Cadmium toxicity and bioaccumulation in freshwater biofilms

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Abstract	A microcosm study was undertaken to examine the effects of dissolved cadmium at various concentrations (0, 10, and 100 $\mu g \cdot L^{-1}$) on biofilm accumulation and diatom assemblages. A natural biofilm sampled from the Riou-Mort River (Southwest France) was inoculated into three experimental systems, where biofilm settled on glass slides. Samples collected after 1, 2, 4, and 6 weeks of colonization were analyzed for metal accumulation (total metal content and intracellular metal content in the biofilm), biomass (as measured through dry weight and ash-free dry matter), and quantitative as well as qualitative analysis of diatom assemblages. There was a positive correlation between cadmium accumulation and dissolved cadmium concentrations and duration of exposure: a linear relationship was found between concentration factors (CFs) of growing biofilms and time (CFs/day = 0.25 and 0.38 under contaminations of 10 and 100 $\mu gCd \cdot L^{-1}$, respectively). Biofilm settlement, more than photosynthetic activity, was affected by high cadmium concentrations: we observed for all stages of settlement a drastic and significant ($p < 0.05$) reduction in biofilm biomass and in diatom densities in the highest cadmium contamination, compared to control and low cadmium concentration units.

Keywords (separated by '-')	Cadmium toxicity - Bioaccumulation - Biofilms - Diatom densities
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Cadmium Toxicity and Bioaccumulation in Freshwater Biofilms

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Abstract A microcosm study was undertaken to examine the effects of dissolved cadmium at various concentrations (0, 10, and 100 $\mu g \cdot L^{-1}$) on biofilm accumulation and diatom assemblages. A natural biofilm sampled from the Riou-Mort River (Southwest France) was inoculated into three experimental systems, where biofilm settled on glass slides. Samples collected after 1, 2, 4, and 6 weeks of colonization were analyzed for metal accumulation (total metal content and intracellular metal content in the biofilm), biomass (as measured through dry weight and ash-free dry matter), and quantitative as well as qualitative analysis of diatom assemblages. There was a positive correlation between cadmium accumulation and dissolved cadmium concentrations and duration of exposure: a linear relationship was found between concentration factors (CFs) of growing biofilms and time (CFs/day = 0.25 and 0.38 under contaminations of 10 and 100 $\mu gCd \cdot L^{-1}$, respectively). Biofilm settlement, more than photosynthetic activity, was affected by high cadmium concentrations: we observed for all stages of settlement a drastic and significant ($p < 0.05$) reduction in biofilm biomass and in diatom densities in the highest cadmium contamination, compared to control and low cadmium concentration units.

Keywords Cadmium toxicity · Bioaccumulation · Biofilms · Diatom densities

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Introduction

Because of their specific ecological preferences (Lange-Bertalot 1979; Steinberg and Schiefele 1988; van Dam et al. 1994), benthic diatoms are commonly used to assess water quality and a great number of methods based on the use of diatoms have been proposed and are applied for the evaluation of eutrophication and organic pollution in rivers (a review of the major indices used in Europe is given in Prygiel et al. 1999). These methods are determined with consideration to nutrients and eutrophic conditions, however, interactions between nutrients and toxicants often occur and are not evidenced through current indices. With the implementation of the European Water Framework Directive (2000/60/EC), there is a need to take into account priority substances such as heavy metals; studies are necessary for the improvement of diatom monitoring of these pollutions.

Aquatic primary producers from polluted sites are generally considered to be passive absorbers of the toxicants present in waters, in which all their vital functions happen: nutrition, respiration, reproduction, excretion, etc. Although a number of studies have assessed the accumulation of cadmium and the biological effects in higher organisms in rivers of Southwest France (Andres et al. 2000; Baudrimont et al. 2003, 2005), cadmium toxicity is often observable from the beginning of the food chain. Previous in situ (Ivorra et al. 1999, 2000; Gomez and Licursi 2003; Morin et al. 2007) and experimental studies (Interlandi 2002; Ivorra et al. 2002; Gold et al. 2003a, b) have underlined the impact of combined nutrients and metals on diatom community structures but interactions between contaminants are likely to bias the sensitivity of species to single factors (Lozano and Pratt 1994; Guasch et al. 1998). To avoid this bias, we propose to set up an

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in vitro experiment to describe biofilm development under metal contamination. Microcosm studies stray from field conditions but allow the control of physicochemical parameters; in order to limit the differences with environmental conditions we worked on natural assemblages of diatom species. In this study, biofilm growth was quantified through dry weight, ash-free dry mass and chlorophyll *a* measurements in order to point out the effects of cadmium on structural as well as functional characteristics of the periphyton.

Therefore, these studies linked shifts in diatom consortia to water metal concentrations and did not reflect the real exposure in the organic matrix. Indeed, periphytic biofilms accumulate metals following three main mechanisms (Holding et al. 2003): (i) absorption in extracellular polymeric substances (EPS), (ii) cell surface adsorption, and (iii) intracellular uptake. Here we propose to discriminate between metal adsorbed to abiotic or biotic materials and intracellular (nonexchangeable) metal by assaying the metal concentrations in the periphyton after no treatment or EDTA washing of periphyton samples (Behra et al. 2002). Moreover, many authors have reported metal-induced deformities of the frustule in polluted streams (McFarland et al. 1997; Shehata et al. 1999; Gomez and Licursi 2003; Nunes et al. 2003), yet correlations between exposure and occurrence of abnormal valves still lack experimental validation. The use of the frequency of abnormal frustules, if pointing to a metal stress, would be worthy for routine biomonitoring of metal contaminations.

The present microcosm study is aimed at characterizing biofilm growth (using measurements of dry weight, ash-free dry mass, and diatom density) under various metal levels and determining the accumulation kinetics of dissolved cadmium in a natural freshwater biofilm, as well as metal toxicity to diatom assemblages and individuals.

Materials and Methods

Field Sampling

The Riou-Mort stream, a small tributary of the river Lot located in the industrial basin of Decazeville (Southwest France; 44°N / 2°E), exhibits polymetallic pollution from its confluence with the Riou-Viou, a stream carrying seepage from a former zinc factory, presenting high levels of dissolved cadmium (Say 1978). In the year 2000, Audry et al. (2004) measured concentrations of 16 $mg \cdot L^{-1}$ dissolved cadmium (average value).

Diatom communities were sampled from the field, at a site on the Riou-Mort located upstream of the contamination source, in March 2006. Microbenthic biofilms were grown on 20 glass slides (300 cm^2 per slide) immerged in the stream in plastic racks for 5 weeks before collection, scraping, suspension in stream water, and transport to the laboratory (Morin et al. 2007).

Experimental Design

In the laboratory, biofilm suspensions were separated into four aliquots; each aliquot was inoculated in the water column of three independent experimental units (EUs). Each system runs in a closed circuit and is composed of replicate artificial streams (60 cm long, 6 cm radius) equipped with six glass substrates, connected in parallel to a 40-L tank (Fig. 1). Using an external pump, water was circulated from the tank to a header tank providing a steady supply of water to the streams.

EUs were placed in an air-conditioned room, at a light intensity of approximately 70 $\mu mol \cdot s^{-1} \cdot m^{-2}$ (10:14 light:dark regime) and under continuous water movement at a velocity of approximately 0.4 $cm \cdot s^{-1}$. The reservoirs were filled with 40 L of modified Woods Hole culture medium (without EDTA and supplemented with silica) diluted fourfold (Gold et al. 2003a). The levels of nitrates and orthophosphates in the systems were typical nutrient concentrations found in the Riou-Mort river in 2004 and 2005. Before the start of the experiment, the microcosms were equilibrated overnight with the culture media, to which the experimental concentrations of cadmium had been added.

During the course of the experiment (6 weeks), physical and chemical variables of the water (temperature, pH, electric conductivity, dissolved oxygen concentration and saturation) (WTW, Weilheim, Germany) were determined daily at the end of the light cycle. Nutrient (orthophosphate, nitrate) concentrations were analyzed weekly at the

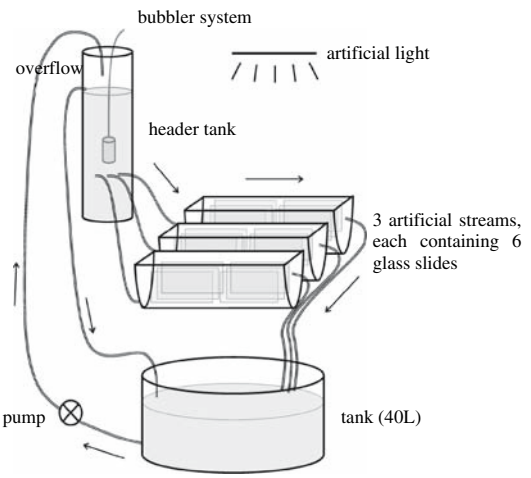

Fig. 1 Schematic representation of one experimental unit. Arrows indicate direction of flow

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laboratory from 1L water samples, according to French and international standards (NF T90-023 and NF EN ISO 13395, respectively). Depending on the results of the analyses, culture medium was added as required to compensate for the decrease of nutrient concentrations due to algal uptake.

Water Column Contamination Protocol

Each individual experimental unit represented a different treatment: control (EU1), low contamination (EU2; 10 μCd·L^{-1}, in accordance with concentrations found in the polluted river Riou-Mort), high contamination (EU3; 100 µgCd·L^{-1}), which approximately corresponds to extreme values recorded in a tributary of the river Riou-Mort, the Riou-Viou, by the GEMA team, University Bordeaux 1 (e.g., more than 60 µgCd·L^{-1} in October 2004).

The EUs were contaminated with a cadmium chloride solution ($CdCl_2$; Merck, Darmstadt, Germany) to reach nominal test concentrations of 10 and 100 µg·L^{-1}. Cadmium concentrations were measured (and, when necessary, corrected) daily during the first week and twice per week during the rest of the experiment. Water samples were filtered and acidified with HNO_3, to determine cadmium concentrations by atomic absorption spectrophotometry (Varian AA400—Zeeman correction, Victoria, Australia). The detection limit was 0.1 µgCd·L^{-1}. The accuracy of the analytical methods was checked periodically using two certified biological reference materials (Tort-2, lobster hepatopancreas; and Dolt-2, dogfish liver; NRCC-CNRC, Ottawa, Canada). Values were consistently within the certified ranges (data not shown).

Biofilm Sampling and Analyses

After a 1- and 2-week colonization, two glass slides were removed at random from each artificial stream; one slide was sufficient for biofilm collection at weeks 4 and 6. The streams were then reset with new glass slides, to preserve identical flow conditions.

Both faces of the glass substrates were carefully scraped with a cutter blade and the biofilm was suspended in a standard volume of 100 mL to form a single sample per stream (i.e., three replicate periphyton samples per experimental unit) per sampling date and per treatment.

Each suspended biofilm sample was separated into aliquots assigned to various analyses. Twenty milliliters was used for particulate matter analysis: biofilm dry weights (DW) were determined following the European standard NF EN 872; after drying and weighing, samples were ashed at 500°C for 1 h in a muffle furnace (Solax Technology Ltd., China) and the results are reported as ash-free dry mass (AFDM). Growth rates inferred from AFDM measurement data were calculated for the exponential phase (Biggs 1990) and were expressed as micrograms of AFDM per unit area of glass substrate per day.

Forty milliliters was put into Teflon jars to assay metal concentrations, following two protocols (Behra et al. 2002): (i) 20 mL was used for determination of total metal in the biofilm, and (ii) 20 mL was washed for 10 min with 4 nM EDTA at pH 8 to determine the intracellular metal content of the periphyton. After filtration and mineralization of each sample (after washing with EDTA or not), cadmium concentrations were measured by atomic absorption spectrophotometry (Varian AA400-Zeeman correction, Victoria, Australia) and by autosampler, with a 0.1 µg·L^{-1} detection limit. Finally, 5 mL was preserved in a formaldehyde solution for countings and diatom identifications to the species level (see Quantitative and Qualitative Analysis of Diatom Assemblages, below).

Quantitative and Qualitative Analysis of Diatom Assemblages

Enumeration was done in each formalin-preserved sample (100 µL) using a Nageotte counting chamber: the total number of cells counted in 10 fields (1.25 µL each, 0.5 mm deep) using light microscopy at 400× magnification (photomicroscope Leica DMRB, Wetzlar, Germany) was then recorded as cells per unit area of sampled substrate (number of diatom cells·cm^{-2}).

The sample fractions devoted to taxonomic analysis of diatom assemblages were prepared according to ANSP protocols (Charles et al. 2002), i.e., digestion in boiling hydrogen peroxide (30% H_2O_2) and hydrochloric acid (35%) followed by three cycles of centrifugation of the sample and pellet rinsing with distilled water. After the last treatment, the pellet was once again resuspended in distilled water and pipetted onto coverslips, which were mounted onto slides after air-drying, using the high refractive index medium Naphrax (Brunel Microscopes Ltd., UK; RI=1.74). Diatom identifications were conducted at a magnification of ×1000; individual microscope fields were scanned until a minimum of 400 valves had been identified using specialized literature (Krammer and Lange-Bertalot 1986–1991). Individual deformities (twisted cells and/or diatoms with deformed valve wall ornamentation) were observed and their frequency determined. Note that the valves of initial cells, which typically have a morphology different from that of

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Table 1 Physical and chemical parameters and nutrient concentrations measured during the 6-week experimental period in the water column of the three experimental systems

		EU1	EU2	EU3
pH	Week 0	7.1 ± 0.0	7.0 ± 0.0	7.0 ± 0.0
	Week 1	7.4 ± 0.1	7.5 ± 0.1	7.4 ± 0.1
	Week 2	7.3 ± 0.0	7.5 ± 0.0	7.2 ± 0.0
	Week 4	7.7 ± 0.0	8 ± 0.0	7.8 ± 0.0
	Week 6	7.9 ± 0.0	7.9 ± 0.0	7.7 ± 0.0
Temperature (°C)	Week 0	18.0 ± 0.2	18.3 ± 0.2	17.8 ± 0.2
	Week 1	18.8 ± 0.1	17.6 ± 0.1	18.0 ± 0.1
	Week 2	18.9 ± 0.1	17.9 ± 0.2	18.2 ± 0.1
	Week 4	18.8 ± 0.1	16.5 ± 0.2	17.4 ± 0.2
	Week 6	18.7 ± 0.1	17.6 ± 0.2	18.1 ± 0.1
Dissolved O_2 ($mg{\cdot}L^{-1}$)	Week 0	8.5 ± 0.2	8.9 ± 0.2	8.3 ± 0.2
	Week 1	8.7 ± 0.1	8.4 ± 0.1	8.3 ± 0.1
	Week 2	8.5 ± 0.1	8.2 ± 0.1	8.2 ± 0.0
	Week 4	9.2 ± 0.1	8.9 ± 0.1	9.2 ± 0.1
	Week 6	8.8 ± 0.0	8.3 ± 0.1	8.2 ± 0.1
Oxygen saturation (%)	Week 0	89.0 ± 1.1	89.1 ± 1.1	87.1 ± 1.1
	Week 1	93.5 ± 0.9	87.6 ± 0.9	86.5 ± 0.9
	Week 2	91.7 ± 0.5	87.9 ± 1.5	85.4 ± 0.9
	Week 4	97.3 ± 0.5	86.9 ± 0.9	97.5 ± 0.7
	Week 6	94.2 ± 0.6	86.3 ± 1.0	84.9 ± 1.4
Conductivity ($\mu S{\cdot}cm^{-1}$)	Week 0	143 ± 6.2	152 ± 6.2	135 ± 6.2
	Week 1	153 ± 1.2	180 ± 2.5	139 ± 1.0
	Week 2	163 ± 1.5	201 ± 3.5	155 ± 2.5
	Week 4	183 ± 1.1	254 ± 2.5	181 ± 2.1
	Week 6	202 ± 2.1	320 ± 5.6	220 ± 3.5
Cadmium ($\mu g{\cdot}L^{-1}$)	Week 0	<d.l.	5.34 ± 0.22	70.38 ± 3.74
	Week 1	<d.l.	8.26 ± 0.59	106.59 ± 4.46
	Week 2	<d.l.	7.87 ± 0.41	92.52 ± 2.48
	Week 4	<d.l.	12.90 ± 1.19	101.65 ± 4.03
	Week 6	<d.l.	9.72 ± 0.14	110.50 ± 2.84
Nitrates ($mg{\cdot}L^{-1}$)	Week 0	33.5	33.3	32.6
	Week 1	27.3	24.4	27.6
	Week 2	25.7	22.5	28.0
	Week 4	22.2	23.6	31.6
	Week 6	18.9	29.6	37.2
Orthophosphates ($mg{\cdot}L^{-1}$)	Week 0	2.9	2.9	2.9
	Week 1	2.5	2.1	2.4
	Week 2	2.3	1.9	2.2
	Week 4	1.7	1.9	1.9
	Week 6	0.9	1.9	2.2

Note. Data are means ± standard deviation. d.l.: detection limit, 0.1 µg $Cd{\cdot}L^{-1}$. The water was changed at the end of weeks 3 and 5

vegetative valves (larger and usually presenting distorted costae) (Round et al. 1990), were not taken into account in diatom counts.

Data Analyses

Data were evaluated statistically in two ways. First, variations in DW, AFDM, density, and abnormal form frequencies with treatments and duration of exposure were examined by means of a linear mixed-effects (LME) model for repeated measurements, with the treatments and dates as fixed effects and the subsamples taken from each artificial channel as random effects. In this case, averaging is only an approximate analysis, and a specific statistical approach adapted to the subsampling design used is needed to avoid inflated test statistics, deflated *p*-values, or incorrect rejection of the null hypothesis. These statistical analyses were computed with the statistical modeling environment R (Ihaka and Gentleman 1996). Second, PC-ORD software (McCune and Mefford 1999) was used for conducting Hierarchical Cluster Analysis. A dendrogram based on relative abundances of the species occurring most (the 80 species that had the highest cumulative relative abundances) was drawn using Ward's method.

Values are mean ± standard deviation.

Results

Physical and Chemical Characteristics of Microcosm Water (See Table 1)

The pH of the water did not differ significantly ($p = 0.77$) among experimental units. A slight increase was observed, ranging from 7 at the beginning of the experiment to 8 on the last dates. Temperature and oxygenation were relatively constant throughout the experimental period and similar between treatments. Temperatures averaged 17.9°C (±0.8°C). Dissolved oxygen concentrations were stable, with a mean value of 8.7 $mg{\cdot}L^{-1}$ (±0.8 $mg{\cdot}L^{-1}$), and oxygen saturation was quite high, at 92% (±4%). The mean conductivity increased regularly in all streams, from 140 $\mu S{\cdot}cm^{-1}$ to reach 200 $\mu S{\cdot}cm^{-1}$ in EU1, 220 $\mu S{\cdot}cm^{-1}$ in EU3, and 320 $\mu S{\cdot}cm^{-1}$ in EU2.

Nitrate and phosphate concentrations were around 33 $mg{-}NO_3{\cdot}L^{-1}$ and 2.9 $mg{-}PO_4{\cdot}L^{-1}$ in all systems at the beginning of the experiment (week 0). Concentrations decreased gradually in all the EUs, until additions of culture medium at the end of weeks 3 and 5 restored nutrient to comparable levels in all systems, followed by stabilization. In EU1, however, a continuous decrease in nitrate and phosphate concentrations occurred.

Cadmium concentrations in the EUs matched the nominal concentrations well. Actual concentrations were below

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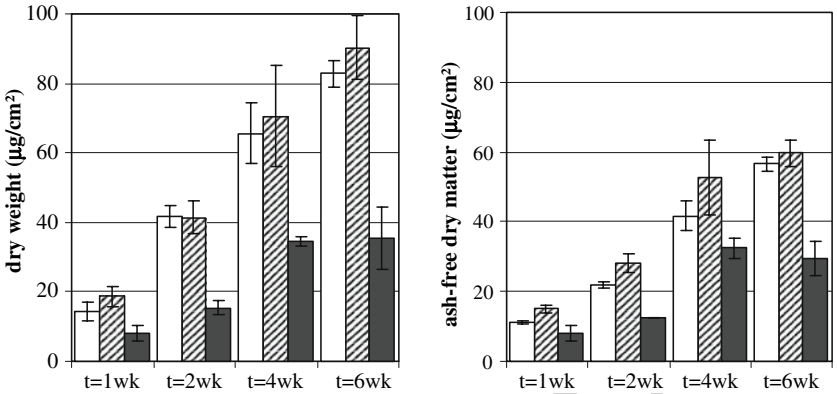

Fig. 2 Composition of the periphytic layer in the experimental units: white bars—EU1, 0 µg $Cd{\cdot}L^{-1}$; hatched bars—EU2, 10 µg $Cd{\cdot}L^{-1}$; gray bars—EU3, 100 µg $Cd{\cdot}L^{-1}$. Error bars: standard deviation of three replicates

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0.1 µg·L^{-1} in EU1, 9.07 ± 2.55 µg·L^{-1} in EU2, and 95.93 ± 14.57 µg·L^{-1} in EU3 during the experiment.

Biofilm Biomass and Metal Content

The mean DW and AFDM of the biofilms increased throughout the 6-week exposure (Fig. 2). In EU3, however, DW and AFDM measurements tended to stabilize between week 4 and week 6. The LME model performed on the dataset confirmed this date effect (mean DW and AFDM ranged from 13.6 and 11.4 µg·cm^{-2}, respectively, at week 1 to 69.5 and 48.5 µg·cm^{-2} at week 6; $p < 0.05$ in both cases) and also underlined a treatment effect. EU1 and EU2 exhibited similar DW values during the whole course of the experiment, whereas EU3 values were half those of EU1 and EU2 for almost all dates. Indeed, the tests did not discriminate EU1 and EU2 but expressed strong differences from EU3 (with $p < 0.01$ for the treatment × date effect at weeks 2 and 6). A slight but statistically significant increase in AFDM ($p = 0.08$ at week 4) was observed between EU1 (mean AFDM = 32.8 µg·cm^{-2}) and EU2 (mean AFDM = 38.9 µg·cm^{-2}). AFDM in EU3 was significantly reduced for all stages of settlement ($p < 0.01$), with an average value of 20.6 µg·cm^{-2}. Growth rates in the exponential phase were comparable for EU1 (1.4 µg·cm^{-2}·day^{-1}; $R^2 = 0.99$) and EU2 (1.6 µg·cm^{-2}·day^{-1}; $R^2 = 0.87$). A cadmium overload of 100 µg·L^{-1} in EU3 induced a slight decrease in growth rate (0.9 µg·cm^{-2}·day^{-1}; $R^2 = 0.73$).

Metal contents in biofilms as measured with both protocols (without and after an EDTA wash) reflected their exposure history. Biofilms from the cadmium-enriched systems (EU2 and EU3) sequestered increasing concentrations per DW unit as the biofilm settled. We observed a logarithmic accumulation of nonexchangeable and total cadmium in periphyton (Figs. 3 and 4). Cadmium contents increased from week 1 to week 4, and then stabilized at around 3 and 5 µg·g^{-1} DW in EU1 for nonexchangeable and total cadmium, respectively, 115 and 330 µg·g^{-1} DW in EU2, and 1350 and 3700 µg·g^{-1} DW in EU3. Within any given EU, cadmium concentrations in periphyton without and after EDTA wash varied during the course of the experiment: the ratio nonexchangeable/total metal was about 0.4 for EU3 and increased in EU2 from 0.2 at week 1 to 0.4 at the end of the experiment. In addition, concentration factors (CFs) of the biofilm for cadmium were calculated according to Foster (1976) by dividing concentrations of cadmium in biofilms (total and nonexchangeable fractions) by those in water, reflecting an increasing accumulation ability of the biofilms with dissolved metal concentrations and duration of exposure (Fig. 4). Gross uptake capacity (i.e., the ratio of total cadmium accumulated to dissolved metal) increased regularly, from 21.5 ± 7.6 at

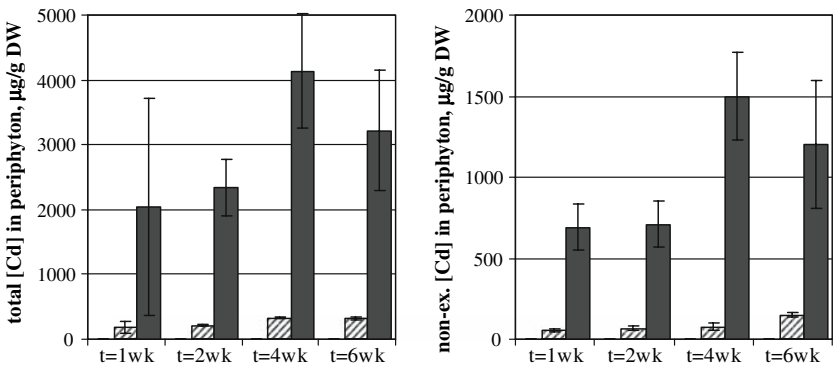

Fig. 3 Biofilm accumulation of total and nonexchangeable cadmium in the experimental units: white bars—EU1, 0 µg $Cd{\cdot}L^{-1}$; hatched bars—EU2, 10 µg $Cd{\cdot}L^{-1}$; gray bars—EU3, 100 µg $Cd{\cdot}L^{-1}$. Error bars: standard deviation of three replicates

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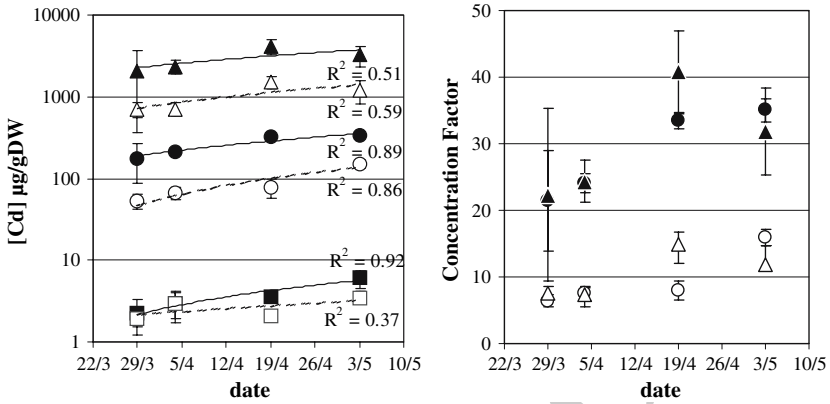

Fig. 4 Non-exchangeable (open symbols) and total cadmium (filled symbols) accumulation in biofilms and concentration factor [(µg·g^{-1}, DW)/(µg·L^{-1})] during the course of the experiment. (□) EU1, 0 µg Cd·L^{-1}; (○) EU2, 10 µg Cd·L^{-1}; (Δ) EU3, 100 µg Cd·L^{-1}. Error bars: standard deviation of three replicates. Note the logarithmic scale on the *Y* axis in the left-hand plot

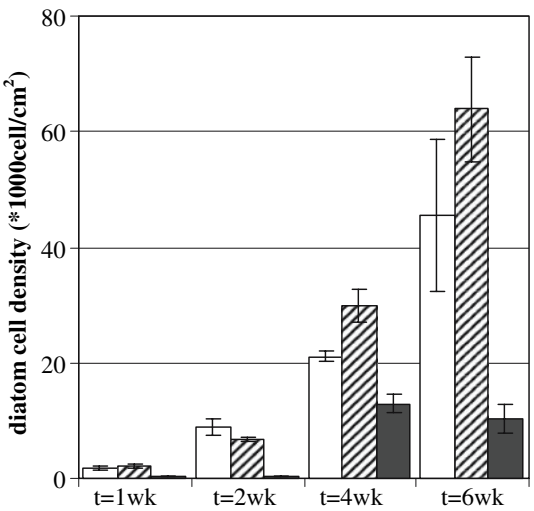

Fig. 5 Diatom cell densities enumerated in the glass substrates of each experimental unit: white bars—EU1, 0 µg Cd·L^{-1}; hatched bars—EU2, 10 µg Cd·L^{-1}; gray bars—EU3, 100 µg Cd·L^{-1}. Error bars: standard deviation of three replicates

week 1 to 35.1 ± 1.7 at week 6 in EU2 (with a regression coefficient of 0.41; R^2 = 0.93) and from 22.3 ± 13.0 at week 1 to 40.9 ± 6.1 at week 4 (regression coefficient, 0.93; R^2 = 0.97) before decreasing to 31.8 ± 6.5 in EU3. The calculations of CF after non-exchangeable cadmium values also expressed an increasing sorption activity of the cells grown under 10 and 100 µg Cd·L^{-1} concentrations: from 6.4 ± 0.9 and 7.6 ± 1.1 (week 1) to 15.9 ± 1.2 (week 6) and 14.8 ± 1.9 (week 4) in EU2 and EU3, respectively, with regression slopes of 0.25 (R^2 = 0.80) and 0.38 (R^2 = 0.91). In EU3, CF values tended to decrease slightly between week 4 and week 6 (CF = 11.9 ± 2.7).

Diatom Assemblages

EU1 and EU2 samples had initial diatom densities of 2000 ± 150 cells·cm^{-2} after 1 week of colonization (Fig. 5). These cell densities increased strongly during the experiment ($p < 0.01$), to 55,000 ± 2700 cells·cm^{-2} at week 6 (average EU1 and EU2 values). Statistically significant differences ($p = 0.07$) in diatom densities between EU1 and EU2 were observed only after a 6-week incubation, and not at the other sampling dates. EU3 displayed dramatically low cell densities for all stages of settlement ($p < 0.01$ at week 6), compared to control and low cadmium contamination units. Data ranged from 350 ± 40 cells·cm^{-2} at week 1 to 10,400 ± 2600 cells·cm^{-2} at week 6. Positive correlations were also observed between diatom densities and AFDM measurements from the three EUs (R^2 = 0.67; $p < 0.01$); analyses of each EU separately showed very similar results.

About 160 different taxa were identified. In Fig. 6, the dendrogram based on relative species abundances showed two major clusters, with one comprising diatom communities grown under 100 µg Cd·L^{-1} contamination after 2, 4, and 6 weeks' exposure, which were strongly dominated by *Nitzschia palea* (Kützing) W. Smith (from more than 50% relative abundances to about 80% at week 6, with a species richness of < 20 taxa). The other cluster was divided into two subclusters: one subcluster included communities from the inoculum (week 0) and week 1 for every EU, and the other subcluster was composed of assemblages from EU1 and EU2 developed after 2, 4, and 6 weeks. Communities from the inoculum (week 0) and from the first sampling date (week 1) were characterized by an association of *Navicula lanceolata* (Agardh) Ehrenberg, *Navicula gregaria* Donkin, *Surirella brebissonii* Krammer & Lange-Bertalot var. *brebissonii*, *Nitzschia dissipata* (Kützing) Grunow, and *Achnanthidium minutissimum* (Kützing) Czarnecki, while the other subcluster, corresponding to EU1 and EU2 communities developed after a 2-week period, contained *Nitzschia palea*, *Eolimna minima* (Grunow) Lange-Bertalot, *Planothidium frequentissimum* (Lange-Bertalot) Lange-Bertalot, *Navicula gregaria*, and *Nitzschia pusilla* (Kützing) Grunow. Naviculaceae presented 40% to 70% relative abundances in the control unit

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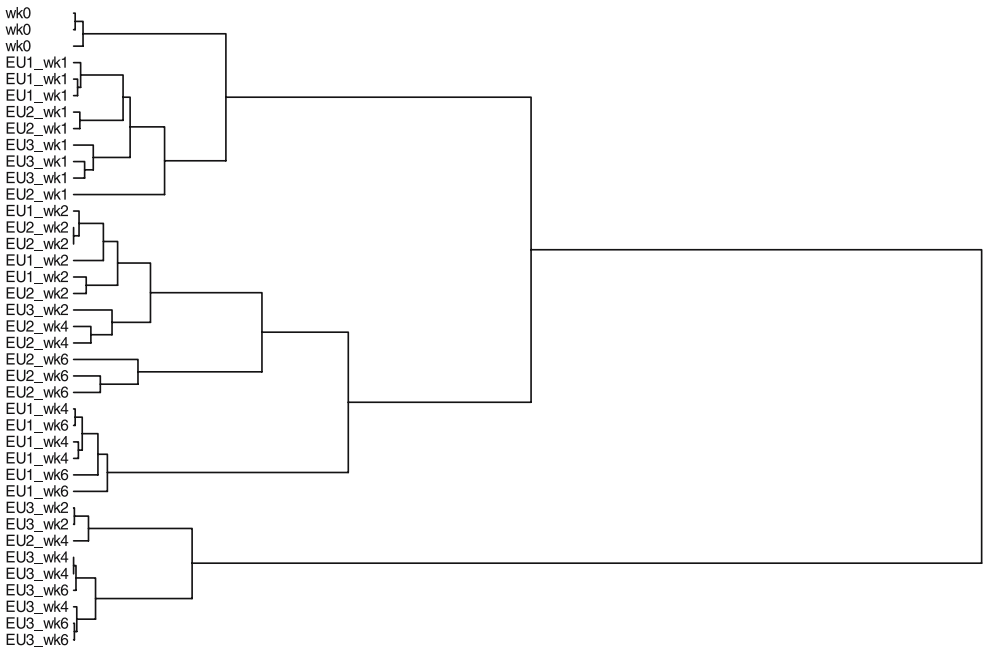
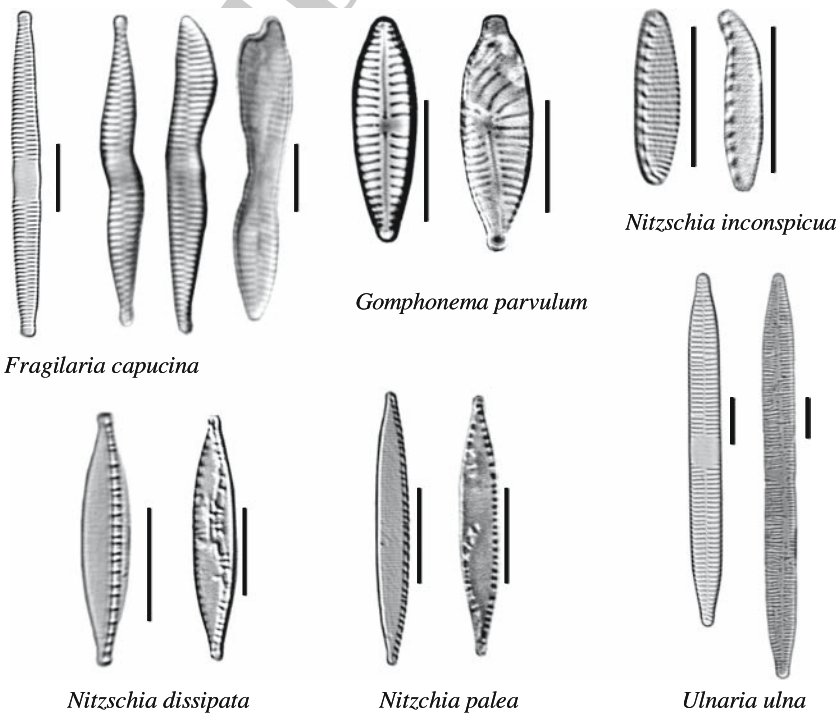


Fig. 6 Dendrogram of assemblages relationships among diatom communities developed under three cadmium concentrations and sampled on five sampling dates. EU, experimental unit; wk, week of sampling

Fig. 7 Normal and deformed diatoms. Scale bar = 10 μm



and tended to decrease under high cadmium exposure, unlike Nitzschiaceae, which tended to increase, representing more than 80% relative abundances at the end of the experiment.

A total of 21 diatom species representing 10 genera exhibited morphological deformities (Fig. 7) and were quantified. Teratological valves were abundant among Raphids (66% of the abnormalities enumerated) belonging

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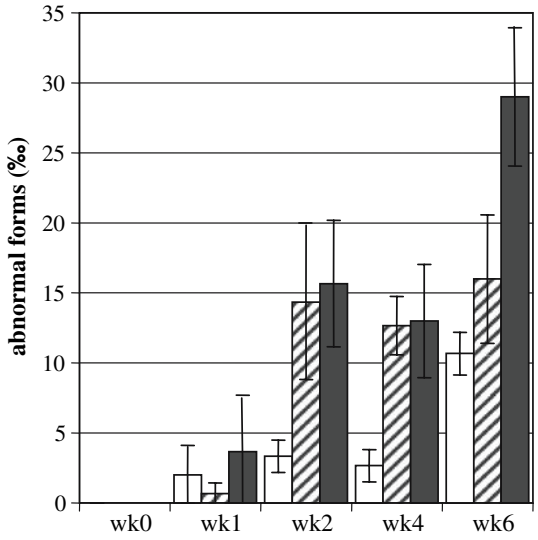

Fig. 8 Average relative abundances of deformed frustules in each experimental unit: white bars—EU1, 0 μg $Cd{\cdot}L^{-1}$; hatched bars—EU2, 10 μg $Cd{\cdot}L^{-1}$; gray bars—EU3, 100 μg $Cd{\cdot}L^{-1}$). Error bars: standard deviation of three replicates

to the genera *Nitzschia* (and, occasionally, to the genera *Caloneis*, *Eolimna*, *Gomphonema*, *Navicula*, *Sellaphora*, and *Surirella*) and Araphids (33%) such as *Fragilaria* sp. and a few occurrences of *Meridion*. Abnormalities were less abundant in Monoraphids from the genera *Planothidium*. In Fig. 8 we observed that no teratological forms were present in the inoculum; in the experimental units, however, some abnormalities tended to appear, with increasing abundances in EU2 and EU3, reaching 16 ± 4.5‰ in EU2 and 35 ± 5‰ in EU3 after a 6-week exposure. The frequency of valve abnormalities in the control units was quite stable between week 1 and week 4 (2.7 ± 0.7‰) and increased to more than 10‰ at week 6, which was, however, lower than the frequencies calculated in EU2 and EU3, in which combined effects between duration and level of exposure were highlighted ($p < 0.05$).

Discussion

Experimental Setup

In this study there were three different experimental units (EUs) that consisted of six different slides each. To ensure that the starting conditions (inoculum, physical and chemical conditions) were the same in the different EUs, water characteristics and diatom community composition were determined at week 0. Every EU received a different treatment (0, 10, 100 $\mu g{\cdot}L^{-1}$), but not all slides from each channel within an EU were completely independent of each other, and they were consequently considered as subsamples. On the other hand, the analyses performed would not have been possible in 3 × 3 systems, because of the time involved, especially in the determination of cadmium concentrations as well as the community composition of the biofilms. Because the treatments were not "truly" replicated, the experimental design required the use of mixed-effects modeling techniques, and the results need to be taken with caution.

It has been reported that cultivated phototrophic biofilms can develop as small independent ecosystems, showing poor reproducibility of community composition between microcosms exposed to comparable treatments (Roeselers et al. 2006). In the present study, some trends were observable in all the systems, e.g., nitrogen and phosphorus decreases or slight increases in pH, likely related to nutrient consumption and photosynthetic activity of the primary producers, respectively. However, conductivity in EU2 increased more than in EU1 and EU3, perhaps reflecting heterogeneity created by the intrinsic complex behavior of the microbial communities (Kangas and Adley 1996; Vandermer et al. 2002).

Cadmium concentrations used in this study were 0, 10, and 100 $\mu g{\cdot}L^{-1}$, which led to different dynamics depending on the nominal values imposed in the systems; however, the absence of experimental data between 10 and 100 μg $Cd{\cdot}L^{-1}$ did not allow any precise evaluation of the cadmium threshold for negative effects on biofilm biomass and diatom community structure.

Sorption Kinetics of Dissolved Cadmium in the Biofilms

Cadmium concentrations in the biofilms differed between EUs and temporally within EUs. Generally, an increase in metal concentrations in periphyton reflected the duration of exposure and the dissolved concentrations of this metal in the culture media of each experimental unit. Hence, the EU3 biofilms had much higher concentrations at any sampling time than those of EU2 and EU1 (Fig. 3). It has been demonstrated that biofilms have a good bioaccumulation capacity (Guanzon et al. 1995; Sunda and Huntsman 1998; Chang and Reinfelder 2000; Hill et al. 2000) and could be used to monitor river pollution equivalent to metal measurements in the sediment and suspended solids (Fuchs et al. 1996). Indeed, heavy metal ions can be entrapped in the organic matrix and occasionally biosorbed by live cells (bacteria, fungi, and algae). As observed in this experiment, large amounts of metals assayed in the biofilms were not actually taken up by the cells; 60% of the metal was rather absorbed on the cell surface (Torres et al. 1998) and hence eliminated by the EDTA wash.

Metal bioaccumulation reflects both passive and active modes (Campbell et al. 2002); most studies dealing with

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the subject describe an initial rapid phase of biosorption (Wang and Dei 2001; Hudson 2005), followed by slower active metal uptake (Perrein-Ettajani et al. 1999); however, most of the studies relative to biofilm bioaccumulation capacities describe short-term sorption kinetics. The logarithmic regressions (Fig. 4) expressed an increasing accumulation of cadmium per unit DW during the first 4 weeks of the experiment, then stabilization at the end of the experiment, indicating some kind of steady state, for total as well as non^exchangeable cadmium. Increasing cadmium sorption (until week 4) with increasing biofilm biomass implied a significant contribution of subsurface cells to metal sorption, these cells providing additional sorption sites (Hill et al. 2000). However, the effectiveness of additional biofilm biomass at removing metal was limited on the last dates of the experiment when a steady state was reached. This may be due to saturation of the metal binding sites in the cells (Di Toro et al. 2001; Rijstenbil and Gerringa 2002), limiting cell absorption capacity, according to the decrease in CF at the end of the experiment. It may also be explained by the three-dimensional architecture of the biofilm, strongly modified under metal exposure. Indeed, in copper-treated biofilms, Teitzel and Parsek (2003) observed an external layer of dead cells and an increased number of live cells toward the substratum. Presumably, most of the actively growing cells were in a "protected" region of the biofilm, where metal penetration was retarded. The protective effect of the organic matrix against pollution by toxins has been underlined by studies comparing the effects of heavy metals on thin vs. thick biofilms (Hill et al. 2000) or on biofilms exposed at various stages of development (Ivorra et al. 2000). This protective role of the matrix has been attributed to local pH and hypoxia conditions in the internal layers of the biofilm (Teissier and Torre 2002), which modify the redox conditions and, possibly, cell bioaccumulation potentials. Moreover, it is generally accepted that exopolysaccharides secreted by algal and bacterial communities (Pistocchi et al. 1997; Decho 2000; Muller et al. 2005) also play a role in binding metals, thus reducing their bioavailability and toxicity toward live cells.

Influence of Metal Contamination Level on Biofilms

In this study, biofilm accumulation was affected by high levels of dissolved cadmium: DW and AFDM were significantly lower in biofilms exposed to a 100 µg $Cd{\cdot}L^{-1}$ contamination (EU3) than those measured in biofilms grown in EU1 and EU2. Growth inhibition at high metal contamination levels, as shown by the decrease in growth rate in EU3, has been widely reported (Conway 1978; Conway and Williams 1979; Wong 1987; Guanzon et al. 1994; Payne and Price 1999; Gold et al. 2003a). Not only is global metabolism affected by metals (Husaini and Rai 1991), but so is cell ultrastructure (endoplasmic reticulum, mitochondria), which seems to be modified by elevated intracellular cadmium concentrations (Wong 1987). Moreover, Guanzon et al. (1994) described perturbations in phosphorus metabolism and cell division over critical concentrations of metal. Takamura et al. (1989) found growth inhibition of freshwater benthic algae at far lower cadmium concentrations than those applied in EU3, and our findings provide added support for the idea that DW and AFDM are better indicators of metal damage to biofilm communities than microbenthic algal photosynthesis (Lehmann et al. 1999; this study, data not shown), on which substances like cadmium and zinc have an unspecific mode of action (Lehmann et al. 1999).

Although dissolved cadmium in EU2 exceeded metal concentrations normally regarded as toxic for chemical waste, biomass as assessed by AFDM was comparable between EU1 (reference) and EU2 (10 µg $Cd{\cdot}L^{-1}$), and DW was even slightly higher in EU2 than in EU1. Nalewajko (1995) showed that moderate additions of cadmium (up to 5 µg $Cd{\cdot}L^{-1}$) may result in some growth stimulation. Nutrient bioavailability would also be likely to interact with the toxicant and slightly favor biofilm development (Lozano and Pratt 1994), nutrient concentrations being higher in EU2 at weeks 4 and 6 compared to the reference (see ^). Ivorra et al. (2002) noted that increased dissolved phosphorus concentrations might mitigate metal toxicity to biofilms or exert a protective role, by making coordination complexes of trace metals with phosphorus; moreover, Barranguet et al. (2002) described nutrient-stimulated biomass accumulation in photosynthetic biofilms affected by metals. This complicates the assessment of toxic effects on living organisms (and especially diatoms) embedded in the biofilm.

Influence of the Metal Contamination Level on Diatom Assemblages

It was expected that differences in cadmium concentrations between the systems would control the quantitative as well as the qualitative characteristics of the diatom assemblages.

Diatom growth played a role in the development of the biofilm by dynamically contributing to the pool of live organisms (export and import of cells) as well as producing polysaccharide exsudates, which are partly responsible for the coherence of the organic matrix. Whatever the stage of colonization, Stevenson and Peterson (1991) estimated substantial daily immigration and emigration rates for diatoms in slow-flowing streams. In the present study, specific growth rates were observed, diatom densities

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Table 2 Metal sensitivity and tolerance of diatom species identified in the experiment, as described in previous mesocosm (M) and field (F) studies

Species	Described as metal-sensitive in[a]	Described as metal-tolerant in[a]
Achnanthidium minutissimum (Kützing) Czarnecki	3 (M), 18 (F)	4 (F), 5 (F-M), 7(M), 8(M), 11 (M), 16 (F), 20 (F)
Amphora pediculus (Kützing) Grunow	18 (F)	20 (F)
Eolimna minima (Grunow) Lange-Bertalot		5 (F-M), 6 (F), 17 (M) , 20 (F)
Fragilaria capucina Desmazières	6 (F)	11 (M), 13 (F), 19 (M), 20 (F)
Gomphonema parvulum (Kützing) Kützing	11 (M)	5 (F-M), 6 (F), 10 (M), 18 (F)
Mayamaea atomus var. *permitis* (Hustedt) Lange-Bertalot		15 (F)
Navicula lanceolata (Agardh) Ehrenberg		20 (F)
Navicula(dicta) seminulum (Grunow) Lange Bertalot		9 (F)
Nitzschia dissipata (Kützing) Grunow	5 (M), 7 (M), 8 (M)	20 (F)
Nitzschia palea (Kützing) W.Smith		1 (M), 2 (M), 8 (M), 12 (F), 14 (F), 18 (F)
Planothidium lanceolatum (Brebisson ex Kützing) Lange-Bertalot		20 (F)
Surirella angusta Kützing		5 (F-M), 6 (F), 21 (M)

[a] Numbers refer to the following papers: 1, Admiraal et al. (1999a, b) ; 2, Admiraal et al. (1999a, b); 3, Blanck et al. (2003) ; 4, Cattaneo et al. (2004); 5, Feurtet-Mazel et al. (2003); 6, Gold et al. (2002); 7, Gold et al. (2003a); 8, Gold et al. (2003b); 9, Ivorra et al. (1999); 10, Ivorra et al. (2002a); 11, Ivorra et al. (2002b); 12, Lai et al. (2003); 13, Lehmann et al. (1999); 14, Medley and Clements (1998); 15, Morin et al. (2007); 16, Nunes et al. (2003); 17, Peres et al. (1997); 18, Sabater (2000); 19, Shehata et al. (1999); 20, Szabó et al. (2005); 21, Takamura et al. (1989)

increasing regularly in EU1 and EU2. In the streams exposed to high cadmium concentrations, growth rates were diminished due to chemical stress affecting cell survival, cell division (Perrein-Ettajani et al. 1999), or reproduction rates (Rott 1991; Peres et al. 1997) or even modifying migration strategies (Peterson 1996). These structural and functional perturbations led to the dramatic reduction we observed in diatom biomass, as described by Paulsson et al. (2000). As shown in Figs. 2 and 5 and indicated by correlations, diatom density increases fit AFDM evolutions well, expressing the elevated quantitative contribution of diatoms to the pool of living cells in the biofilms (Stevenson 1996). These approximate covariations may, moreover, be assigned to a limited mortality of the cells; thus a thin layer of dead cells would probably be enough to exert a protective role on diatoms in the inner parts of the biofilms exposed to metals.

The diatom assemblages present during the first week were similar to the common available pool of species present in the inoculum. Then the assemblages differentiated according to the competitive ability to grow of the species under elevated metal exposure. However, most of the dominant taxa identified in this experiment (including the control unit) had already been reported in metal-contaminated environments (see Table 2), even though the status of many of them is still under discussion. The use of microcosms enabled us to eliminate external agents such as grazing or other disturbances, and the only factor that was liable to mitigate toxicity was resource competitive ability (Tilman 1986). Nitrogen and phosphorus are prerequisites for growth and development of autotrophic organisms, but diatoms are found with a broad range of nutrient availability, conductivity, etc. (Stevenson and Pan 1999). In EU2, especially, the joint effects of metal contamination and interspecific resource competition on the composition of the ultimate community structure must be taken into consideration. Saprophilous species like *Nitzschia palea* and, to a lesser extent, *Gomphonema parvulum* (Kützing) Kützing may have been favored by the nutrient availability, mitigating the effects of the cadmium at low concentrations (Barranguet et al. 2002; Morin et al. 2007).

The shifts observed in diatom species in EU3 (high-level cadmium) were also consistent with data found in the literature. The increased abundance of *Nitzschia palea* in EU3 coincided with the reported tolerance of this species to metal pollution (Table 2). The predominance of *N. palea* concomitant with the effect of high contamination detrimental to most of the species initially present (decline in diatom abundances, decrease in species richness, elimination of many species from the inoculum) is in agreement with the observations of Ivorra et al. (2002). A few studies of metal-tolerant algae, cited by Foster (1982), have shown that metal resistance is sometimes correlated with decreased metal uptake. This assumption may provide an additional piece of explanation for the decrease in the ability to accumulate cadmium, strong contamination

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creating a selective environment where the shifts in community structure toward dominance of a particular species probably led to reduced accumulation capacity of the ultimate biofilms.

Observations of Diatom Deformities

The communities inoculated into the three experimental units showed no morphological abnormalities. In EU1 at the beginning of the experiment, aberrant cells occurred at low rates (<3‰), similar to the rates of "naturally occurring" deformities. From week 2 in EU2 and EU3, however, there was a larger percentage of valve abnormalities, and cadmium exposure was identified as the leading stressor responsible for their increased occurrence. Abnormal cells had already been noted in sediment cores at various stages of the geological record from metal-polluted environments (Ruggiu et al. 1998; Cattaneo et al. 2004), as well as in fresh-water (Yang and Duthie 1993; McFarland et al. 1997; Nunes et al. 2003; Szabó et al. 2005; Morin et al. 2007) and in seawater (Thomas et al. 1980; Dickman 1998). In this experiment, concentrations of dissolved cadmium were above the "bad status" thresholds (3 µg $Cd{\cdot}L^{-1}$) proposed by the French freshwater quality evaluation system (SEQ-eau). As the percentage of abnormal cells increased with increasing cadmium concentrations in the streams, and with duration of exposure, it seems that cadmium is the causative agent for deformed diatoms in this experimental study. Moreover, the experimental units used very soft water, with a neutral pH, which would have enhanced metal bioavailability. The frequency of deformities in diatom communities exposed to cadmium contaminations confirmed the observations described previously, in systems that were mostly contaminated by metals such as Cd, Cu, and Zn. However, the percentages of deformities recorded here were very high compared with those in other studies. Maybe the high values found in this experiment indicated a more attentive consideration to slight alterations overlooked in other studies.

The reasons underlying the appearance of large numbers of deformed diatoms in EU1 after 6 weeks were suspected not to involve heavy metals. Anomalous diatom cells have commonly been described under culture conditions (Estes and Dute 1994), in relation to the fact that the cells had probably exceeded the lower limits for successful sexual reproduction and were on their way to extinction; however, deformities happen in roughly 2- to 12-year cycles. Here we would be inclined to single out some physical damage during cell development, related to crowding on the substrate. Cells with mechanically induced deformities may also be replicated in clonal populations as the cells divide, keeping the frequency of aberrations stable or increasing.

The great number of diatom-based indices used in Europe (Prygiel et al. 1999) are efficient tools for monitoring quality in rivers: trophic or saprobic levels (Lange-Bertalot 1979; Sládeček 1986; Schiefele and Schreiner 1991; Kelly and Whitton 1995), acidification (Håkansson 1993), salinity (Ziemann 1991), or general water quality (Coste, in Cemagref 1982; Dell'Uomo 1996; Lenoir and Coste 1996). However, these methods are not suitable for the assessment of metal pollution. A number of diatom species identified as metal-tolerant in this study can withstand a wide range of environmental conditions, and further investigations are needed to provide a pertinent classification of taxa depending on their resistance, sensitivity, or indifference to metal contamination. Diatom deformities are certainly a more specific indicator of high metal concentrations, as emphasized by many field studies (Dickman 1998; Medley and Clements 1998; Morin et al. 2007). This experiment confirmed the increased occurrence of such abnormalities as a distinctive characteristic of growing diatom biofilms exposed to cadmium. The percentage aberrant diatoms is already used in the United States (Stevenson and Bahls 1999) as a metrics of presumptive metal pollution. In France an update to the SPI (Specific Pollution Sensitivity Index [Coste, in Cemagref 1982]) is currently under test: it takes into account the relative abundances of deformities (abnormal diatoms having the worst pollution sensitivity values) in order to get a better estimate of general water quality.

Conclusions

It is very difficult to assess the effects of metal pollutions on diatom biofilms in field experiments. Therefore, this study was carried out to obtain an approximation of the toxicity of cadmium at different stages in the development of the community using experimental microcosms. It is difficult to account for the differences between treatments because the physicochemical variables exhibited similar trends for all treatments, with the exception of conductivity, which increased more in one system (EU2). Despite this, it was observed that: (i) diatom biofilms have a good accumulation capacity, and their growth seems to be lowered by cadmium exposure, (ii) shifts in species composition and decreases in species richness may be attributed to cadmium exposure, and (iii) changes in diatom morphology (deformities) are much more frequently observed in cadmium-treated microcosms.

The present work reveals how diatom communities can be used to provide an assessment of metal pollutions. Field validation of the observed effects remains an interesting subject for further investigations.

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