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Water-Column, Sediment, and *in Situ* Chronic Bioassays with Cladocerans

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In situ **bioassays are becoming very popular for laboratory test validation and field extrapolation. Natural conditions, including environmental variables, affect the "behavior" of contaminants and, consequently, their toxicity. This work aimed to develop protocols for *in situ* chronic bioassays with cladocerans and to assess the ecological relevance of conventional protocols for laboratory testing (water-column and sediment). An aquatic system impacted with acid mine drainage was chosen for the study. At some stations that were contaminated with heavy metals, water-column bioassays did not reveal any toxicity, while sediment and, especially *in situ* bioassays, revealed a delay in reproduction and a reduction in fertility. Thus, both laboratory bioassays underestimated the actual toxicity of studied sites: sediment, besides being an important contaminant source, became significantly altered with sampling, transport, storage, and manipulation. Therefore, an extra caution is needed when interpreting laboratory results, since the extrapolation to natural systems could be a very problematic step.** © 2000 Academic Press

Key Words: in situ **bioassay; test development;** ***Daphnia magna; Ceriodaphnia dubia;*** **acid mine drainage.**

INTRODUCTION

Chronic bioassays, under controlled conditions, supply useful information for estimating the potential adverse effects of contaminants on aquatic organisms in order to develop an understanding of causal relationships (Rand and Petrocelli, 1985). While the objective of most laboratory studies is not the simulation of field conditions, the extrapolation to natural ecosystems is the most important and problematic step. Environmental factors (including physical, chemical, and biological factors) that can affect the concentration, bioavailability, toxicity, distribution, and fate of a contaminant must be carefully considered when making conclusions about the risk posed by a specific contaminant in aquatic ecosystems (Rand and Petrocelli, 1985). Some of these processes and factors are difficult to replicate and cannot be accurately reproduced in the laboratory environment. This study aims to develop and adapt ecotoxicological tools allowing a sounder extrapolation from laboratory to the field, essentially suitable for ecotoxicological risk assessment at contaminated sites. Ecological relevance of standard sublethal tests is addressed here through the comparison of results obtained under controlled and field conditions, using water-column, sediment and *in situ* bioassays. Since sediments could act as source, sink, and cycling center for toxicants, sediment toxicity bioassays have been increasingly recommended and used (Burton, 1991; Stemmer *et al*., 1990a). Sediment sampling, manipulation, and exposure design variables have been found to affect laboratory toxicity results (Stemmer *et al*., 1990a,b). *In situ* bioassays are becoming a very popular tool for laboratory test validation and field extrapolation (Clark, 1989; Sasson-Brickson and Burton, 1991; Ireland *et al*., 1996). *In situ* bioassays can be used to confirm or validate predicted responses based on laboratory studies to those obtained in the field (Cairns, 1984).

In situ bioassays began to be conducted with acute exposures of fish (Kelly, 1991). Recently, some *in situ* bioassays have been successfully developed with a great variety of organisms such as estuarine crustaceans (Baughman *et al*., 1989), periphyton (Lewis *et al*., 1993), phytoplankton (Munawar and Munawar, 1987), mussels (Salazar *et al*., 1995; Warren *et al*., 1995), amphipods (Crane *et al*., 1995), benthic invertebrates (Hare *et al*., 1994; Chappie and Burton, 1997), mysids (Clark *et al*., 1987), oligochaetes (Monson *et al*., 1995), and also cladocerans (Sasson-Brickson and Burton, 1991; Ireland *et al*., 1996; Ojala *et al*., 1995). Snyder-Conn (1993) conducted chronic bioassays *in situ*, but only with locally collected cladocerans. Most of these studies have found that *in situ* toxicity responses provide an effective means of assessing water and sediment quality.

This work had three principal objectives: (i) to develop protocols for *in situ* chronic bioassays with cladocerans, (ii) to evaluate the toxicity of a contaminated site using three different bioassays (water-column, sediment, and *in situ* bioassays); and (iii) to assess the ecological relevance of

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water-column and sediment laboratory bioassays and their use to make valid field extrapolations in a particular ecosystem impacted by an acid mine effluent.

Low pH of acid mine drainage (AMD) can stress aquatic organisms, which can result in "acidemia" (Kelly, 1991) or even the death of some organisms. Some of the physiological effects of low pH include the denaturation of cellular components, the alteration of ionic balance across organism membranes, and increased exposure to toxic metals (Maltby *et al*., 1987). AMD is characterized by low pH and high concentrations of heavy metals. Variables such as pH, water hardness, alkalinity, and temperature have been reported to affect heavy metal toxicity in invertebrates (Kelly, 1991). These variables reveal fluctuations in nature very difficult to simulate in the laboratory.

MATERIALS AND METHODS

Study Site

The present work was conducted in an aquatic system located in southeastern Portugal (Fig. 1), impacted by a mine effluent that still flows, though the mineral exploitation ended in 1966 (Custódio, 1996). Through several centuries, S. Domingos Mine was an important source of iron, copper, and sulfur (Rodrigues and Cunha, 1992). With the end of the exploitation the mine pit was inundated. The actual heritage of S. Domingos Mine includes the mine effluent, a system of channels and acids ponds (that were built in the last century for the effluent treatment), an abandoned sulfur factory, and many ruins and mine tailings. The mine effluent, with very acid pH and high concentrations of heavy metals (e.g., iron, copper), is discharged into the Chança Reservoir, after an insignificant dilution by the Mosteirão Stream (Lopes *et al*., in press) (Fig. 1).

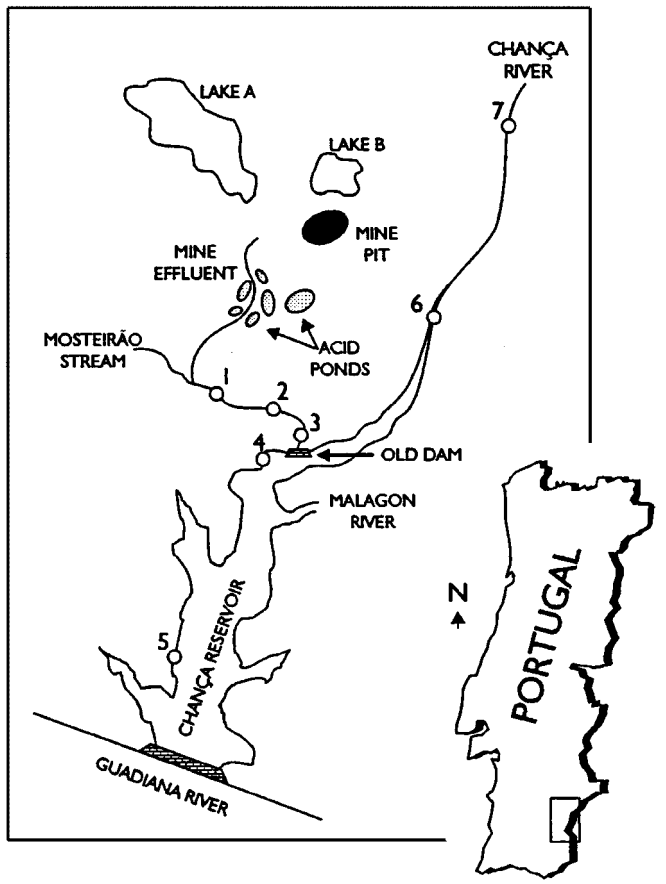

FIG. 1. Study site: location of sampling stations and test sites (stations 1 through 7).

Sampling Stations

The locations of the study site and the sampling points are illustrated in Fig. 1. The mining company constructed Lakes A and B to provide water for their operations. The former still contributes to the flow of the mine effluent (Pereira *et al*., 1995). Stations were selected to represent a gradient of contamination, with station 1 potentially the most contaminated site, and station 7 the reference station.

Test Animals and Culture Conditions

Stock cultures of *Ceriodaphnia dubia* Richard were maintained in groups of 20–30 individuals in 1000 mL of reconstituted hard medium (ASTM) (Weber, 1991). *Daphnia magna* Straus were maintained in individual cultures, using 175-mL beakers, with 100 mL of ASTM medium. An organic supplement, Marinure 25 (supplied by Pann Britannica Industries Ltd, Waltham Abbey, U.K.) was added every second day before medium exchange. This supplement is an extract of the alga *Ascophyllum nodosum* (Baird *et al*., 1989). Organisms were fed daily with the alga *Selenastrum capricornutum* (1.5×10^5 cells/mL). The cultures were maintained at $20 \pm 1^{\circ}$C, with a 16:8 light:dark cycle. Neonates, 6 to 24 h old and from the third to fifth broods, were used to initiate bioassays.

In Situ Bioassays

In situ chronic bioassays were conducted in fall with *D. magna*. They were performed in chambers constructed for this purpose, which were previously tested for their suitability for acute testing. At each site, two types of *in situ* bioassays were run: one (T) using test chambers allowing the flow-through of water, and the other (C) using closed chambers without contact with the surrounding medium. The test chambers were 50-mL polypropylene beakers that had three windows covered by a 50-μm mesh. Closed chambers were also 50-mL polypropylene beakers without windows and filled with site water, previously filtered with a 50-μm mesh.

To guarantee a tight fit, the caps were wrapped with a piece of laboratory film. The bioassay conducted in closed chambers (C) had the purpose of eliminating the stress factor of low food availability in the field. The differences between the exposure conditions in this bioassay and those conducted in the laboratory were thus restricted to light, photoperiod, and temperature.

Control treatment (0) consisted of ASTM medium (Weber, 1991), with the organic supplement Marinure 25 and food, in closed chambers, deployed *in situ*. Organisms in the closed chambers (from C bioassay and control animals) were fed *S. capricornutum* (3.0×10^5 cells/mL) every second day, simultaneously with the change of medium. In closed chambers, a small volume of air was kept to maintain a sufficient concentration of dissolved oxygen in the water, which was regularly monitored. Before beginning the bioassay, all neonantes from stock cultures were removed with a plastic pipet to a glass vessel containing ASTM medium. From this vessel, one organism was transferred to each *in situ* chamber, previously filled with filtered site water or ASTM medium in the case of the control. This was done in the laboratory, and chambers were then transported to the field inside plastic containers filled with filtered site water. Ten to twelve replicates, with one organism each, were run at each station. The chambers were placed in the field, attached to a plastic structure, which was kept in the bottom near the sediment with a stone on it. This allowed the maintenance of the right position of test chambers, with the net of the cap in contact with the sediment. These structures with the chambers were placed approximately 0.5–1 m deep and 2–4 m from the margin. Chambers were removed every day, and the nets were cleaned, to avoid a decrease in water flow-through inside the chamber. Chambers were checked for broods every day. At the end of the bioassay, all chambers were collected and transported to the laboratory. In test chambers and closed chambers, eggs/embryos present inside the marsupial chamber at the end of the bioassay were counted as a brood (generally the second broad, N2), and the number of days until this brood was extrapolated by adding 1 or three days (respectively for embryos or eggs) to the bioassay duration. The criteria followed for the validation and for the bioassay ending were as recommended by Weber *et al.* (1989), for the 21-day test, with some modifications: (i) the bioassay ended when more than 60% of control females had the eggs corresponding to the third brood in the marsupial chamber; (ii) the bioassay was considered valid if the control survival was at least 80%, and the total number of juveniles per surviving female was equal to or higher than 40, on average (Gersich and Milazzo, 1990).

Laboratory Bioassays

Polyethylene beakers were used to collect water-column samples from each site. Composite sediment samples, collected with a plastic scoop from each site, were placed in black airtight plastic containers and were maintained in the laboratory at 4°C until bioassay start. Sediment samples consisted approximately of the upper 6 cm of the sediment surface.

Summer bioassays were conducted only in laboratory using *C. dubia*, while in fall laboratory and *in situ* bioassays were performed with *D. magna*. Laboratory bioassays basically followed USEPA protocols (Weber *et al.*, 1989). Neonates of *D. magna* and *C. dubia*, less than 24 h old, from third to fifth broods, were used. Two types of bioassays were run for each site: water-column and sediment. *C. dubia* bioassays were performed with 30 mL of site water for the water-column bioassay and with 15 mL of of sediment and 15 mL of site water for the sediment bioassay, both in 42-mL beakers. *D. magna* bioassays were performed in 175-mL beakers, using 50 mL of site water for the water-column bioassay and 50 mL of sediment and 50 mL of site water for the sediment bioassay. For each site and bioassay, 10 to 12 replicates were used, with one organism per vessel. The control consisted of ASTM medium with the organic supplement. All test organisms were fed every day with *S. capricornutum* (1.5×10^5 cells/mL), and the medium was changed every second day. This concentration of algae corresponds to 55 mg/m^3 chlorophyll *a*, measured according to APHA (1989). Renewal of media for the sediment bioassays was always done 24 h before, to allow the fine particles to settle. Before beginning the bioassays, all neonates from stock cultures were removed with plastic pipets to a glass vessel containing ASTM. Broods were checked and registered every day.

System Parameters

All laboratory bioassays were conducted at 20 ± 1°C, with a 16:8-h light:dark cycle. Physical and chemical parameters (pH, temperature, dissolved oxygen, and conductivity) were monitored every second day, in the laboratory and in the field. Measurements of pH and temperature were performed with a WTW 537 pH meter. Dissolved oxygen concentrations and conductivity were measured with WTW OXI 92 and WTW LF 92 meters, respectively.

Water samples were collected in polyethylene beakers, transported and maintained at 4°C, for chemical analysis of metals (total recoverable), hardness, alkalinity, and chlorophyll *a*. Except for metals, Hach (1993) protocols were followed. Heavy metals (total recovered) in water-column samples were determined by a combination of inductively coupled argon plasma (ICP) and atomic absorption spectroscopy (AAS) (after acidification with HCl 37%, until a pH equal to or lower than 2). Mercury was analyzed by AAS, without flame, with Hg vapor, and arsenic was analyzed by AAS, with a hydrate generator.

Endpoints

The responses used in the analysis were survival, fertility (mean number of juveniles per surviving female), mean number of days until the first and second reproductions, and total body length (from the top of the head to the base of the tail spine) of laboratory animals. Body size was measured with a calibrated eyepiece micrometer, to an accuracy of 0.01 mm.

Data Analysis

Fisher's exact test was used to determine the sites where survival of *C. dubia* and *D. magna* was significantly reduced. Data from chronic bioassays were analyzed by ANOVAs (Model I). The multicomparison Student–Newman–Keuls test was used if significant differences were found ($P \leq 0.05$). Whenever heteroscedasticity or nonnormality of data was pronounced, a nonparametric analysis of variance was employed (Kruskal–Wallis test), followed by nonparametric multiple comparison testing (Zar, 1984). To make two-sample comparisons, the *t* test was used.

RESULTS

Water Quality

The highest metal concentrations were detected in summer, especially at station 1, for Al, Cd, Cu, Mn, Ni, Pb, Co, and Zn (Table 1). As, Cr, and Hg were present in concentrations below detection limits in all stations. Metal concentrations were generally lower in fall than in summer, Ni, Hg, and Co being below detection limits at all sampling sites (Table 2). Stations 1 and 3 were the only sites where Al and Cu were detected, and had the highest values of Fe, Mn, and Zn.

TABLE 1
Concentration (mg/L) of Metals (Total Recoverable) in Surface Water Samples from the Aquatic System of S. Domingos Mine, in Summer

Metal	1	4	5	6	7
A1	11.3	0.116	< 0.1	< 0.1	0.17
As	< 0.005[a]	< 0.005	< 0.005	< 0.005	< 0.005
Cd	0.0286	0.00056	0.00082	0.00129	0.00091
Cr	< 0.015	< 0.015	< 0.015	< 0.015	< 0.015
Cu	1.7	< 0.015	0.027	< 0.015	< 0.015
Fe	1.64	0.0271	0.129	0.392	0.301
Mn	3.07	< 0.03	0.081	< 0.03	< 0.03
Ni	68	0.032	< 0.03	0.033	< 0.03
Pb	4.60	0.0033	0.640	0.00506	0.00788
Hg	< 0.002	< 0.0002	< 0.0002	< 0.0002	< 0.0002
Co	90	< 0.03	< 0.03	< 0.03	< 0.03
Zn	12.10	0.088	0.288	0.2	0.064

[a]Values preceded by the "less than" symbol are below the detection limit.

TABLE 2
Concentrations (mg/L) of Metals (Total Recoverable) in Surface Water Samples from the Aquatic System of S. Domingos Mine, in Fall

Metal	4	5	6	7
A1	< 0.05[a]	< 0.05	< 0.05	0.0817
As	0.00091	0.00069	0.00083	0.00053
Cd	< 0.020	< 0.020	< 0.020	< 0.020
Cr	0.0060	< 0.006	0.0076	< 0.006
Cu	< 0.020	< 0.020	< 0.020	< 0.020
Fe	0.0141	0.0189	0.0945	0.0937
Mn	0.0299	0.0313	0.0765	0.0412
Ni	< 0.005	< 0.005	< 0.005	< 0.005
Pb	< 0.1	< 0.1	< 0.1	< 0.1
Hg	< 0.00071	< 0.00071	< 0.00071	< 0.00071
Co	< 0.015	< 0.015	< 0.015	< 0.015
Zn	0.0225	0.0337	0.0265	< 0.020

[a]Values preceded by the "less than" symbol are below the detection limit.

Considering all sampling stations, water temperatures ranged from 26.1 to 27.2°C in summer and from 20.7 to 21.9°C in fall (Table 3). Conductivity and pH values measured in the field followed a spatial gradient, with the highest values of conductivity and the lowest pHs being recorded at station 1, in the mine effluent. In summer, the system was recently inundated, due to discharges from a dam located in the Malagon River (Spain). The old dam was submersed, failing to separate the two water bodies. The highest value of conductivity registered in the summer was 681 μS/cm at station 7 (the reference station), when the Chança River was not flowing there, being reduced to small ponds. Dissolved oxygen was almost always higher than 8 mg/L, in both field campaigns.

In the laboratory, in water-column bioassay beakers, the physical and chemical parameters were very similar to *in situ* values (Table 3). In the sediment bioassay, there was a general decrease in pH and dissolved oxygen and an increase in conductivity values. In summer, at station 1, the pH decreased by more than 1 unit and the conductivity increased by more than 100 units. In fall, pH and conductivity in the laboratory were similar to *in situ* measurements. Still, in the laboratory, the water exhibited a strong decrease in dissolved oxygen, especially in the sediment bioassay. The lowest value was 4.4 mg/L for station 6.

In summer, the sample from station 7 had the highest value of hardness (181 mg/L of $CaCO_3$), and all other stations had moderately soft water (about 80 mg/L of $CaCO_3$) (Table 3). In fall, the highest value of hardness was registered at station 6 (136 mg/L of $CaCO_3$). Stations 4, 5, and 7 had moderately soft water. There was an evident spatial gradient of alkalinity, with the highest values at the reference station

TABLE 3
Physical and Chemical Parameters (Mean Values) and Chlorophyll a Levels Measured in Summer and Fall, in the Aquatic System of S. Domingos Mine (*in Situ*) and in the Respective Laboratory Tests (Water-Column and Sediment Bioassays)

	Summer						Fall					
Parameter/Stations	1	3	4	5	6	7	1	3	4	5	6	7
In situ												
Temperature (°C)	26.1	26.9	26.8	26.5	26.7	27.2	21.7	21.7	21.4	20.8	21.9	20.7
pH	7.15	7.34	7.57	7.42	7.87	8.30	3.45	3.45	7.14	7.32	7.81	7.16
Conductivity (μS/cm)	312	309	276	271	291	681	505	437	292	283	296	307
Dissolved oxygen (mg/L)	8.7	7.5	9.1	8.9	9.1	8.9	9.2	9.1	8.9	8.9	10.0	8.0
Water-column												
pH	7.03	6.28	7.72	7.79	8.00	8.41	—	—	7.27	7.46	7.39	7.78
Conductivity (μS/cm)	323	321	281	290	299	743	—	—	298	289	300	368
Dissolved oxygen (mg/L)	9.5	9.4	9.5	9.3	9.4	9.1	—	—	8.1	8.3	8.0	8.2
Sediment												
pH	6.03	6.02	7.29	7.70	7.03	8.04	—	—	6.98	6.93	7.02	7.69
Conductivity (μS/cm)	472	408	343	477	322	726	—	—	320	308	349	403
Dissolved oxygen (mg/L)	6.8	6.8	7.3	6.4	5.3	7.9	—	—	5.5	5.4	4.4	6.9
Hardness (mg $CaCO_3$/L)	85.7	88.4	86.0	89.7	87.5	180.7	106.7	107.4	85.1	75.3	135.5	92.2
Alkalinity (mg $CaCO_3$/L)	6.5	7.8	32.4	27.0	40.7	131.3	—	—	21.8	16.4	28.6	57.91
Chlorophyll *a* (mg/m^3)	0.80	2.06	0.92	1.71	3.80	1.97	0.34	0.16	0.90	0.67	2.27	0.25

(131 mg/L $CaCO_3$ in summer and 58 mg/L $CaCO_3$ in fall) and the lowest values in the mine effluent (bellow 10 mg/L $CaCO_3$ in summer). The highest levels of chlorophyll *a* were registered at station 6, with 3.80 mg/m^3 in summer and 2.27 mg/m^3 in fall. The levels of chlorophyll *a* were higher in summer, in all the test stations. The lowest value was measured at station 1, with 0.80 mg/m^3 chlorophyll *a*. In fall, station 7 had the lowest value of chlorophyll *a*, 0.25 mg/m^3.

Summer Experiments

The duration of chronic laboratory bioassays with *C. dubia* was 10 days. Fisher's exact test only revealed lethal toxicity in the sediment bioassay of the two most contaminated sites, stations 1 and 2, where no surviving organisms were registered at the end of the bioassay (Table 4).

The highest value of fertility was recorded at station 7, in the sediment bioassay, with 37.4 juveniles per surviving female (Fig. 2). Fertility in the water-column bioassay at stations 1 and 4 was significantly different from that at the control and reference site (station 7) (ANOVA: $P < 10^{-3}$, $F(6,55) = 5.493$). In the sediment bioassay, fertility at the reference site (station 7) was significantly different from that at all other stations, even from control (ANOVA: $P < 10^{-3}$, $F(4,39) = 7.148$). The sediment bioassay at the two most contaminated sites (stations 1 and 2) demonstrated lethal toxicity, with 100% mortality, but station 4 did not reveal any toxicity (Fig. 2). Fertility values in the sediment bioassay were higher than in the water-column bioassay at stations 4, 5, and 7 (t tests: $P < 10^{-3}$, $t(15) = -4.94$; $P < 10^{-4}$, $t(16) = -5.95$; $P < 10^{-3}$, $t(16) = -4.43$, respectively).

TABLE 4
Survival of *Ceriodaphnia dubia* (in Summer) and *Daphnia magna* (in Fall) in Chronic Bioassays Performed in the Laboratory[a]

	Summer		Fall			
Station	W	S	W	S	C	T
0	100%	100%	100%	100%	100%	100%
1	70%	**0%**	—	—	—	—
2	80%	**0%**	—	—	—	—
4	90%	80%	100%	100%	100%	100%
5	90%	90%	90%	100%	100%	100%
6	90%	80%	90%	90%	91.7%	83.3%
7	90%	90%	100%	**60%**	83.3%	100%

[a]W, water-column fraction; S, solid fraction; and *in situ*; C, closed chambers; T, test chambers. 0 = control. Value in boldface indicate a significant reduction in survival (Fisher exact test).

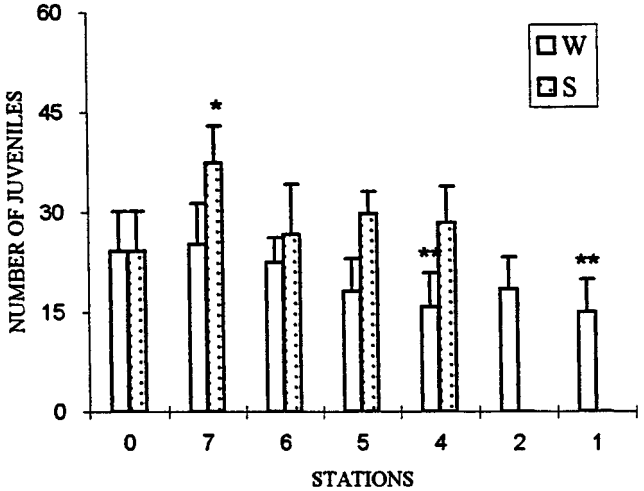

FIG. 2. Fertility (average and standard deviation) of *Ceriodaphnia dubia*, in a chronic laboratory test, conducted in the summer. W, water-column test; S, solid-phase test. 0 = control. *Significantly different from all other stations; **significantly different from control and reference station.

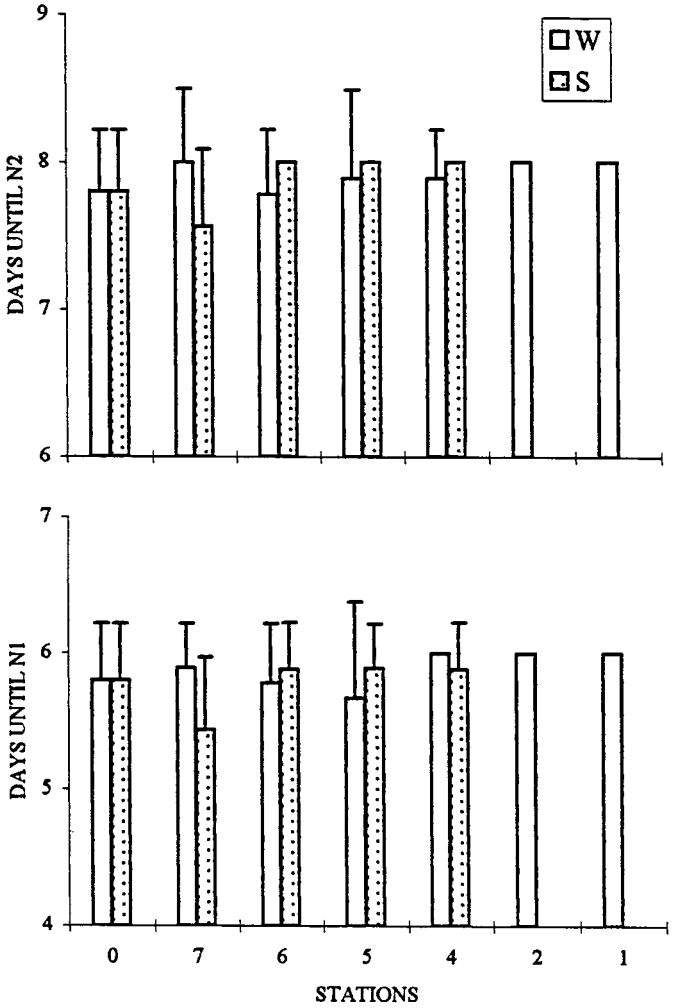

FIG. 3. Age until the first and second reproduction (average and standard deviation) of *Ceriodaphnia dubia* females, in a chronic laboratory test, conducted in the summer. W, water-column test; S, solid-phase test. 0 = control.

Despite a small delay until the first reproduction observed in the mine effluent (6.0 days), this was not significantly different from the control (5.8 days), in any of the tested sites, for water-column and sediment bioassays (Fig. 3).

In the water-column bioassay, there were no significant differences in the number of days until N2, which ranged from 7.8 days in the control to 8 days at the three stations located in the mine effluent. A Kruskal–Wallis test detected significant differences in the mean number of days until N2 in the sediment bioassay ($P = 0.0236$, $N = 44$, $H = 11.3$); however, the multiple comparison test was not able to discriminate these differences. At stations 4, 5, and 6 the mean was 8 days, against 7.6 days for the reference site (Fig. 3).

The smallest animals were the ones from control (1.04 mm) and from the water-column bioassay at stations 1 and 2 (1.01 and 1.08 mm, respectively) (Fig. 4). The largest animals were those from station 6 in the water-column bioassay, with 1.21 mm, which was significantly different from that of the animals in the sediment bioassay at the same station (1.18 mm) (t test: $P = 0.0147$, $t(16) = 2.74$). Analysis of variance performed for the measurements of laboratory animals (total body length) revealed that for the water-column bioassay, the control animals were smaller, having significant differences from the animals from stations 4, 5, 6, and 7 (ANOVA: $P < 10^{-4}$; $F(6,62) = 11.025$). The animals from the most contaminated site (station 1) were smaller than all others, except those from the control site and station 2. In the case of sediment bioassays, no statistical differences were found between the measurements of *C. dubia* at all stations, but all the animals were larger relative to control animals (Kruskal–Wallis: $P < 10^{-4}$, $N = 52$, $H = 30.9$) (Fig. 4).

Fall Experiments

Fall chronic bioassays were conducted with *D. magna*. Stations 1 and 2 were not tested for sublethal toxicity, because in summer, these two sites presented lethal toxicity for *C. dubia*. Fisher's exact test revealed lethal toxicity only at station 7, in the sediment bioassay (Table 4). In this precise bioassay and station, the highest fertility was recorded, with a mean of 101.5 juveniles per surviving female (Fig. 5). The lowest values were obtained in test chambers, at the most contaminated sites: stations 4 and 5 with 5.5 and 5.4 juveniles, respectively.

Fertility results demonstrated that there was a strong reduction when the control was conducted under field

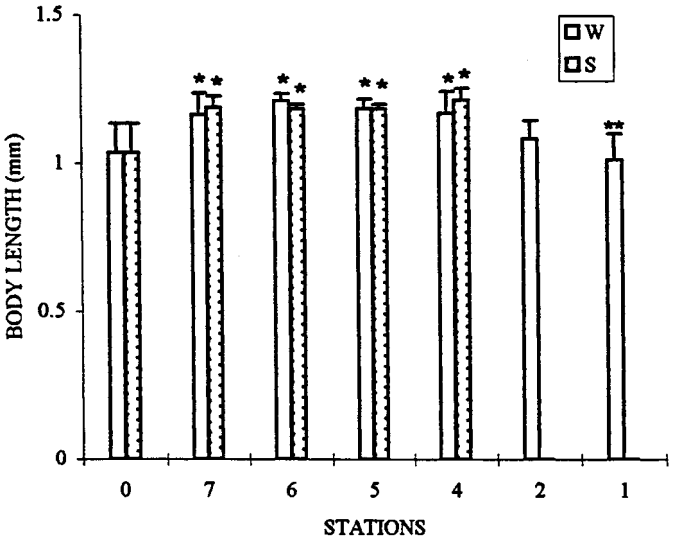

FIG. 4. Total body length (mm, average and standard deviation) of Surviving *Ceriodaphnia dubia* females at the end of a chronic laboratory test, conducted in the summer. W, water-column test; S, solid-phase test. 0 = control. *Significantly different from control; **significantly different from all other stations, except control and station 2.

conditions (almost 50%). At the reference site (station 7), the females in closed chambers had an average number of 53 juveniles, a value close to 46 juveniles recorded in the control conducted *in situ*. In the test chambers at station 7, females had a mean number of 19 juveniles. This corresponds to more than 80% fertility reduction against the value recorded for the sediment bioassay, at the same station. At the contaminated sites, the reduction was even more pronounced, being almost 95% at stations 4 and 5. In the laboratory, water-column bioassays did not reveal toxicity at any station, and in the sediment bioassay only at the most contaminated site (station 4) was a significant reduction in fertility recorded (ANOVA: $P = 0.0013$, $F(4,40) = 5.452$).

In the water-column bioassay, the number of days until N1 at stations 4, 5, and 6 was significantly higher than in the control (Kruskal–Wallis: $P = 0.0013$, $N = 49$, $H = 17.9$) (Fig. 6). At the second reproduction, the age ranged from 11.0 days in the control to 11.7 days at stations 4, 5, and 6, the differences being significant (Kruskal–Wallis: $P = 0.0106$, $N = 48$, $H = 13.1$) (Fig. 6). In the sediment bioassay, organisms at station 6 reproduced, both for the first and second times, significantly later than control organisms (Kruskal–Wallis: $P = 0.0011$, $N = 45$, $H = 18.3$, and $P = 0.0021$, $N = 45$, $H = 16.8$, respectively). *In situ*, in closed chambers, there was a delay until the first reproduction at stations 4 and 5 relative to control (Kruskal–Wallis: $P < 0.0001$, $N = 64$, $H = 26.5$). The mean number of days until N1 at station 4 was 10.1, against 8.25 in the control. Still in the closed chambers, the number of days until N2 was significantly different from that of the contol at stations 4, 5, and 7 (Kruskal–Wallis: $P < 10^{-4}$, $N = 63$, $H = 43.9$). In the case of test chambers, the delay was more pronounced, and the mean number of days until N1 at stations 4 and 5 was 11.8 and 12, respectively, being significantly different from control and from stations 6 and 7 (Kruskal–Wallis: $P < 10^{-4}$, $N = 65$, $H = 53.2$). The age at second reproduction in the test chambers followed almost the same pattern of differences as the age at first reproduction (Fig. 6). The mean number of days until N2 at station 4 was 14.8, being significantly different from the numbers for the control and stations 6 and 7 (Kruskal–Wallis: $P < 10^{-4}$,

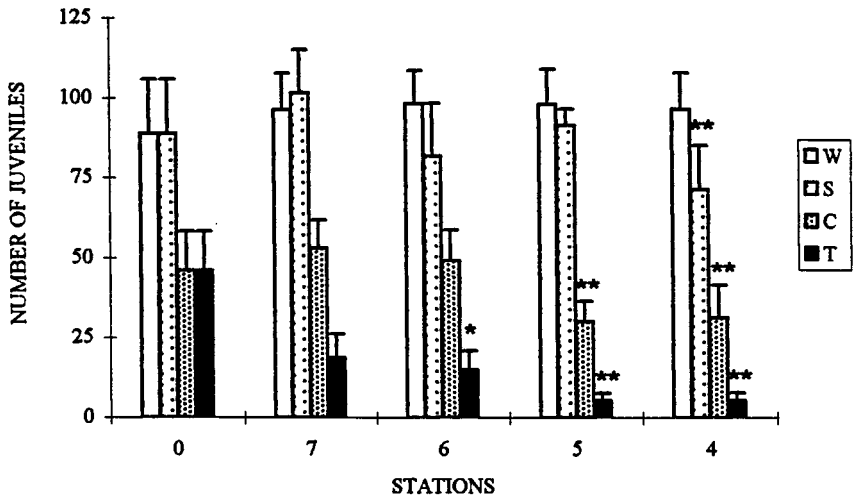

FIG. 5. Fertility (average and standard deviation) of *Daphnia magna*, in chronic tests (in the laboratory and *in situ*), conducted in the fall. W, water-column test; S, solid-phase test; F, filtrate test (closed chambers); T, *in situ* test (test chambers). 0 = control; *Significantly different from control; **significantly different from control and reference site.

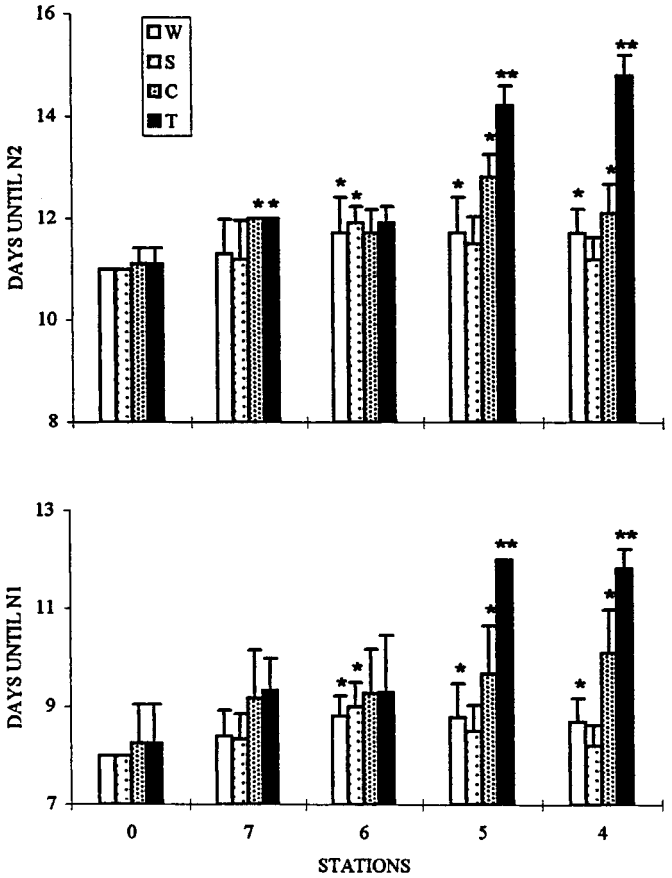

FIG. 6. Age until the first and second reproduction (average and standard deviation) of *Daphnia magna* females in chronic tests conducted in the fall. W, water-column test; S, solid-phase test; F, filtrate test (closed chambers); T, *in situ* test (test chambers); 0, control. *Significantly different from control; **significantly different from control and from reference station.

$N = 65$, $H = 59.1$). All stations, except station 6, presented a significant delay relative to control.

The largest animals were those from the control with a mean total body length of 4.16 mm and the smallest were from station 4 at 3.43 mm, at the need of the bioassay (Fig. 7). In the water-column laboratory bioassay, control animals were larger than those from test stations (ANOVA: $P < 10^{-4}$, $F(4,43) = 17.63$). In the case of the sediment bioassay (S), animals from all stations were significantly smaller than control animals, and animals at stations 4, 5, and 6 were also significantly smaller than reference site animals (ANOVA: $P < 10^{-4}$, $F(4,39) = 59.06$).

DISCUSSION

In the summer, all stations had a pH higher than 7. In the laboratory, after 24 h, the pH of the sediment bioassay dropped more than one unit at stations 1 and 2. Inversely, conductivity was higher in the sediment samples than *in situ*, the difference being more pronounced, again, at station 1. At this station, pH in the field decreased almost 4 units and conductivity increased almost 200 units, from summer to fall.

Stations located upstream the old dam were the most impacted by the acid mine drainage (AMD), presenting the lowest values of pH and the highest values of conductivity and metals. Chança Reservoir has moderately soft water and low alkalinity, which is reflected in the impact of the acid mine effluent on this reservoir: acidity characteristic of AMD is not quickly annulled when the dilution is made. This fact justifies the lethal toxicity registered at stations 1 and 2 and the sublethal toxicity at station 4, located just downstream the old dam. Stations 1 and 2 exhibited lethal toxicity in the sediment bioassay, which proved that the sediments from these two stations are effectively toxic. The levels of several metals (e.g., Al, Cd, Cu, Ni, Zn) at station 1 were very high, leading to the expectation of strong mortality. However, this was registered only in the sediment bioassay; in the water-column bioassay only a significant reduction in fertility was observed. Stations 1 and 2 were not tested in fall for sublethal toxicity, since they exhibited lethal toxicity in the summer sediment bioassay.

One of the major problems in the impact assessment of acid mine drainage is the complexity of these kinds of effluents. In addition to the acidity itself, there may be problems from precipitation of iron(III) oxide, from high concentrations of suspended solids, and from elevated concentrations of heavy metals (Kelly, 1991). The toxicity of many divalent metals may be controlled by the acid-volatile

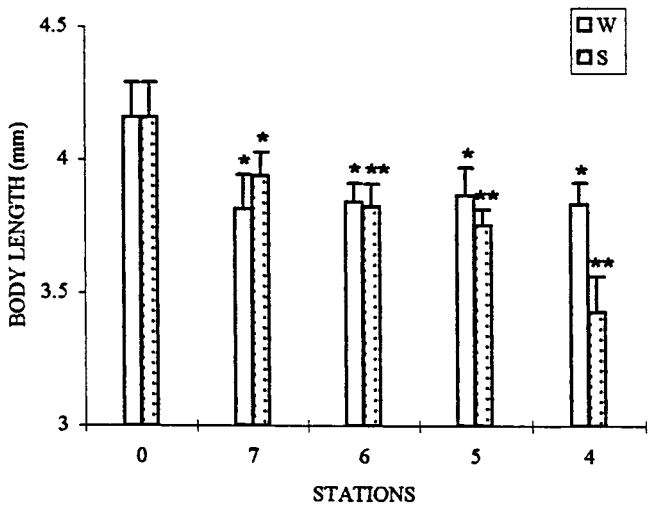

FIG. 7. Total body length (mm, average and standard deviation) of Surviving *Daphnia magna* females at the end of chronic tests conducted in the fall. W, water-column test; S, solid-phase test; 0 = control. *Significantly different from control; **significantly different from control and from reference station.

sulfide (AVS) phase of sediments (Di Toro *et al*., 1990). However, the AVS model has several potential limitations, as demonstrated by Hare *et al*. (1994).

The toxicity of contaminants in sediments is influenced by the extent to which the contaminant binds to sediment (Kemble *et al*., 1994). Metals exist in water in equilibrium between particulate, colloidal, dissolved, organic, and inorganic forms (Depledge *et al*., 1994). Organic ligands, temperature, hardness, redox potential, and pH are important factors affecting metal speciation and bioavailability, giving rise to a dynamic equilibrium between metals ions, dissolved metal, and organic and inorganic complexes (Depledge *et al*., 1994). Several authors have demonstrated the importance of the chemical form of metals in assessing their bioavailability and toxicity (Penttinen *et al*., 1995; Rieuwerts and Farago, 1995). Most metals are generally more toxic at lower pH values, which is usually attributed to a greater concentration of free metal ions (Cummins, 1994). Some of these factors that affect the metal speciation and bioavailability indicated differences between laboratory and field values. This could help to explain at least part of the lack of agreement between the toxicity registered in laboratory and *in situ* bioassays.

Most studies of the toxicity of metals have been concerned primarily with the toxicity of individual metals; however, as in the case of acid mine drainage, it is common that several metals, occur in the same environment. The chemical and biochemical complexities involved when organisms are exposed to mixtures of metals have not been sufficiently understood, and the interpretation of results is difficult (Holdway, 1992). While the interactions are most commonly additive, all possible interactions due to the presence of a complex chemical mixture in a sediment are not known (Hoke *et al*., 1993). However, antagonistic and synergistic effects could be expected in the expression of chemical mixtures (Hoke *et al*., 1993), as has been found for mixtures of only two or three metals (Jak *et al*., 1996). The ecological relevance of most studies of heavy metal toxicity could be questionable, as factors that modulate *in situ* toxicity usually are not taken into account by current test procedures (Depledge *et al*., 1994).

Laboratory summer bioassays revealed strong contamination of sediments from stations 1 and 2, since on the second day all *C. dubia* from the sediment bioassay in these two stations were dead. At all other stations fertility was higher in the sediment than in the water-column bioassay, probably due to the presence of extra food supplied by sediments. Food was added *ad libitum* to all flasks. However, it consisted of only one algal species: *S. capricornutum.* it can only be hypothesised that bacteria present in the sediments of these stations provide a better energy supply than algae alone. Bacteria are not a primary resource for cladocerans; however, their extracellular metabolites could contribute to increase survival/reproduction of cladocerans, especially during the first 4 to 5 days of life (Knight and Waller, 1992). Sometimes, a poorly understood phenomenon called *hormesis* seems to occur; this generally consists of a stimulation of reproduction/growth/survival at low concentrations of contaminants that reduces these parameters at higher concentrations (Kooijman and Bedaux, 1996). The higher fertility of *C. dubia* found in the summer sediment bioassay at stations 4, 5, and 6 could also be explained on the basis of this phenomenon. However, in the fall, a higher fertility in sediment bioassays was not recorded. On the contrary, at station 4 there was a significant reduction of fertility in the sediment bioassay, which indicates sediment contamination.

The mean number of days until N1 and N2 of *C. dubia* was revealed to be a nonsensitive parameter in summer bioassays. In the fall, age at both N1 and N2 of *D. magna* was sensitive enough to discriminate impacted stations from the control. Measurements of summer-tested animals revealed that the smallest were those from control site and stations 1 and 2, in the water-column bioassay. This similarity between control and contaminated sites most probably represents two distinct realities, i.e., a compromise between somatic growth and a strong energy investment toward reproduction versus a growth deceleration due to chemical stress. This emphasizes the need to be very careful when using growth as a toxicity endpoint. On the contrary, in fall bioassays with *D. magna*, control animals were larger than all others, and animals from stations 4 and 5 were smaller than control and reference site animals, which corroborated results relative to reproduction. Organisms at all stations had similar body length in both water-column and sediment bioassays, with the exception of *C. dubia* at station 6 in the summer: females in the sediment bioassay grew less than those in the water-column bioassay. This was the station with the lowest values of oxygen, with a mean of 5.3 mg/L, in the sediment bioassay. Their sediments apparently had more organic matter, which could consume some oxygen from the water column, resulting eventually in a stressful condition for tested animals. However, that value is above 4 mg/L, which is the recommended minimum level for toxicity testing with these organisms (Weber, 1991).

Sediments play an important role in aquatic ecosystems, since they serve as source, sink, and cycling centre for nutrients, organic matter, and chemicals, including contaminants (Stemmer *et al*., 1990b). Often, sediments may accumulate contaminants to higher concentrations than in overlying waters (Stemmer *et al*., 1990a). Most protocols for sediment bioassays generally recommend that the manipulation of sediment samples (e.g., sieving and mixing) be minimal, to maintain the chemical equilibrium of potential contaminants associated with the sediments (Reynoldson *et al*., 1994). Chemical, physical, and biological alterations of sediments in polluted aquatic ecosystems can affect contaminant bioavailability (Burton, 1991). Sediment collection

may induce some changes, including alterations in chemical speciation due to oxidation and reduction, chemical equilibrium disruption, and changes in biological activity (Burton, 1991). Such changes may modify toxicity results, making the extrapolation to the field inaccurate. However, in this study, sediment results were more similar to *in situ* results.

In situ bioassays did not confirm laboratory results, with an evident sublethal toxicity at stations 4 and 5. These results seem to indicate that the sediments of these stations were a source of contaminants, effectively toxics. However, this was not reflected in the water-column quality. It seems that, in this particular system, laboratory exposures following classic methodologies represent unrealistic field conditions and underestimated the real toxicity. This difference between laboratory and *in situ* results could be partially explained by additional stress induction due to handling in the field. Physical conditions such as light and temperature affect the reproductive success of organisms, as has been proved in many works (Goss and Bunting, 1983; Cooney *et al*., 1992a; Korpelain, 1986). Reproduction of *D. magna* depends on energy reserves and body size and, thus, indirectly on food availability and feeding rates (Kooijman and Bedaux, 1996). Feeding rates affect not only reproduction but also survival and growth of cladocerans. These responses depend on the quantity and nutritional value of food available (Groeger *et al*., 1991; Martínez-Jerónimo *et al*., 1994; LaRocca *et al*., 1994), on temperature (Goss and Bunting, 1983; Korpelain, 1986), on photoperiod (Cooney *et al*., 1992a; Korpelain, 1986), and on water quality (Cooney *et al*., 1992b), which were conditions not under control in the field. In practice, not only these parameters but also the environmental concentrations of contaminants fluctuate and vary continuously in time (Mance, 1987). This seems to be the most probable reason for the differences found between laboratory and *in situ* results. Cooney *et al*. (1992a) conducted a study in which they found that photoperiod and renewal frequency were the test conditions with a higher impact on survival and reproduction. Temperature, beaker size, and amount of food had only minimal effects on reproduction and none on survival (Cooney *et al*., 1992a). However, as pointed out by Groeger *et al*. (1991), the influence of food quality available to zooplankton is important in determining its success in a particular environment, since it regulates growth and reproduction. In the fall, the lowest levels of chlorophyll *a* were found at stations 1, 4, and 5. At station 7, the fertility reduction in test chambers relative to closed chambers can probably be explained on the basis of the food available (quality and quantity). The concentration of chlorophyll *a* in ASTM medium, with the addition of *S. capricornutum* (1.5×10^5 cells/ml), is about 55 mg/m^3. At station 7 the level of chlorophyll *a* detected in a fall sample was 0.25 mg/m^3. This value is very low in comparison with the optimal level of laboratory conditions. In a study conducted by Jak *et al*. (1996), the sensitivity of cladoceran species to a metal mixture seemed to increase with susceptibility to low food concentrations. Hardness and alkalinity values were low in water samples from stations 1, 3, 4, and 5. In addition to organic ligands, temperature, and pH, alkalinity and hardness can also affect the toxicity of some toxicants such as heavy metals, since they influence metal speciation (Cummins, 1994). Studies conducted with *D. magna*, *Tubifex tubifex*, and two species of gastropods indicated that water hardness reduces the toxicity of zinc (Mance, 1987). The general tendency is to have lower toxicity of metals with higher values of hardness (Rand and Petrocelli, 1985; Penttinen *et al*., 1995).

If growth is affected, feeding rate is altered as well and consequently reproduction too, not only by a reduction in allocation to reproduction, but also via a delay in the onset of reproduction (Kooijman and Bedaux, 1996). In the laboratory work, in the fall, females from station 4 (sediment bioassay) were smaller and had lower fertility than control and reference site females. However, there was no delay in the onset of reproduction. In the case of *in situ* bioassays, the delay in the onset of reproduction had great reprecussions in fertility values. At the end of the bioassay, a reduced number of females reached maturity in test chambers, and the eggs found in the marsupial chamber were counted as N1. At station 4, the age at second reproduction needed to be extrapolated in some cases for the closed chambers and always for the test chambers.

The presence of indigenous animals in test chambers was not checked, despite some occasional observations. Reynoldson *et al*. (1994) proved that the presence of indigenous organisms could greatly affect chronic endpoints in sediment bioassays. It has been suggested that caged animals be prevented from using avoidance behavior, which contributes to an overestimation of the actual toxicity (Baughman *et al*., 1989). Probably, in this study this limitation is not as important as it will be with caged fish. However, this emphasized the fact that *in situ* caged animals were tested under semiartificial conditions. Some works aimed to investigate if cladocerans are able to avoid toxicants by adaptative swimming behavior (e.g., Laurén-Määttä *et al*., 1997). There is a need to understand these complex effects when the simulation of field conditions intends the extrapolation to natural systems.

CONCLUSIONS

This study attempted to establish a direct link with laboratory information by *in situ* bioassays, and proved that caution is needed when interpreting laboratory bioassay results. Whenever there is a suspicion of sediment contamination, sediment bioassays should be performed, since results are more closely related to field situations.

In the laboratory, fluctuations of physical–chemical parameters and toxic concentrations do not occur, and the toxic

exposure is made under very controlled conditions, especially in the case of water-column bioassays, without the presence of an important source of contaminants: the sediments. In opposition, *in situ* results can be very difficult to interpret because of all the variations in bioassay conditions associated with field exposures. It has been emphasized that ecotoxicological extrapolation from laboratory to the field could be even more difficult than the extrapolation from animal data to humans (Koeman, 1982). The extrapolation to natural systems is a very complex step, and *in situ* bioassays seem to be an important aid.

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REFERENCES

APHA (American Public Health Association) (1989). *Standard Methods for the Examination of Water and Wastewater*, 17th ed. APHA, Washington, DC.

Baird, D. J., Soares, A. M. V. M., Giriling, A., Barber, M. C., and Calow, P. (1989). The long term maintenance of *Daphnia magna* Straus for use in ecotoxicity tests: Problems and prospects. In *Proceedings of 1st European Conference on Ecotoxicology* (H. Lokke, H. Tyle, and F. Bro-Rasmussen, Eds.), pp. 144–148. Lyngby, Denmark.

Baughman, D. S., Moore, D. W., and Scott, G. I. (1989). A comparison and evaluation of field and laboratory toxicity tests with fenvalerate on an estuarine crustacean. *Environ. Toxicol. Chem.* **8,** 417–429.

Burton, G. A., Jr. (1991). Assessing the toxicity of freshwater sediments. *Environ. Toxicol. Chem.* **10,** 1585–1627.

Cairns, J., Jr. (1984). Factors moderating toxicity in surface waters. In *The Fate of Toxic in Surface and Ground Waters*, Proc. 2nd Nat. Water Conference (J. Wilson, Ed.), pp. 49–64. Acad. Nac. Sci. Philadelphia.

Chappie, D. J., and Burton, G. A., Jr. (1997). Optimization of *in situ* bioassays with *Hyalella azteca* and *Chironomus tentans*. *Environ. Toxicol. Chem.* **16,** 559–564.

Clark, J. R. (1989). Field studies in estuarine ecosystems: A review of approaches for assessing contaminant effects. In *Aquatic Toxicology and Hazard Assessment* (U. M. Cowgill and L. R. Williams, Eds.), Vol. 12, ASTM STP 1027, pp. 120–133. Am. Soc. for Testing and Materials, Philadelphia.

Clark, J. R., Borthwick, P. W., Goodman, L. R., Patrick, J. M., Jr., Lores, E. M., and Moore, J. C. (1987). Comparison of laboratory toxicity test results with responses of estuarine animals exposed to fenthion in the field. *Environ. Toxicol. Chem.* **6,** 151–160.

Cooney, J. D., DeGreave, G. M., Moore, E. L., Lenoble, B. J., Pollock, T. L., and Smith, G. J. (1992a). Effects of environmental and experimental design factors on culturing and toxicity testing of *Ceriodaphnia dubia*. *Environ. Toxicol. Chem.* **11,** 839–850.

Cooney, J. D., DeGreave, G. M., Moore, E. L., Palmerv, W. D., and Pollock, T. L. (1992b). Effects of food and water quality on culturing of *Ceriodaphnia dubia*. *Environ. Toxicol. Chem.* **11,** 823–837.

Crane, M., Delaney, P., Watson, S., Parker, P., and Walker, C. (1995). The effect of Malathion 60 on *Gammarus pulex* (L.) below watercress beds. *Environ. Toxicol. Chem.* **14,** 1181.

Cummins, C. P. (1994). Acid solutions. In *Handbook of Ecotoxicology*. (P. Calow, Ed.), Vol. 2, pp. 21–44. Blackwell Scientific, Oxford.

Custódio, J. (1996). Sistemas de lavra na Mina de S. Domingos (1854–1966). In *Mineração do Baixo Alentejo* (Câmara Municipal de Castro Verde, Ed.), pp. 174–185. Gráfica Maiadouro, Portugal.

Depledge, M. H., Weeks, J. M., and Bjerregaard, P. (1994). Heavy metals. In *Handbook of Ecotoxicology* (P. Calow, Ed.), Vol. 2, pp. 79–105. Blackwell Scientific, Oxford.

Di Toro, D. M., Mahony, J. H., Hansen, D. J., Scott, K. J., Hicks, M. B., Mayr, S. M., and Redmond, M. (1990). Toxicity of cadmium in sediments: The role of acid volatile sulfide. *Environ. Toxicol. Chem.* **9,** 1487–1502.

Gersich, F. M., and Milazzo, D. P. (1990). Evaluation of a 14-day static renewal toxicity test with *Daphnia magna* Straus. *Arch. Environ. Contam. Toxicol.* **19,** 72–76.

Goss, L. B., and Bunting, D. L. (1983). *Daphnia* development and reproduction: Responses to temperature. *J. Therm. Biol.* **8,** 375–380.

Groeger, A. W., Schram, M. D., and Marzolf, G. R. (1991). Influence of food quality on growth and reproduction in *Daphnia*. *Freshwater Biol.* **26,** 11–19.

Hach (1993). *Dr/2000 Spectrophotometer Handbook*. Hach Co., Loveland, CO.

Hare, L., Carignan, R., and Huerta-Diaz, M. A. (1994). A field study of metal toxicity and accumulation by benthic invertebrates: Implications for the acid-volatile sulfide (AVS) model. *Limnol. Oceanogr.* **39,** 1653–1668.

Hoke, R. A., Giesy, J. P., Zabik, M., and Ungers, M. (1993). Toxicity of sediments and sediment pore waters from the Grand Calumet River — Indiana Harbour, Indiana area of concern. *Ecotoxicol. Environ. Saf.* **26,** 86–112.

Holdway, D. A. (1992). Uranium mining in relation to toxicological impacts on inland waters. *Ecotoxicology* **1,** 75–88.

Ireland, D. S., Burton, G. A., Jr., and Hess, G. G. (1996). *In situ* toxicity evaluations of turbidity and photoinduction of polycyclic aromatic hydrocarbons. *Environ. Toxicol. Chem.* **15,** 574–581.

Jak, R. G., Mass, J. L., and Scholten, M. C. Th. (1996). Evaluation of laboratory derived toxic effect concentration of a mixture of metals by testing fresh water plankton communities in enclosures. *Water Res.* **30,** 1215–1227.

Kelly, M. (1991). *Mining and the Freshwater Environment*. Elsevier Science, Essex.

Kemble, N. E., Brumbaugh, W. G., Brunson, E. L., Dwyer, F. J., Ingersoll, C. G., Monda, D. P., and Woodwards, D. F. (1994). Toxicity of metal-contaminated sediments from the upper Clark Fork River, Montana, to aquatic invertebrates and fish in laboratory exposures. *Environ. Toxicol. Chem.* **13,** 1985–1997.

Knight, J. T., and Waller, W. T. (1992). Influence of the addition of Cerophyl® on the *Selenastrum capricornutum* diet of the cladoceran *Ceriodaphnia dubia*. *Environ. Toxicol. Chem.* **11,** 521–534.

Koeman, J. H. (1982). Ecotoxicological evaluation: The eco-side of the problem. *Ecotoxicol. Environ. Saf.* **6,** 358.

Kooijman, S. A. L. M., and Bedaux, J. J. M. (1996). Analysis of toxicity tests on *Daphnia* survival and reproduction. *Water Res.* **30,** 1711–1723.

Korpelain, H. (1986). The effects of temperature and photoperiod on life history parameters of *Daphnia magna* (Crustacea: Cladocera). *Freshwater Biol.* **16,** 615–620.

LaRocca, C., Francisco, D. E., and DiGiano, F. A. (1994). Effects of diet on survival, reproduction and sensitivity of *Ceriodaphnia dubia*. *Water Environ. Res.* **66,** 905–911.

Laurén-Määttä, C., Kleiven, O., and Kiviranta, J. (1997). Horizontal distribution of *Daphnia pulex* in response to toxic and non-toxic algal extracts. *J. Plankton. Res.* **19,** 141–148.

Lewis, M. A., Pittinger, C. A., Davidson, D. H., and Ritchie, C. J. (1993). *In situ* response of natural periphyton to an anionic surfactant and an environmental risk assessment for phytoplankton effects. *Environ. Toxicol. Chem.* **12,** 1803–1812.

Lopes, I., Ribeiro, R., Gonçalves, F., and Soares, A. M. V. M. (in press). Field validation of specific tools for aquatic systems impacted with acid mina drainage. *Int. J. Environ. Stud.*

Maltby, L., Calow, P., Cosgrove, M., and Pindar, L. (1987). Adaptation to acidification in aquatic invertebrates: Speculation and preliminary observations. *Ann. Soc. R. Zool. Belg.* **117,** 105–114.

Mance, G. (1987). *Pollution Threat of Heavy Metals in Aquatic Environments.* Elsevier Science, Essex.

Martínez-Jerónimo, F., Villaseñor, R., Rios, G., and Espinosa, F. (1994). Effect of food type and concentration on the survival, longevity, and reproduction of *Daphnia magna. Hydrobiologia* **287,** 207–214.

Monson, P. D., Ankley, G. T., and Kosian, P. A. (1995). Phototoxic response of *Lumbricus variegatus* to sediments contaminated by polycyclic aromatic hydrocarbons. *Environ. Toxicol. Chem.* **14,** 891–894.

Munawar, M., and Munawar, I. F. (1987). Phytoplankton bioassays for evaluating toxicity of *in situ* sediment contaminants. *Hydrobiologia* **149,** 87–105.

Ojala, A., Kankaala, P., Kairesalo, T., and Salonen, K. (1995). Growth of *Daphnia longispina* L. in a polyhumic lake under various availabilities of algal, bacterial and detrital food. *Hydrobiologia* **315,** 119–134.

Penttinen, S, Kukkonen, J., and Oikari, A. (1995). The kinetics of cadmium in *Daphnia magna* as affected by humic substances and water hardness. *Ecotoxicol. Environ. Saf.* **30,** 72–76.

Pereira, E. G., Moura, I., Costa, J. R., Mahony, J. D., and Thomann, R. V. (1995). The S. Domingos Mine: A study of heavy metal contamination in the water column and sediments of the Chança River basin by discharge from an ancient cupriferous pyrite mine (Portugal). *Mar. Freshwater Res.* **46,** 145–151.

Rand, G. M., and Petrocelli, S. R. (1985). *Fundamentals of Aquatic Toxicology*. Hemisphere, Washington, DC.

Reynoldson, T. B., Day, K. E., Clarke, C., and Milani, D. (1994). Effect of indigenous animals on chronic end points in freshwater sediment toxicity tests. *Environ. Toxicol. Chem.* **13,** 973–977.

Rieuwerts, J. S., and Farago, M. E. (1995). Lead contamination in smelting and mining environments and variations in chemical forms and bioavailability. *Chem. Speciation Bioavail.* **7,** 113–123.

Rodrigues, R., and Cunha, A. (1992). Vinte e cinco anos de solidão. *Grande Reportagem.* **13,** 60–71.

Salazar, M. H., Duncan, P. B., Salazar, S. M., and Rose, K. A. (1995). *In situ* bioassays using transplanted mussels: II. Assessing contaminated sediment at a Superfund site in Puget Sound. In *Environmental toxicology and risk assessment — Third Symposium* (J. S. Hughes, G. R. Biddinger, and E. Mones, Eds.), ASTM STP 1218, pp. 242–263. Am. Soc. for Testing and Materials, Philadelphia.

Sasson-Brickson, G., and Burton, G. A., Jr. (1991). *In situ* and laboratory sediment toxicity testing with *Ceriodaphnia dubia. Environ. Toxicol. Chem.* **10,** 201–207.

Snyder-Conn, E. (1993). *In Situ Toxicity Testing with Locally Collected Daphnia*, Biol. Rep. 15. U.S. Department of the Interior, Fish and Wildlife Service, Washington, DC.

Stemmer, B. L., Burton, G. A., Jr., and Leibfritz-Frederick, S. (1990a). Effect of sediment test variables on selenium toxicity to *Daphnia magna. Environ. Toxicol. Chem.* **9,** 381–389.

Stemmer, B. L., Burton, G. A., Jr., and Sasson-Brickson, G. (1990b). Effect of sediment spatial variance and collection method on cladoceran toxicity and indigenous microbial activity determinations. *Environ. Toxicol. Chem.* **9,** 1035–1044.

Weber, C. I., Peltier, W. H., Norberg-King, T. J., Horning, W. B., Kessler, F. A., Menkedick, J. R., Neiheisell, T. W., Lewis, P. A., Klemm, D. J., Pickring, Q. H., Robinson, E. L., Lazorchak, J. M., Wymer, L. J., and Freyberg, R. W. (Eds.) (1989). *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms*, EPA/600/4-89/001. U.S. EPA, Office of Research and Development, Cincinnati, OH.

Weber, C. I. (Ed.) (1991). *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*, 4th ed., EPA/600/4-90/027. U.S. EPA, Office of Research and Development, Cincinnati, OH.

Warren, L. W., Klaine, S. J., and Finley, M. T. (1995). Development of a field bioassay with juvenile mussels. *J. North Am. Benthol. Soc.* **14,** 341–346.

Zar, J. H. (1984). *Biostatistical Analysis*, 2nd ed. Prentice–Hall, Englewood Cliffs, NJ.