioassays are given in Fig. 1. In summer, the site AMD-pH 4.4 dried out

Diatom taxonomy and community analysis

In June and October diatoms were sampled by scraping 10 cobbles of approximately 1 dm^2 surface with a knife, collected at a depth of 30–70 cm in the euphotic zone, into a bucket of stream-water. After washing the cobbles, the water was gently poured away through a sieve with 100 μm mesh size, and the retained wet material was fixed with formaldehyde at final concentration of 5%. In the laboratory the samples were cleaned by boiling in 35% peroxide, oxidation with K_2CrO_7 and embedded in Naphrax-resin with refractive index of 1.7 (Krammer and Lange-Bertalot 1986–1991; Schiefele and Kohmann 1993). Slides were made for cell counts and a minimum of 400 cells was enumerated and identified under light microscopy at a magnification of 100–1,000×. Identifications were made to the lowest taxonomic level possible by using several taxonomic keys (Hustedt 1930, 1977, 1985; Krammer and Lange-Bertalot 1986–1991; Krammer 2000; Lange-Bertalot 2001). Additionally, scanning electron microsocopy was used to confirm some species.

Phytotoxicity test

Laboratory bioassay with AMD or acid-only

The duckweed was collected in a lacustrine drainage ditch near Aveiro (N. Portugal, 40°33′15″ N/08°45′10″ E). The ditch water served (1) as culture medium, (2) as reference water for the laboratory and the field exposures (see below). The duckweed was cultured in a climate room (18°C, light: dark = 16:8 h) as an axenic culture (manual cleaning) in ditch water.

The bioassays took place in September. Glass beakers of 100 ml unfiltered water from the respective field sites were inoculated with four plants containing a total of 12 fronds, replicated 3× per pH treatment under static conditions in a climate room at 18°C with 16 h light/8 h dark photoperiod. No additional nutrients were added to the test water. The exposures were carried out in water (1) taken directly from the AMD (pH 6.4, 5.5, 5.0, 4.4, 3.3), (2) from the reservoir (pH 6.8) situated in the same catchment but unaffected by direct AMD-inflow, however affected by atmospheric input of arsenic and (3) from the Aveiro ditch (unpolluted reference where *L. gibba* originated from, pH 7.2), and lasted 7 days (OECD guideline 221 2002). The choice of using ditch water for reference, culture and medium water rather than water artificially enriched with ions was dictated by the necessity to remain as close as possible to field conditions and ecological relevance. It is known that chemical composition of the medium might exert great influence on the experimental outcome (Mkandawire and Gert 2005).

Another set of exposures was carried out with reference ditch water acidified with HNO_3 (30%) to the same pHs as in the AMD-treatments (pH 6.4, 5.5, 5.0, 4.4, 3.3) and compared with exposures in reference ditch water.

We chose the mine tailings gradient as an unambivalent model in order to validate a combination of biological methods for developing new methods of risk assessment, based on phytobiological quality criteria as required for the EU Water Framework Directive. Therefore, of course, the pollutional context is set or known a priori.

Field test

In October 2–3 replicate polypropylene artificial channels (4 l, 40 × 16.5 × 15 cm^3) were inoculated with 8–10 plants containing each three to four fronds and exposed for 4 days in selected field sites (ditch, reservoir, AMD pH 4.4 and AMD pH 3.3). Since temperature and rainfall in this ecoregion can change very fast in field conditions (Gerhardt et al. 2004), exposure during 4 days instead of 7 days minimized the risk of natural stressors affecting the test results, as well as disturbance and vandalism. Moreover, 4 days exposure was estimated to be sufficient for gaining dose-dependent responses, as supported by the laboratory tests.

Data analysis

Diatoms

Seasonal shifts and species differences of diatoms among the sites were graphically represented in a non-metric multidimensional scaling analysis (estimation convergence after 55 iterations, Guttman–Lingoes start configuration) on the matrix of site correlations based on the relative abundances of the 55 most common diatom taxa. In order to identify taxa which are typical for the respective sites, the individual taxa (log (p + 1)) were ordinated by means of the first three principal component axes of a Principal Components Analysis (Podani 2000). The scores of the first principal component were linearly regressed against pH and concentrations of heavy metals.

The practical diatom index (PDI) (Prygiel and Coste 1996) was chosen as a pragmatic method derived from the more extensive polluo-sensitivity index (Prygiel and Coste 1996) and transformed to vary from 1 to 20 by using a formula proposed by Descy and Coste (1988), in order to enable comparison with similar studies. Although the PDI is essentially an index of trophy, and hence not suited to detect AMD, it was tested on our data to ensure that the observed differences were indeed independent of background trophy. Further, the presence of indicator species for salinity (Schönfelder 2000), trophy, alcalinity, acidity and mines (Alles 1998), as well as carbonates and silicates (Schaumburg et al., 2005) was screened per site. Moreover, the Shannon diversity index ($H = -\Sigma P_i \ln P_i$, where P_i = proportion of species i, Begon et al. 1986) and the Jaccard similarity index ($J = a/(a + b + c)$, where a is number of species common to two samples, b = number of species only present in one sample, c = number of species only present in the other sample, Podani 2000) were calculated in order to get an appreciation of respective species richness and species overlap among sites.

Lemna gibba

After ensuring that logarithmic growth of *Lemna* in the controls followed a monotonous linear pattern without time lags, average specific growth rate was calculated to obtain percentage inhibition of growth rate (% Ir) compared to the reference site (OECD 2002). Growth rates were normally distributed and subjected to a one-way repeated measures ANOVA with pH as independent variable and repeated measures over different days. The EC_{50} for % Ir was determined by regressing its probit-transformed values with pH. Number of yellow (chlorosis) and dead (necrosis) fronds were counted daily, and expressed as percentage of the actual number of fronds which were at least 1-day old. Chlorosis and necrosis percentages were transformed to $\text{Arcsine}(p)^{0.5}$ for one-way repeated measures ANOVA at a significance level of 5%, followed by post hoc Tukey tests for equal sample size. When distribution frequency was not normal (Chi-square goodness of fit, $p < 0.05$), the non-parametric Friedman ANOVA test was applied to test pH or time effects. Linear relationships among metals in the

water and in the tissues were expressed by means of (multiple) linear regressions and correlations by Spearman rank correlation analysis. All statistics was performed with the software 'Statistica for Windows 4.5'.

Multimetric index

A multimetric index "MI_{auto}" is developed, analogous to the development of the 'chemical stress index' in Niyogi et al. (2002), to 'METR' in Schmidt et al. (2002), and to a similar approach based on macroinvertebrates in AMD (Gerhardt et al. 2004), where the multimetric index "MI" integrated parameters of macroinvertebrate community and a behavioural toxicity assay with a freshwater shrimp (*Athyaephyra desmarestii*):

$$MI = [BMWP_{st} + EPT_{st} + (100 - \text{predators\%})_{st} + A5_{st}]/4,$$

where BMWP = Biological Monitoring Working Party, EPT = % Ephemeroptera + % Plecoptera + % Trichoptera; $(100\text{-Predators\%})_{st}$ represented the percentage of animals classified as non-predatory, A5 = % of time spent by shrimp on locomotory activity after 5 h of exposure, and quotient 4 being the number of parameters. Suffix "st" indicated a standardisation of each of the parameters to a scale of 10 points, the value of 10 being the "ideal" score in a control site.

The multimetric index for autotrophs (MI_{auto}) proposed in the present work was derived on the basis of parameters extracted from the diatom community assessment and the *Lemna gibba* bioassay, in order to rank the sites in a weight-of-evidence multimetric risk assessment at the autotrophic level. All parameters in the formula of MI_{auto} were standardised ("$parameter_{st}$") as a function of the control, which was set at 10 points (=no pollution), then summed up, and divided by the number of parameters, like in MI (Gerhardt et al. 2004):

$$MI_{auto} = [S_{st} + (100 - \%\ \text{acid})_{st} + (100 - \%Ir)_{st}]/\ 3,$$

where S = number of diatom species as % of species in control, (100 – % acid) = % of diatom taxa which are not indicators of acidity, % Ir = % inhibition of average specific growth rate of *Lemna* sp. after exposure during 4 days or 7 days, whereby $(100 - \%\ Ir)_{st}$ for the reference site = 10 or the maximum growth rate. The formula is divided by the number of used parameters to obtain a value between 0 and 10.

The river Vascão was taken as control for both the diatoms and the *Lemna* bioassay for sake of comparison with the AMD-sites. Moreover, this pristine reference site, characterized with excellent chemical water quality (Table 1), has previously been used for animal bioassays and biomonitoring of macroinvertebrates and chironomids (Gerhardt et al. 2004, 2005a, b; Janssens de Bisthoven et al. 2004, 2005, 2006), and for the calculation of the MI for macroinvertebrates.

For example, with the reference site on River Vascão having 39 taxa and % Ir = 0%, MI_{auto} for AMD-pH 4.4 after 7 days is:

$$\begin{aligned} MI_{auto} &= [S_{st} + (100 - \%\ \text{acid})_{st} + (100 - \%Ir)_{st}]/\ 3 \\ &= ((18/39 \times 10) + (15/39 \times 10) + (100 - 70)/10) \\ &= (4.6 + 3.8 + 3)/3 = 3.8 \end{aligned}$$

At this stage of development of new biological assessment methods, the MI_{auto} is calculated for the AMD sites, a well defined and well-known pollution background, as it needs to be calibrated. Further validation on sites with black box pollution background needs to be undertaken in order to provide wider acceptance of the proposed methods.

Results

Chemical analysis

Results of the chemical analysis of four essential and four non-essential metals, together with other elements in the respective sites and seasons are summarised in Table 1. Metal concentrations in the water and in *Lemna* tissues taken from the laboratory bioassays are illustrated in Fig. 1. Arsenic was the most important metal in the reservoir (Table 1). Aqueous and duckweed metal concentrations tended to increase with decreasing pH of the AMD, except for arsenic, cadmium, cobalt or zinc (Spearman Rank, non-significant (n.s.), linear regression, n.s.). Lead (Pb_{Lem}) and copper (Cu_{Lem}) increased log-linearly within the tissues of *Lemna* with decreasing pH (Spearman Rank $r > 0.74$, $p < 0.05$) (Fig. 1):

$$\ln[Cu_{Lem}] = 5.90 + 0.54\ln[Cu_{wa}], \quad r^2 = 0.83, p = 0.01$$

$$\ln[Pb_{Lem}] = 6.25 + 0.70\ln[Pb_{wa}], \quad r^2 = 0.70, p = 0.04$$

Manganese showed a sudden increase in body burden above a threshold value of 8 mg/l, and iron tended to decrease with increasing pH, due to precipitation reactions (visual observation of orange crusts and flocculations in the AMD).

The non-metal chemical field data showed similar negative correlations to pH (Table 1). Most metals scored a factor 2–3× higher in the field experiment (October)

compared with the laboratory experiment (September), due to weather related differences in water flow and temperature. The choice of river Vascão as an unpolluted control site was confirmed by the lower metal concentrations.

Diatom community

In total, 82 taxa of diatoms were identified. River Vascão and the reservoir had the highest diversity with each 39 taxa, followed by 18 taxa or less in the respective AMD-sites (complete taxa lists available on request). The multidimensional scaling (MDS) on diatoms clearly distinguished three distinct site groups, being river Vascão, reservoir and the two low pH AMD-sites. Seasonal differences within the sites played a secondary role in the total variance (Fig. 2): in river Vascão a shift occurred from October to June from the dominant *Epithemia sorex* to *Mastogloia smithii*, the latter already having been the second most common species in October. In the reservoir, a shift occurred from *Fragilaria pinnata* dominating in October to *Cyclotella stelligera* in June, with *F. pinnata* still remaining the second most dominant species. In AMD-pH 3 *Pinnularia subcapitata* remained dominant at both sampling dates, and was second in AMD-pH 4 (only autumn), where *Nitzschia capitellata* was the dominant species.

The taxa exclusively occurring in AMD were mainly ordinated by the first principal components axis (p1), the ones occurring mainly in river Vascão by p2, and taxa typical for the reservoir by p3 (% of total variance of p1 = 35.7, p2 = 23.4, p3 = 20.8). These site-specific and/or dominant (> 8%) taxa are listed in Table 2. The first axis of the PCA, hence representing typical AMD-taxa, correlated negatively with pH and positively with Cu, Cd, Co, Pb, Zn, S, Ca and K (Spearman Rank $r = 0.99$, $p < 0.05$). A stepwise multiple regression analysis retained for the first principal component $p1_{diatoms}$ as independent variable, the metal copper and the element sodium: $p1_{diatoms} = -0.99 + 1.06[Cu] - 0.08[Na]$, $r^2 = 0.99$, $p < 0.0001$.

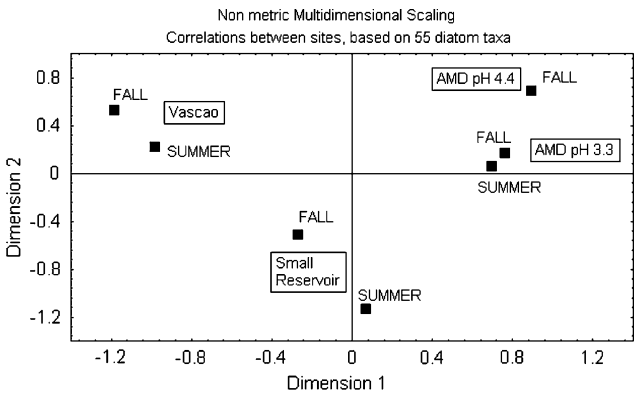

Fig 2 Non-metric multidimensional scaling (MDS) of the correlations between sites for % abundance for 55 selected diatom taxa collected in June and October in river Vascão, reservoir, acid mine drainage (AMD) at pH 4.4 and pH 3.3

With 17 species or less, the diversity in the mine (at the low pH sites) was 50% of that in river Vascão, and the values for Shannon index reflected these differences (Table 3). The practical diatom index did not reflect the pollution stress caused by AMD, as the PDI scores remained very high in all sites (good quality: PDI = 13–17; high quality: PDI = 17–20). Screening for indicator taxa known from the literature showed that salinity indicators (halophilic and halobiontic) were found in all sites. However, more taxa known to prefer freshwater conditions were found in river Vascão and reservoir than in the AMD sites. Saproby and trophy indicators were found in low numbers at all sites, hence did not discriminate well. Acidity indicators were only found in the AMD sites, whereas alkalinity indicators were more frequent in the river Vascão and the reservoir. Mine, silicate and carbonate indicators were found at all sites (Table 3).

Phytotoxicity test with *Lemna gibba*

AMD laboratory assay

In the laboratory *Lemna gibba* was more affected by AMD than by acid-only exposure. The inhibition of growth rate (% Ir) showed a dose–response relationship as a function of the AMD-gradient ($p = 0.00002$, no time and pH × time effects), with after 4 days a pH-EC_{50} = 5.9 (95% confidence limits C.I.: 5.7–6.0, $r^2 = 0.69$, $p < 0.0001$) and after 7 days a pH-EC_{50} = 5.0 (C.I. = 4.8–5.2, $r^2 = 0.35$, $p < 0.005$). After 2 days (for comparison with AMD bioassays of 48 h with animals), necrosis was only observed at pH ≤ 4.4. After 4 days, chlorosis and necrosis (Friedman: pH: no effect, time, $\chi^2_{21,6} = 19.60$, $p = 0.003$) became significant in the treatments with pH ≤ 6.4, setting the 4 days-LOEC at pH 6.4 and the 4 days-NOEC at >pH 6.4. The mortality did not reach 50% and the dose–response regression was not significant due to low necrosis in AMD-pH 3.3. Therefore, a 4 or 7 days-LC_{50} could not be computed.

Acid-only

The duckweed was more tolerant to acid-only conditions from pH 5.0 upwards, where growth was either equal to the control, or was stimulated relatively to the control

Table 2 Relative abundances (%) of selected diatom species collected in October and June in the river Vascão, the reservoir (Res.), acid mine drainage at pH 4.4 (AMD4, only October) and acid mine drainage pH 3.3 (AMD3)

Taxa	October				June		
	R. Vascão	Res.	AMD4	AMD3	R. Vascão	Res.	AMD3
Achnanthes minutissimaaxa Kützing var. *min.*	20.2	0.5	0.9	2.8	5.7		0.5
***Cyclotella stelligera* Cleve and Grunow**		0.8			0.6	**42.4**	
Cymbella microcephala Grunow	3.8	0.3			8.8	1.9	
Cymbella affinis Kützing	6.6				1.3		
***Epithemia sorex* Kützing**	**25.1**				4.4		
***Mastogloia smithii* Thwaites**	16.4				**44.7**		
Nitzschia sublinearis Hustedt	0.5		3.7	0.5	8.2	2.6	1.4
Nitzschia rostellata Hustedt						2.2	
***Nitzschia capitellata* Hustedt**			**51.4**	16.2			
Rhopalodia gibba (Ehrenberg) O. Müller	6.6	12.6	0.4	0.5	13.8	0.4	
Anomoeoneis vitrea (Grunow) Ross		0.5				4.8	
Fragilaria nanana Lange-Bertalot						2.9	
***Fragilaria pinnata* Ehrenberg**		**79.1**				31.4	
Pinnularia acoricola Hustedt			5.1	0.5		0.4	5.3
***Pinnularia subcapitata* Gregory**			33.0	**74.1**		0.4	**86.1**
Eunotia exigua (Brébisson) Grunow			3.2	1.9			3.8
Cocconeis placentula Ehrenberg var. *placentula*	5.5		0.4				

The most dominant taxa per site are put into bold

Table 3 Diatom assemblage community metrics and bioindicator values in different sites of the mine and control river

Metrics	River Vascão (V)	Reservoir (R)	AMD4 (4)	AMD3 (3)
Number of species	39	39	18	15
Shannon index	2.2 (A), 1.9 (S)	0.9 (A), 1.6 (S)	1.3 (A)	0.9 (A) 0.6 (S)
Jaccard index	V/R: 15.4	R/4: 14.3	pH 4/3: 34.4	
	V/4: 8.9	R/3: 5.5		
	V/3: 14.8			
Practical diatom index	13.8–17.4	16.7–15.7	18.9	18.5–18.5
Number of indicators[a]				
For high salinity (>1,000 mg/l)	2	0	1	0
For low salinity (<1,000 mg/l)	4	4	2	2
For saproby	3 (III)	2(II)	3(III)	3(III)
For trophy	2 (eu)	1(ol)	1 (eu)	1 (eu)
For acidity	0	1	2	3
For alkalinity	5	6	3	4
For mines	1	1	2	2
For carbonate	2	2	1	1
For silicate	1	3	2	2

A = autumn, S = spring, eu = eutrophic, ol = oligotrophic

[a] From Alles (1998), Schönfelder (2000) and Schaumburg et al. (2005)

($p < 0.0001$, no time and pH × time effects). The inhibition of growth rate (% Ir) had after 4 days a pH-EC_{50} = 4.4 (C.I. = 4.3–4.5, $r^2 = 0.71$, $p < 0.001$) and after 7 days a pH-EC_{50} = 4.2 (C.I. = 4.1–4.3, $r^2 = 0.94$, $p < 0.0001$). Chlorosis was low (Friedman: pH effect, $p = 0.01$; time effect, $p = 0.04$), but necrosis reached higher values at pH 4.4 and especially pH 3.3 of acid-only (repeated measures ANOVA: pH, $F_{5, 12} = 27.0$, $p < 0.0001$, time, $F_{6, 72} = 11.2$,

$p < 0.0001$, pH × time, $F_{30,\ 72} = 17.3$, $p < 0.0001$), setting the LOEC at pH 4.4 and the NOEC at pH 5.0. Mortality did not reach 50% and the regression was not significant due to low necrosis in AMD-pH 3.3. Therefore, a 4 or 7 days-LC_{50} could not be computed.

Field assay and comparison with laboratory

Only in the ditch *Lemna* showed growth, but neither in the reservoir nor in AMD-pH 3.3 and 4.4. The *in situ* growth in the reservoir was different from laboratory growth, since the number of fronds exposed in the reservoir consistently diminished over time (rotting process due to high day temperatures or wind drift might be responsible).

Chlorosis was not significantly different among the sites and among the days of exposure. However, necrosis showed significant differences among sites and days of exposure: (Friedman: site and time, $p < 0.05$), showing similar trends as in the laboratory. Necrosis was however much more pronounced *in situ* (AMD) than in the laboratory (Friedman test: pH 3.3 and pH 4.4, $\chi^2 = 6.0$, $p = 0.01$; reservoir, not significant).

Multimetric index

The MI_{auto}, when calculated with the laboratory *Lemna* bioassay after 4 days, ranked as follows: reservoir (10.6) > river Vascão (estimated 10) > AMD-pH 4.4 (3.2) > AMD-pH 3.3 (3.0). After 7 days of exposure in the *Lemna* bioassay, the MI_{auto} ranked reservoir (11.5) > river Vascão (estimated 10) > AMD-pH 4.4 (3.8) > AMD-pH 3.3 (2.6). The MI_{auto}, when calculated with the data from the field bioassay (4 days) ranked the sites as follows: Vascão (estimated 10) > reservoir (6.7) > AMD-pH 4.4 (2.8) > AMD-pH 3.3 (2.3), reflecting the higher stress in the field, compared with the laboratory.

Discussion

Mine drainage exerts chemical stress (low pH, dissolved metals) as well as physical stress (deposition of metal oxides) on stream biota (Niyogi et al. 2002). DeNicola and Stapleton (2002) elegantly demonstrated in field exposures of various treatments of substrates with and without precipitates (mainly Al and Fe) that precipitate on the substrata did neither affect macroinvertebrate nor periphyton density and species composition. Therefore, they suggested that the aqueous chemical environment of AMD had a greater effect than the coating of precipitate. This hypothesis was based on an AMD-impacted creek of relatively high pH (6.7) and hence might not apply to the much more acidic AMD-environment in the present study (pH 3.3–4.4 AMD-gradient), where most of the metals are expected to be in solution (Stumm and Morgan 1981).

For As (Table 1), the values in river Vascão, the reservoir (June), ditch and AMD-pH 3.3 (June) are around or lightly exceeding drinking water guidelines in Germany or recommended by the World Health Organisation (10 µg/l) (Sunderman et al. 1991; WHO and World Health Organization, 1996). However, As in the reservoir (October) and in AMD-pH 4.4 (October) is 5–12 times higher. Cadmium, iron, copper, zinc and lead exceed the limit values (resp. 3, 300, 2,000, 3,000 and 10 µg/l) in both AMD sites. Manganese only exceeds the guideline of 100 mg/l in AMD-pH 3.3 (October). Sulphur, being the main cause for the acidity, is exceeding guidelines in all AMD sites. The other elements don't exceed the water quality guidelines, except for chloride in the ditch, showing a relatively salty water due to the neighbouring salt lagune.

These comparisons show that the study covers the whole range of values, going from drinking water quality (river Vascão, reservoir and ditch for most metals) to situations where heavy metals are higher by a factor going from 1.5× to more than 10×, and hence provides a realistic framework to cover sublethal to lethal responses.

As shown for copper after 12 h exposure in Akhtar et al. (2005), and in the present study, *L. gibba* accumulated lead and copper log-linearly with decreasing pH. Hutchinson and Czyrska (1975) found in *Lemna* sp. similar trends at pH 6.8 for lead, iron, manganese and cadmium. They found high accumulation for copper and nickel, postulating the existence of some threshold for copper at ±0.1 ppm Cu, a value which is exceeded in the present study. Uptake and toxicity of metals in *Lemna trisulca* were enhanced by the presence of other metals in Huebert and Shay (1991). Cadmium contents above 116 µg Cd/g dry weight caused growth reduction in *L. trisulca* (Huebert and Shay 1991), a value not reached in the present study. However, the observed growth reduction might have been caused by synergistic effects or the high Cu, Zn or Pb concentrations.

A concentration of over 8,000 µg/l Copper causes growth inhibition in the diatom *Cyclotella* (Cairns et al. 1978), but already at ±800 µg/l, the EC_{50} for growth is reached for the diatom *Nitzschia* (Patrick et al. 1968), showing species-specific sensitivities. The AMD sites clearly exceed such copper values of 800 µg/l but remain under 3,000 µg/l, indicating a copper range where still certain diatom species can survive, as we have seen in the present study.

Although copper is high in the AMD sites, the high calcium hardness will tend to mitigate the toxic effects, setting expected EC_{50} values for *Lemna* at around 250 µg/l (e.g. Stanley 1974), a value exceeded by a factor 10 in the

AMD. However, in the other sites, copper concentrations are much lower than the expected EC_{50}, giving appropriate conditions to cover the whole range of responses in *Lemna*. At values of Cu > 10 mg/l and Cd > 0.5 mg/l, the antioxidant system in duckweed disintegrates, values not reached in the present study (Hou et al. 2006). Copper starts having a decolourising effect on *Lemna gibba* at 1 µg/l, Fe at 10 µg/l, Zn at 100 µg/l and Mn at 1 µg/l (Tsatsenko and Malyuga 2002), all these values being exceeded in one or more sites in our study, confirming the chlorosis effects observed.

A recent multivariate analysis of the chemical data from the same AMD-sites (Gerhardt et al. 2004; Janssens de Bisthoven et al. 2005) discriminated all sites as a function of pH and heavy metals.

Relating chemical with biological parameters by regression is usual practise in, e.g., reconstructing or predicting pH or phosphorus from lakes (e.g. Dixit and Smol 1994; Battarbee et al. 1999) or streams (Pan et al. 1996). In the present study, a similar analysis involving the first principal component axis of a PCA on diatoms retained pH, especially copper and other metals, sulphur, calcium and sodium as important factors correlating with the diatom community structure in AMD. In a case-study on industrial pollution, Ruggiu et al. (1998) found copper to be very disruptive for diatom communities, responsible for the disappearance of *Cyclotella* and *Fragilaria* species. This supports our findings, where these genera were present in river Vascão and reservoir, but not in AMD. However *Achnantes* spp. proved to be tolerant to Cu (Ruggiu et al. 1998; Cattaneo et al. 2004), like in the present study, where it was found in the AMD-sites.

As trophy indicator (Prygiel et al. 1996), the PDI proved inappropriate for AMD-sites. Contrary to our study, Nunes et al. (2003) found the PDI appropriate for bioindication of mining pollution in northern Portugal, where trophy and metal pollution might be correlated. Diatom assemblages in the present study shifted with increasing acidity from higher to lower richness (containing more acidophilic or acidobiontic species), hence mirroring the decrease of pH and increase in metal concentrations. This decrease, also observed in other studies (e.g. Pan et al. 1996; Verb and Vis 2005; DeNicola 2000) was more pronounced than for the macroinvertebrates (Gerhardt et al. 2004) and chironomids (Janssens de Bisthoven et al. 2005), where sensitive species also disappeared, but were replaced by more tolerant species, often being opportunistic and/or fugitive predators. Charles (1985) or Stewart et al. (1987) also stressed the importance of pH in determining diatom community structures. Part of the differences observed between floristic and faunistic responses might be due to the inherent immobility of phytobenthos.

Whitton and Satake (1996) mention that diatoms of acidic environments (<pH 3) mostly belong to *Eunotia*, *Pinnularia*, *Navicula* and *Nitzschia*, genera also found to be dominant in the AMD-sites of the present study. Out of 19 'true inhabitants of highly acidic water' (DeNicola 2000), six species were found in the AMD of the present study, being *Achnanthes minutissima*, *Eunotia exigua*, *Nitzschia capitellata*, *N. subcapitellata*, *Pinnularia acoricola* and *P. subcapitata*. The diatom *P. acoricola* dominated brown patches in an acidic stream in southwestern Spain as well (Sabater et al. 2003). The observed differences in diatom community structure are due to a combined action of low pH and highly soluble heavy metals.

Five species from the reservoir and five species from river Vascão were classified by an extensive study (Arcadis Euroconsult 2005) as respectively typically lentic and typically lotic, with *Achnanthes minutissima* being very common in both. However, four species found in river Vascão were classified as being 'lake species' by the same study, while no reservoir species fell into the category 'lotic species'.

Growth inhibition of *Lemna gibba* was dose-dependent and therefore included in the multimetric index for autotrophs. This endpoint measured after respectively 4 or 7 days differed only very slightly, hence pleading in favour of a more cost-effective 4 days biotest. However, *in situ* exposure of *L. gibba* was not very consistent with the laboratory results. The stronger reactions observed in the field might be due to variations of natural stressors such as temperature and water flow amplitude. Field validation is typically a compromise between higher ecological relevance and lower scientifical inference and accuracy (Gerhardt 1999). However, it pinpoints problems easily overseen in controlled laboratory experiments, allowing for a more real world interpretation of ecotoxicological thresholds or endpoints. Duckweed was tested *in situ* as non-target aquatic vegetation after herbicide spraying and already reacted with reduced growth after 48 h (Gardner and Grue 1996). In a multispecies mesocosm study (Coors et al. 2006), it became clear that biotic interactions (nutrient limitation, competition) played a role, compared to single species laboratory studies, hence stressing the need to approach real world conditions.

Growth inhibition and chlorosis were also found in *Lemna minor* exposed to acidic mining lakes of pH = 4.0 and high Al and Zn concentrations, with the first sublethal reactions already appearing after 2 days (Fomin et al. 2000).

Reports about the sensitivity of duckweed to metals are sometimes contradictory and no clear trend emerges from the literature (Lewis 1995). Clark et al. (1981) found *Lemna perpusilla* to be tolerant to a coal-ash retaining

basin with elevated metal concentrations. On the other hand, laboratory tests (Wang 1986) suggested the common duckweed to be nearly as sensitive to metals as fish species, provided the exposure period is prolonged, which is confirmed, when comparing present data (after 4 days) with results on the moskitofish after 48 h (Gerhardt et al. 2005b). Wang and Williams (1990) reported necrosis, lesion and chlorosis to occur after only 24–96 h, with a typical near-linear or sigmoïdal dose–response pattern, depending on the type of effluent. The phytoxicity test with *Lemna gibba* provided a screening of the overall phytotoxicity of AMD sites. However, the analysis of the resident diatom assemblages gave a more ecologically relevant understanding of the complex multispecies processes in a long-term trend biomonitoring perspective.

Comparing our data on chlorosis, necrosis and growth rate inhibition with previous studies on animals exposed to the same AMD (Gerhardt et al. 2005a, b; Janssens de Bisthoven et al. 2005, 2006), the organisms are ranked according to sublethal sensitivity (LOEC) as follows: *Daphnia magna* (48 h test) > *Lemna gibba* (4 days test), *Athyphaera desmarestii* (freshwater shrimp, 48 h test), *Tetrahymena thermophyla* (ciliate, 24 h test) > *Gambusia holbrooki* (moskitofish, 48 h test) > *Chironomus* gr. *thummi* (24 h test) > *Choroterpes picteti* (Ephemeroptera, 48 h test). Sublethal reactions of *Lemna gibba* occurred in a pH-range similar to the ones obtained with animals of intermediate tolerance, even though they were delayed in time. Comparing chlorosis or necrosis in *Lemna* after 48 h only, the test period used for most of the bioassays on animals, it appeared that only AMD pH 3.3 and 4.4 showed first signs of stress, hence degrading *Lemna* to the group of least sensitive organisms, showing the unsuitability of such a short test period for the *Lemna* bioassay.

The MI_{auto} after 4 or 7 days scored for the AMD relatively similarly to the MI for macroinvertebrates in Gerhardt et al. (2004) (river Vascão = 10, reservoir = 8.9, AMD-pH 4.6 = 4.8 and AMD pH=3.3–2.9), in spite of different exposure times for the macroinvertebrates (behavioural reactions after 5 h in a test period of 48 h) and *Lemna* (4 or 7 days). Eventual differences with the MI were small, e.g. the MI_{auto} scored a bit higher for the reservoir, leading to the conclusion that the multimetric index for autotrophs attributes a slightly better quality to the reservoir. It should be emphasized that both indices were in the first place based on data issued from laboratory bioassays. In the present study we have seen that MI_{auto} based on field bioassay generated lower values, reflecting the higher stress encountered in the field.

The use of acid mine drainage to test an assessment approach combining phytotoxicity (*Lemna*) and phytoassessment (diatoms) provided a reference framework within high metal concentrations and a broad pH spectrum. This enabled a first calibration of this multimetric approach on sites which were *a priori* known to be toxic. The next step in the development would be to apply this method on sites with suspected pollution of various types, and evaluate the behaviour of the multimetric index.

Schmidt et al. (2002) were equally successful at ranking AMD sites of extreme and intermediate toxicities with the use of a modified ecotoxicological rating (METR), synthesizing integrative bioassessment data into a single number ranging from 0 to 100. The METR integrates mean conductivity, Asian clam *in situ* survival, Al an Mn in water and habitat, yielding a METR ranging from reference water = ±95 (converted to MI for macroinvertebrates or MI_{auto} scale (0–10), this would be 9.5) to 15–38 for AMD (after scaling, comparable with our MI = 2.9–4.8 and MI_{auto} = 2.6–3.8). In line with all these studies, our study supports the multimetric approach for aquatic risk assessment of acid mine drainage.

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