Metal induced succession in benthic diatom consortia

Ivorra i Castella, N.

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Chapter V

DIFFERENCES IN THE SENSITIVITY OF BENTHIC MICROALGAE TO ZN AND CD REGARDING BIOFILM DEVELOPMENT AND EXPOSURE HISTORY

Abstract. Microbenthic biofilms are consortia of autotrophic and heterotrophic organisms imbedded in a matrix of polymers and particles. As biofilms develop, internal cycling of materials might predominate and dependence on external conditions is reduced. The mature biofilm structure may act as a barrier against deleterious effects of metals on microphytobenthos. To validate this hypothesis, biofilms from two lowland streams near the Dutch-Belgian border, the extremely Zn and Cd polluted Eindergatloop (EP) and the relatively clean Keersop (R) in the River Dommel subsystem, were collected after 2 weeks ("young") and 6 weeks ("old") of colonisation. Young and old biofilms from both sites were subsequently exposed in the laboratory to Zn and Cd concentrations mimicking that of the heavily polluted stream for a period of two weeks. Diatom composition, chlorophyll *a*, total carbohydrates, Zn and Cd concentrations, minimal chlorophyll fluorescence (F_0) and photon yield (ϕ_p) demonstrated more pronounced metal effects on the young than on the old reference biofilms. In contrast, colonisation time had less effect on the overall response of the extremely polluted biofilms. Therefore, biofilms in an early colonisation stage are more vulnerable than mature biofilms to metal exposure, and exposure history determines the response of biofilms to metals.

INTRODUCTION

Freshwater algal ecotoxicity tests, recommended by regulatory bodies, are commonly restricted to the use of planktonic species such as the green algae *Selenastrum capricornutum* (recently renamed as *Raphidocelis subcapitata*) (EPA 1971). Microphytobenthic species have not been included in these recommendations, but in rivers, benthic diatoms have been valuable indicators of organic pollution (Descy and Coste 1991), eutrophication (Kelly and Whitton 1995) and acidification (Pan *et al.* 1996). Several studies have also demonstrated that metal pollution in rivers might affect the species composition of the different algal groups in the microbenthic assemblages (Deniseger *et al.* 1986; Genter and Amyot 1994). In a previous field study, we found that natural periphyton communities originating from streams with differing Zn and Cd concentrations showed marked differences in their diatom composition and metal accumulation (Ivorra *et al.* 1999), demonstrating the indicator value of benthic algae.

The development of a biofilm starts with adhesing bacteria. The primary algal colonisers are frequently pennate diatoms, in later stages, are followed by other, sometimes planktonic species (Hudon and Bourget 1983; Roemer *et al.* 1984). As colonisation proceeds, extracellular polymeric substances of algae and bacteria, such as polysaccharides (Hoagland *et al.* 1993; Decho 1990), might increase, forming a substantial biofilm having a limited exchange with the surrounding water. Internal cycling of materials may predominate in dense films (*e.g.*, among autotrophs and heterotrophs). Under these conditions, biofilm organisms may be more or less protected from external stress conditions (Freeman and Lock 1995).

The capacity to accumulate metals in higher concentrations than those found in the water column (Ivorra *et al.* 1999; Vymazal 1984; Wong and Tam 1998) is one of the singular properties of biofilms. Transport through this barrier occurs by molecular diffusion modified by sorptive capacities of particulates and polymers. Hence, mature (*i.e.*, thicker) biofilms could be less influenced than younger biofilms by variations of water quality. The diffusion and the bioavailability of metals, nutrients and other ions from the water column into the microbenthic mats, and *vice versa*, might be

influenced by an increase of density and complexity of the biofilms with aging (Stevenson and Glover 1993; Johnson *et al.* 1997; Rose and Cushing 1970). The aim of the present study, therefore, was to investigate a possible protective role of the biofilm to microorganisms against deleterious effects of Zn and Cd. We compared both young and mature communities originating from a metal-polluted stream and communities originating from a relatively unpolluted river. In addition to classic parameters, like chlorophyll content and species composition, the pulse amplitude modulated (PAM) fluorometry was used to assess the photosynthetic response to metals in biofilms.

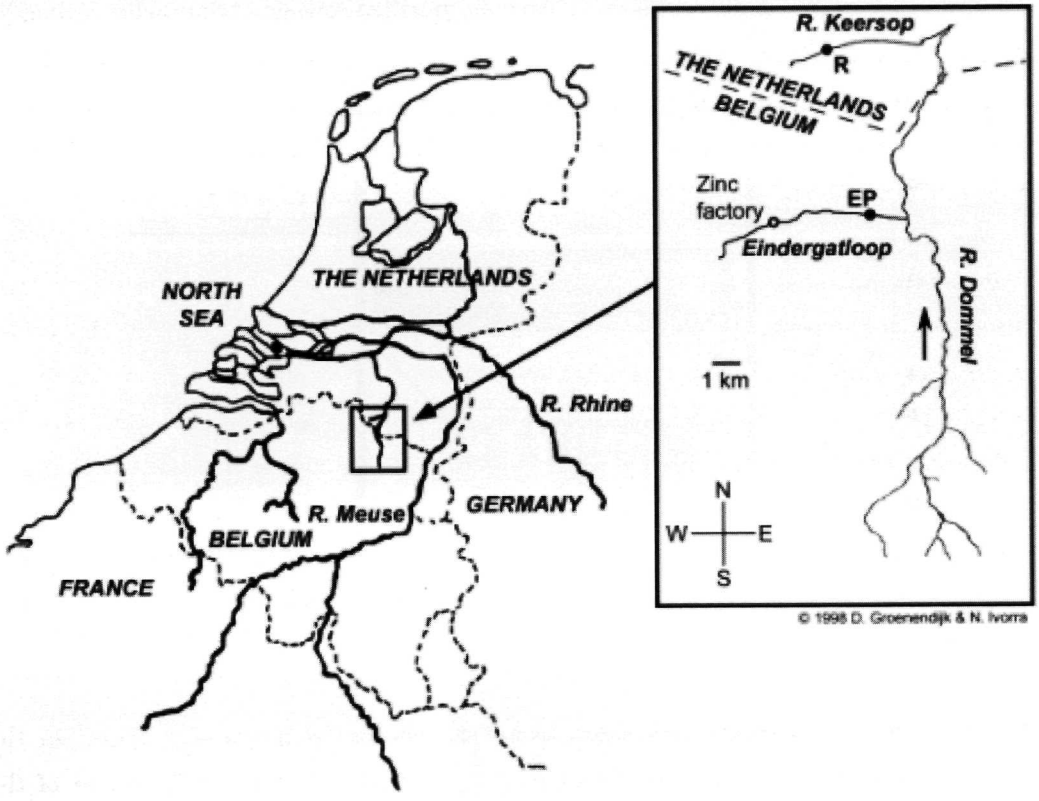

Figure 1. Location of the Keersop (R) and Eindergatloop (EP) stream sites.

MATERIAL AND METHODS

Streams

Microbenthic biofilms were obtained from two neighbouring Belgian-Dutch lowland streams from the River Dommel subsystem, a tributary of the River Meuse (Fig. 1). Reference (R) biofilms came from the Keersop (51° 20′ N/ 5° 24′ E), a relatively clean stream with low metal contents but that is subjected to nutrient runoff from agricultural source. Polluted biofilms were collected from the Eindergatloop (51° 15′ N/ 5° 25′ E), a stream extremely polluted (EP) with Zn and Cd by the seepage of a former Zn factory and exposed also exposed to partially purified sewage (Admiraal *et al.* 1999).

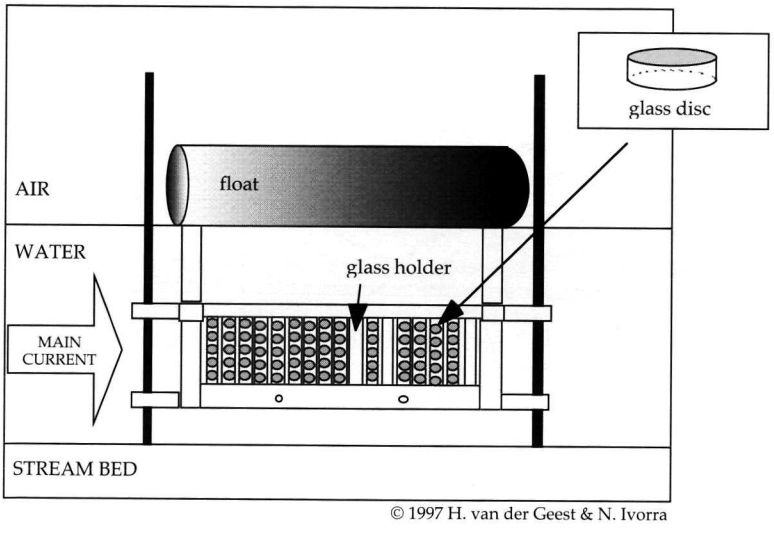

Figure 2. Rack containing glass discs for colonisation of microphytobenthic biofilms.

Microbenthic biofilms

Biofilms were grown on sandblasted glass discs (1.5 cm^2). Twenty polyethylene racks, each supporting a total of 170 discs (Fig. 2) according to the methods of Blanck (1985) were simultaneously placed in the Keersop (R) and in the Eindergatloop (EP) in January 1997. Discs were vertically submerged at a depth of 20 cm, parallel to the

current. Racks were collected after 2 weeks ("young" biofilms) for the first laboratory experiment and after 6 weeks ("old biofilms") for the second experiment. Intact racks were transported vertically in cool boxes that were completely filled with site water to minimise shear. Before use, all material was cleaned with a 1% HNO_3 (reagent grade) solution and rinsed with double distilled water.

Laboratory experiments

Two consecutive laboratory experiments were performed, one with the young biofilms and one with the old biofilms, for a period of 2 weeks each. Both experiments were conducted under identical conditions and treatments. Microbenthic biofilms were incubated in 6 glass aquaria under continuous water mixing, supplied by paddles moving at a speed of approx. 0.1 $m \cdot s^{-1}$ and at a light intensity of approx. 130 μmol photon$\cdot m^{-2} \cdot s^{-1}$ (10h/14 h light: dark regime) and at a temperature of 10 °C (average winter temperature). The aquaria were equilibrated overnight with the test solutions before the start of the experiments. Rack holders containing a total of 120 discs were placed vertically in each aquarium and all aquaria were filled with 6 L of reference (Keersop) water. Both Zn and Cd were added to obtain a final nominal concentration of 3000 μg $Zn \cdot L^{-1}$ and 100 μg $Cd \cdot L^{-1}$ to 4 aquaria, two containing biofilms from the Keersop (R+) and two containing biofilms from the Eindergatloop (EP+). These metal concentrations equaled those measured during the study period in the polluted location (EP). In the remaining two aquaria, reference (R) biofilms were incubated in reference water. Water from the reference site was collected weekly and renewal of metals and water in the aquaria was performed twice a week.

Characterisation of stream water

When the racks were brought to and collected from the field, water samples were taken, and conductivity, temperature, light attenuation (K), oxygen concentration and current velocity were determined *in situ*. Alkalinity, pH, total and reactive soluble phosphate (molybdenum blue method; Murphy and Riley 1962), total and dissolved inorganic and organic carbon (TIC-TOC and DIC-DOC; Total Carbon Analyser 700 C, OI, College Station, TX, USA) and metal (Flame 1100B and Graphite Furnace 5100 with

background correction [Perkin Elmer 1100B and 5100, Norwalk, CT, USA] Atomic Absorption Spectrophotometer [AAS]) determinations were performed in the laboratory. Quality control of the metal analysis was performed using reference material (NIST SRM 1643, National Institute of Standards and Technology, Gaithersburg, MD, USA); the measured values deviated less than 10% from certified values.

Carbon (total, dissolved, organic and inorganic) and phosphate (total and reactive soluble) concentrations of the R water were determined before each water renewal. Before and after each renewal of test solutions in the laboratory, samples were taken for determination of the pH and the actual metal concentrations.

Biofilm composition

Biofilms were detached from discs by sonication in the cold and were suspended in double distilled water for determinations of chlorophyll *a* (Chl. *a*), dry weight (DW), total carbohydrates and metal accumulation. A minimum of three discs (n) per treatment were collected for each determination as described here.

For Chl. *a* extraction, microbenthic suspensions (n= 6-10) were filtered through glass fiber filters (pore size 1.2 μm). Next, 5 ml 80% ethanol was added to the filters and pigments were extracted at 75 °C for five minutes. Absorbances at 750 and 665 nm were read before and after acidification with HCl (0.4 M). Chl. *a* concentrations were calculated according to the method of Vollenweider (1969) and expressed as μg Chl. *a* $\cdot cm^{-2}$.

Suspensions of 10 discs were freeze-dried for determination of DW, total carbohydrates and metal accumulation. The DW values were expressed as μg $DW \cdot cm^{-2}$. Total carbohydrates (n=5) were determined with the phenol-sulfuric acid method (Dubois *et al.* 1956), absorbances were read at 488 nm, and total concentrations were expressed as μg $glucose \cdot cm^{-2}$. Metal concentrations in biofilms were determined after digestion of samples (n= 3-5) in 70% HNO_3 (Ultrex, Baker, Paris, KY, USA) using a microwave oven equipped with temperature and pressure control program (MDS-200, CEM laboratories, Matthews, NC, USA). Metal analyses were performed with the Flame and Graphite

Furnace atomic absorption spectrophotometer. Quality control of the metal analysis was carried out by analysing digestion blanks and reference material (NIST SRM 2704, Buffalo River Sediment, National Institute of Standards and Technology). The measured values deviated less than 10% from the certified values and digestion blanks were near detection limits. Metal concentrations were expressed as μg metal$\cdot g^{-1}$ DW.

Algal species composition

Three discs per treatment were used for enumerating diatom cells. Organic material was eliminated with H_2O_2 (30%) and $KMnO_4$ according to the method of Van der Werff (1955), and cleaned diatom frustules were later mounted in Naphrax (refractive index, 1.69). At least 300 frustules were identified and counted from three different slides according to the classification of Krammer and Lange-Bertalot (1986-1991).

The percentage contribution of seven preselected diatom species, with different ecological and growth features, was established: *Achnanthes minutissima, Cymbella minuta, Fragilaria capucina, F. capucina* var. *vaucheriae, F. ulna, Gomphonema parvulum, Melosira varians*. This simple species set, which we also used previously (Ivorra *et al.* 1999), allows for detection of major changes in the communities. Samples preserved samples in 4% formaldehyde were used to check microscopically for the occurrence of green algae and filamentous microorganisms.

Fluorescence measurements

The minimal chlorophyll fluorescence in dark adapted cells (F_0) and the photochemical efficiency of PSII per absorbed photon or photon yield (ϕ_p) were measured during the experiment using a PAM 101-103 fluorometer (Walz Mess. Regeltechniek, Effeltrich, Germany) as described in Hofstraat *et al.* (1994). The terminology of the fluorescence parameters used was that of Van Kooten and Snel (1990). The settings of the PAM (measuring light, gain and damping) were optimised and fixed before proceeding with the measurements. On days 1, 2, 6 and 14 of experiment 1 and days 1, 2, 3, 7, 11 and 15 of experiment 2, intact colonised glass discs (2-3 per aquarium) were sampled for fluorescence measurements. Discs were individually placed on the bottom of transparent glass vials filled with 10 ml of water

from the corresponding treatment with the colonised side facing upward. The vials were then placed above the sensor of the PAM fluorometer.

Intact discs were adapted to darkness during 30 minutes to remove any energy-dependent quenching and to open all photosynthetic reaction centers before proceeding with the measurement of F_0 using the PAM. The F_0 was measured at a low-intensity, modulated measuring light that was unable to induce photochemical changes, was provided by a pulsed light-emitting diode (LED) peaking at 650-660 n m and was monitored by a photodiode detector.

Thereafter, intact discs were exposed to actinic light provided by fluorescence tubes from the same quality and intensity as those in the experimental setup for 60 minutes before measurement of ϕ_p. The ϕ_p gives a good estimation for the efficiency of the linear electron flow in the photosynthetic apparatus among light adapted cells (Hofstraat *et al.* 1994, Genty *et al.* 1989). The ϕ_p is defined as $(F_m'-F)/ F_m'$, where F is the actual fluorescence and F_m' is the maximal fluorescence in light adapted cells. F_m' is measured after exposure to a high-intensity saturating pulse capable of closing all reaction centers and of disabling any photochemical reaction. The saturating light pulses (10000 μmol photon$\cdot m^{-2}\cdot s^{-1}$) were provided by a KL-1500 lamp and controlled with the PAM 103 unit. The pulses had a duration of 600 ms and they were applied each 30 s for 3 min. The ϕ_p was calculated as the average of 5 successive pulses per disc, on two or three replicate discs.

Statistics

Quantitative differences between young and old R and EP field colonised biofilms were analysed using student's *t* tests (Sokal and Rohlf 1981). Differences between duplicates from treatments, within an experiment, were analysed using one-way analysis of variance (ANOVA; Sokal and Rohlf 1981) before proceeding with further statistical tests. Differences between treatments within an experiment were tested using one-way ANOVA. Student-Newman-Keuls tests were applied *a posteriori*. Log transformation or arc sine transformation (of species abundance) data was applied when needed. When the assumptions for ANOVA were not met, the nonparametric Kruskal-Wallis test was performe. Interactions between colonisation time and treatment were tested

with two-way ANOVA (Sokal and Rohlf 1981). A 5% significance level ($p<0.05$) was used for rejection of null hypothesis in all cases.

RESULTS

Field colonisation conditions

Colonisation of the discs took place under much higher Zn (approx. 29 fold) and Cd (approx. 578 fold) water concentrations than those in the reference (R) stream. The EP location was also characterised by higher conductivity, temperature, organic and inorganic carbon concentrations, and lower irradiance and oxygen concentrations (Table 1) than the reference stream.

	Stream		Reference water	
parameter[b]	Keersop (R)	Eindergatloop (EP)	Exp. 1	Exp. 2
current velocity ($m \cdot s^{-1}$)	0.28 (0.12)	0.27 (0.09)		
alkalinity ($meq \cdot L^{-1}$)	1.0 (0.3)	2.0 (0.9)		
conductivity ($\mu S \cdot cm^{-1}$)	488 (16)	945 (137)		
K (m^{-1})	1.9 (0.3)	5.0 (7.8)		
pH	6.8 (0.2)	7.0 (0.1)		
O_2 ($mg \cdot L^{-1}$)	11.4 (1.1)	7.6 (0.2)		
T (°C)	5.8 (2.5)	9.5 (0.5)		
Cd ($\mu g \cdot L^{-1}$)	0.14 (0.05)	81 (31)		
Zn ($\mu g \cdot L^{-1}$)	76 (57)	2216 (975)		
Fe ($\mu g \cdot L^{-1}$)	253 (186)	619 (812)		
Total P ($\mu g \cdot L^{-1}$)	208 (192)	1336 (1854)	103 (22)	70 (10)
Ortho-P ($\mu g \cdot L^{-1}$)	42 (27)	1224 (1827)	22 (4)	24 (0)
TIC ($mg\ C \cdot L^{-1}$)	18.5 (2.9)	32.3 (13.6)	16 (2)	16 (1)
TOC ($mg\ C \cdot L^{-1}$)	6.5 (1.9)	29.5 (35.0)	6 (1)	8 (1)
DIC ($mg\ C \cdot L^{-1}$)	17.5 (2.3)	31.3 (14.5)	16 (1)	16 (1)
DOC ($mg\ C \cdot L^{-1}$)	6.4 (1.9)	22.4 (31.6)	7 (2)	8 (1)

[b]Phosphate and carbon concentrations of the reference water from experiments (Exp.) 1 and 2. DIC= dissolved inorganic carbon, DOC= dissolved organic carbon, TIC= total inorganic carbon, TOC= total organic carbon.

Table 1. Physical and chemical characteristics of water from the reference (R) and extremely polluted (EP) streams during the colonisation period of the biofilms. Values are mean (standard deviation).

Experimental conditions

Phosphate and carbon concentrations in the reference water used for renewal in both laboratory experiments were very similar and rather constant (Table 1). Almost 100% of the total organic and inorganic carbon in the water was present in the soluble form (Table 1).

The actual metal concentrations in the reference water and hence, the concentrations in the control R treatments of each experiment were always low (Table 2). Actual Zn and Cd concentrations in the exposed R+ and EP+ treatments (Table 2) reached the desired exposure levels and were very comparable between experiments. However, metal concentrations before each water renewal indicated a greater decrease of Zn and Cd levels in the water from the old R+ treatments (*i.e.*, second experiment). Therefore, additional water samples were taken for a period of 5 hours immediately after water and metal renewal. Within 4 hours after renewal, a decrease of approx. 40% of the Zn and Cd in the water was observed in the old R+ treatment compared with an approx. 10% decrease in the old EP+ treatment. This seemed to indicate that the old exposed reference (R+) biofilms removed more metals from the water than the young exposed reference (R+) biofilms and the old (EP+) biofilms.

	Zn (μg Zn·L^{-1})		Cd (μg Cd·L^{-1})	
Sample[b]	Exp. 1	Exp. 2	Exp. 1	Exp. 2
R	47 (33)	84 (58)	0.2 (0.2)	0.3 (0.2)
	41 (27)	86 (57)	0.1 (0.1)	0.3 (0.1)
R+	2547 (663)	2375 (329)	82 (7)	82 (9)
	3007 (200)	2291 (251)	85 (12)	82 (6)
EP+	2824 (100)	2643 (211)	87 (7)	81 (20)
	2939 (118)	2633 (208)	89 (8)	85 (19)

[b]EP+= exposed extremely polluted, R= reference, R+= exposed reference.

Table 2. Measured metal concentrations in the water of different (duplicate) treatments, after water renewal and metal addition, from experiments (Exp.) 1 (young biofilms) and 2 (old biofilms). Values are mean (standard deviation).

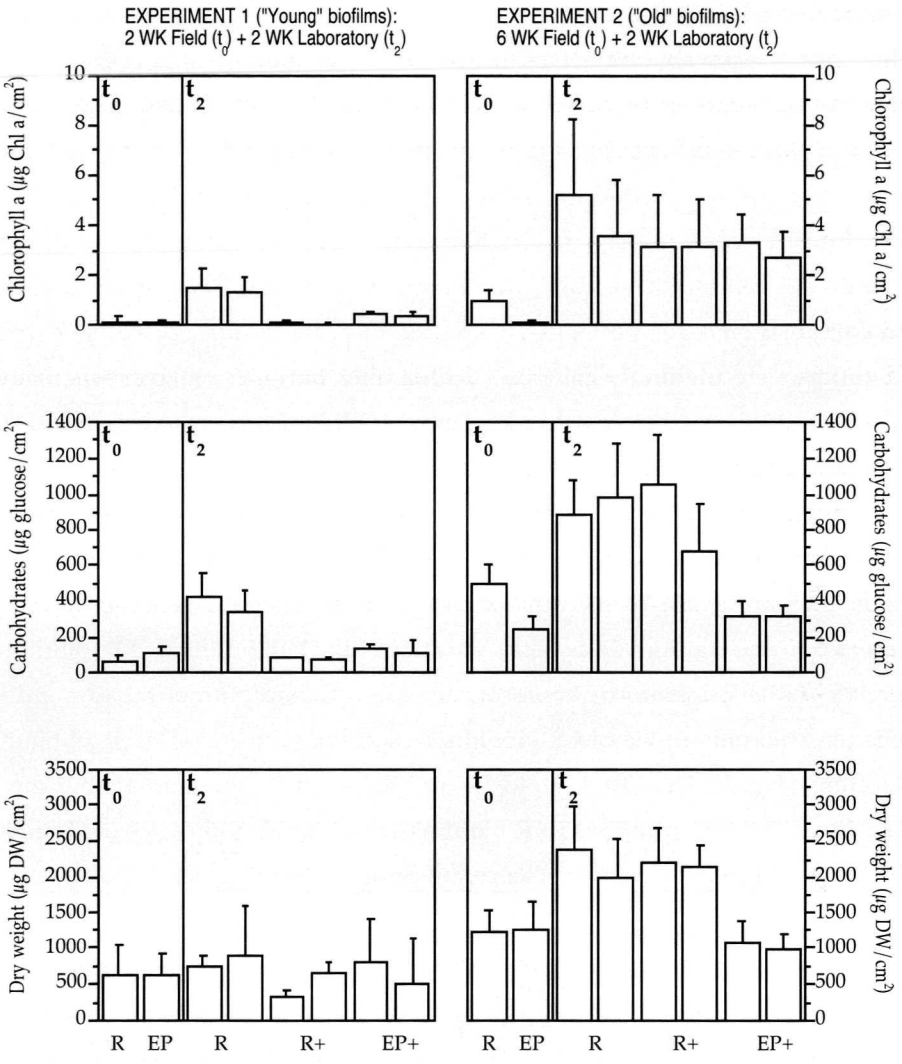

Figure 3. Mean chlorophyll a (Chl. a), total carbohydrate concentrations and dry weights (DW) as determined in young (left graph of left panels) and in old biofilms (right graph of left panels) before laboratory exposure (t_0) and in the young (left graph of right panels) and old (right graph of right panels) control reference (Keersop; R), exposed R (R+), and exposed extremely polluted (EP+) biofilms after the 2-week laboratory exposure (t_2). Bars represent the standard deviation.

After water and metal renewal, the pH in treatments of the first experiment ranged from 7.1 (metal-exposed) to 7.7 (control). The pH values in treatments of the second

experiment ranged from 7.3 (metal-exposed) to 7.5 (control). In the period between water and metal renewals, the pH increased in all treatments, ranging from *ca.* 7.5 (in the exposed treatments of the first experiment) to 8.1 (in all controls and in exposed treatments of the second experiment). The higher pH observed in control R treatments and in old biofilms was related to the increase of biomass and photosynthetic activity in the biofilms.

Biofilm composition

Old R biofilms were uniformly colonised with a thick dark-brown layer whereas young R biofilms were thinner and patchy. In contrast, EP biofilms, whether young or old, were thin and greenish.

The large differences in metal concentrations to which biofilms were exposed in the field were not reflected in the DW, Chl. *a* or carbohydrate accrual of the young EP and R biofilms after two weeks of colonisation in the streams (Fig. 3). After six weeks in the field, the DW and carbohydrate concentrations in both R and EP biofilms had significantly ($p<0.05$) increased, however, the Chl. *a* concentrations had significantly ($p<0.05$) increased only in the old R biofilms, indicating an increase of algal biomass in these biofilms (Fig. 3). The DWs of old EP and R biofilms were similar, but the Chl. *a* and carbohydrates concentrations in R biofilms were significantly ($p<0.05$) higher than those in EP biofilms after the six weeks colonisation period (Fig. 3).

The response of biofilms after two weeks metal exposure in the laboratory was markedly different between those of different age and origin. After incubation, the Chl. *a* and carbohydrates concentrations in young exposed R+ and EP+ biofilms were significantly ($p<0.05$) lower than those in control R biofilms (Fig. 3). The young R+ biofilms had even significantly ($p<0.05$) lower Chl. *a* concentrations than the young EP+ biofilms (Fig. 3). In contrast, the old R+ biofilms seemed to be unaffected by the metals; the Chl. *a* and carbohydrate concentrations in these biofilms were not significantly different from the concentrations in the control R biofilms (Fig. 3).

The Chl. *a* concentrations in the old polluted EP+ biofilms increased considerably and were not significantly different from the concentrations in control R biofilms, but the

DW and carbohydrate concentrations of the old EP+ biofilms remained significantly ($p<0.05$) low (Fig. 3).

	Field (t_0)		Laboratory (t_2)		
	R	EP	R	R+	EP+
Young	12(3)	21(12)	47(13)	18(8)	30(22)
Old	42 (7)	20(3)	44(10)	40(12)	31(3)

Table 3. Carbohydrates as a percentage of the total dry weight of young and old reference (R) and extremely polluted biofilms (EP) before (t_0) and after (t_2) laboratory exposure (+). Values are mean (standard deviation).

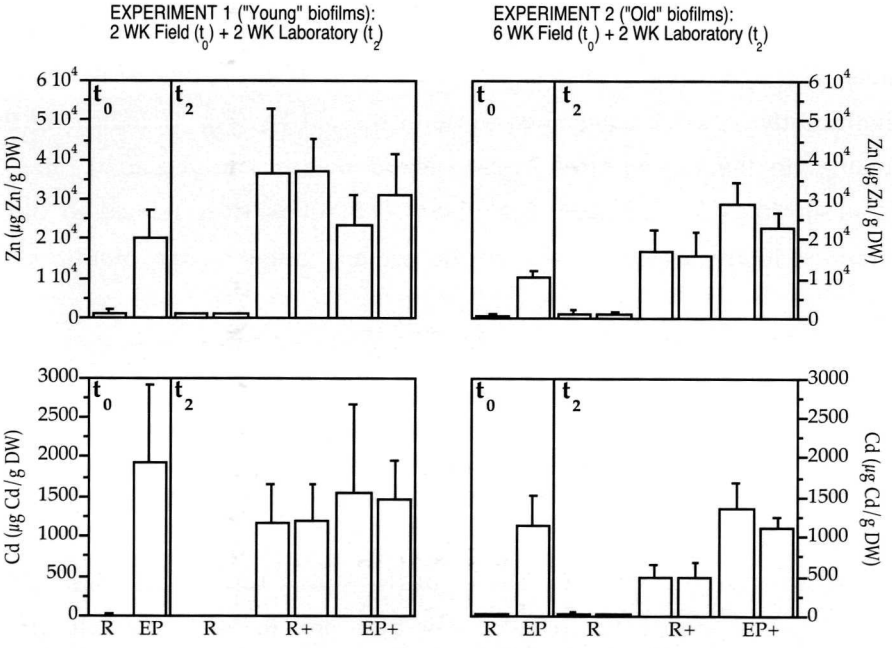

Figure 4. Mean Zn and Cd concentrations determined in the young (left graph of left panels) and in old biofilms (right graph of left panels) before laboratory exposure (t_0) and in the "young" (left graph of right panels) and in old (right graph of right panels) control reference (Keersop; R), exposed R (R+), and exposed extremely polluted (EP+) biofilms after the 2-week laboratory exposure (t_2). Bars represent the standard deviation.

The contribution of the carbohydrates fraction to the total DW was higher in old R field biofilms than in the young ones, but it remained low in the old EP field biofilms (Table 3). Old R and R+ biofilms had similar carbohydrate fraction after metal exposure in the laboratory, but that of the young R+ biofilms after metal exposure in the laboratory was considerably lower than that of the young R biofilms (Table 3).

Metal concentration in biofilms

The Zn and Cd concentrations in the biofilms were clearly related to the dissolved Zn and Cd concentrations in the water. Consequently, the EP biofilms contained a 13 fold more Zn and a 100 to 200 fold more Cd than the R biofilms (Fig. 4). A longer field colonisation time did not cause great changes in the Zn and Cd concentrations in either R or EP biofilms. In the laboratory, addition of Zn and Cd also led to significant ($p<0.05$) higher Zn and Cd concentrations in R+ and EP+ biofilms than in the control R biofilms (Fig. 4). However, the Zn and Cd concentrations in the young R+ biofilms were significantly ($p<0.05$) higher than those in the old R+ (Fig. 4), indicating that the accumulation in the old biofilms lagged behind that in the young biofilms. Thus, metal concentrations in the laboratory exposed biofilms were related to the metal concentrations in the water, but also to the age and origin of the biofilm (two-way anova; $p<0.001$).

Minimal chlorophyll fluorescence (F_0) and photon yield (ϕ_p)

If measured under fixed measuring light intensity, sample geometry and optics, might be proportionally related to the amount of chlorophyll in the sample. Indeed, F_0 at the end of both experiments reflected the differences in Chl. *a* concentrations between the different treatments (Fig. 3 and 5). The F_0 of the young control R biofilms and EP+ biofilms increased significantly ($p<0.05$) over time and reached its maximum at the end of the experiment. The metal exposure of the young R+ biofilms provoked a total inhibition ($p<0.05$) of the F_0 increase over time (Fig. 5).

In contrast to the young biofilms, the normal evolution of the F_0 in the old R+ indicated that microalgal growth in these biofilms was not impaired by metals. At the end of the second experiment, the F_0 values of the old biofilms were equally high (Fig. 5).

The ϕ_p of young control R biofilms was, from the second day onwards, always high. In contrast, the ϕ_p of the young R+ and EP+ biofilms remained significantly ($p<0.05$) lower and recovered only after 14 days exposure to values equal to those of control R biofilms. Metal exposure did not affect the ϕ_p of the old biofilms, old R+ had an equally high yield as the control R biofilms at the start of the experiment (Fig. 5).

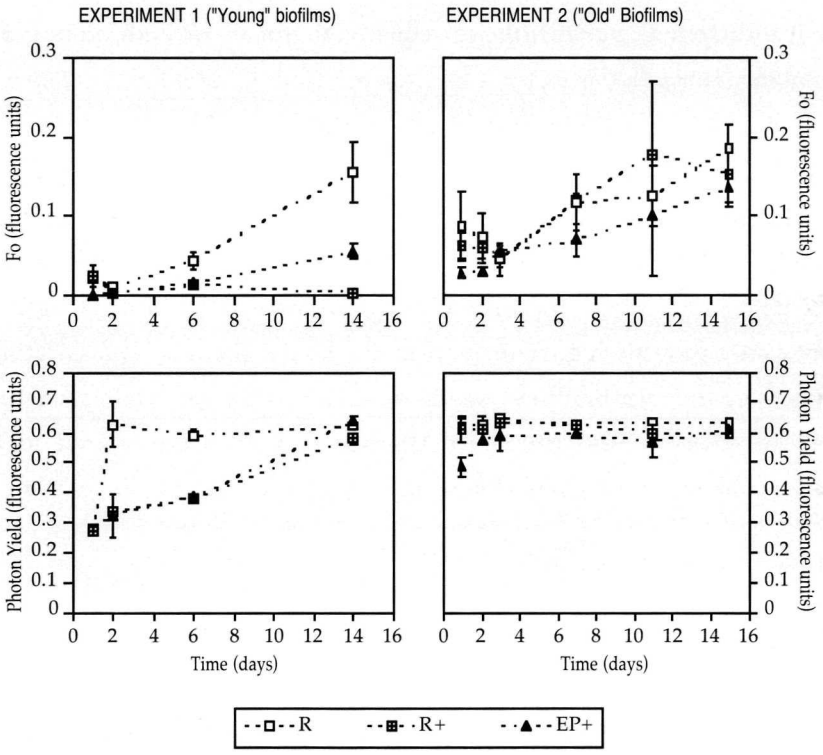

Figure 5. Mean minimal chlorophyll fluorescence (F_0) and photon yield (ϕ_p) of the young (left) and old (right) control reference (Keersop; R), exposed R (R+), and exposed extremely polluted (EP+) biofilms during the course of the laboratory experiments. Bars represent the standard deviation.

Algal species composition

After two weeks of colonisation, *C. minuta*, *F. capucina* and *F. ulna* were significantly ($p<0.05$) more abundant in the R assemblages and *A. minutissima* and *F. capucina* var. *vaucheriae* were significantly ($p<0.05$) more abundant in the EP assemblages (Table 4). After six weeks of colonisation time, the main changes in these diatom assemblages consisted in a significant ($p<0.05$) decrease of *F. ulna* together with the dissapearance of *C. minuta* in the old R and of *F. capucina* var. *vaucheriae* in the old EP assemblages (Table 4). The diatom *G. parvulum* was equally abundant in both, young and old, R and EP biofilms (Table 4).

	Young			Old		
Diatom species	R	EP	$p<0.05$ [a]	R	EP	$p<0.05$
Achnanthes minutissima	-	19	*	1	26	*
Cymbella minuta	2	-	*	-	-	
Fragilaria capucina	3	-	*	2	-	*
F. capucina var. vaucheriae	1	4	*	2	-	
F. ulna	10	-	*	2	-	*
Gomphonema parvulum	8	9		16	9	
Melosira varians	6	-		2	-	

[a] *= significant ($p<0.05$) differences between R and EP.

Table 4. Mean relative abundance (%) of diatom species in young and old reference (R) and extremely polluted (EP) field assemblages.

Metal exposure in the laboratory caused a significant ($p<0.05$) decrease of the filamentous diatom *M. varians* in both young and old R assemblages (Table 5). In contrast, the relative abundance of the other diatoms as *F. ulna*, and *A. minutissima*, in the young R+ and *F. capucina* var. *vaucheriae*, in the old R+ remained the same, or even increased, after metal treatment (Table 5).

A. minutissima was significantly ($p<0.05$) more abundant in the young and old EP+ assemblages. *G. parvulum* became equally abundant in all old biofilms (Table 5).
At the end of the second laboratory experiment, the development of the filamentous green algae *Ulothrix* sp. and *Stigeoclonium* sp. was observed on the exposed old R+ and EP+ discs.

Diatom species	Experiment 1			Experiment 2		
	R	R+	EP+	R	R+	EP+
Achnanthes minutissima	-	5*	17*	-	1	53*
Cymbella minuta	2*	2*	-	4*	3*	-
Fragilaria capucina	4*	5*	-	10*	10*	-
F. capucina var. vaucheriae	9*	12*	1	4	10*	-
F. ulna	1	5*	-	2*	2*	-
Gomphonema parvulum	14	8	29*	17	13	11
Melosira varians	9*	2	-	5*	1	-

*Significant ($p<0.05$) higher relative abundance.

Table 5. Mean relative abundance (%) of diatom species in young and old reference (R), exposed reference (R+) and exposed extremely polluted (EP+) laboratory assemblages.

DISCUSSION

Our results clearly demonstrated that *in situ* levels of Zn and Cd were detrimental to microphytobenthos, but also that the effects of metal exposure vary with the developmental stage of the biofilms. Inmature biofilms from a clean stream were very sensitive to Zn and Cd, whereas mature biofilms were not. Differences in biomass between young and old biofilms could explain the observed differences in sensitivity. Riber and Wetzel (1987) found that in poorly colonised substrata the exchange of substances between the water column and biofilms predominates. Conversely, an increased density might lead to increased recycling of nutrients and gasses within the biofilms (Riber and Wetzel 1987) and to reduced dependence on conditions outside the biofilm. In dense (*i.e.*, algal) biofilms, the thickness of the abiotic materials, and of the biomass, and of the boundary layer are limiting factors for transfer of natural substances (Stevenson and Glover 1993) and contaminants (Lau 1990, Liehr *et al.* 1994) into the biofilms.

Penetration of metals into biofilms is likely to be strongly influenced by the local pH. In thick biofilms, strong pH and redox gradients are observed because of the intense autotrophic and heterotrophic activities of microorganisms (Jørgersen and Revsbech 1985). The increase of pH observed in the water surrounding old biofilms seemed to be

in accordance with the increase of biomass and photosynthetic activity. Liehr *et al.* (1994) found that metal removal from the water was favoured by higher pH and lower alkalinity in the biofilms resulting from high photosynthetic activity. Using autoradiographic techniques, Rose and Cushing (1970) demonstrated that Zn penetration was restricted to the upperstory of biofilms and that this might result from sequestering driven by algal activity. The combined observations of a lower sensitivity for the old biofilms and their low overall metal concentrations and higher DWs, indicate a limited penetration of Zn and Cd into the thick biofilms.

In biofilms, metals might bind to carboxyl and sulfhydril groups of polymers and humic substances. Polymers are secreted by bacteria and algae coexisting in natural biofilms (Decho 1990). Indeed, relationships between the production of algal and bacterial polysaccharide exudates and metal binding have been found (Pistocchi *et al.* 1997; Loaëc *et al.* 1997). Our observation regarding the accumulation of polysaccharides in older R+ biofilms (≤40% DW) compared with that in younger R+ biofilms (18% DW) indicates that carbohydrates play a role in the sorption of metals and may have partly caused the reduced sensitivity of microorganisms in the old biofilm to metals.

Both species composition and architecture of the biofilm play a role in the response of algal asemblages to disturbances (Blenkinsopp and Lock 1994; Biggs *et al.* 1995). Therefore, differences in the sensitivity of species to metals could be strongly related to their position in the biofilm structure. The observed decrease in both young and old assemblages of the filamentous *M. varians* and the significant development of *A. minutissima* in the exposed assemblages seem to provide indications for the role of biofilm architecture. Typically, *M. varians* is a loosely attached and filament forming diatom, is sensitive to shear (Biggs and Thomsen 1995) and to low light (Hudon and Bourget 1983), and grows preferentially in the upperstory of biofilms. Burkholder *et al.* (1990) found higher nutrient uptake rates in loosely attached microalgae than in algae imbedded in biofilms. Moreover, sensitivity of *M. varians* to metal exposure has been reported (Medley and Clements 1998; Genter *et al.* 1987). Thus, the higher degree of metal exposure and, possibly, also an inherent metal sensitivity of the filamentous *M. varians* most likely caused its dissapearance after metal exposure.

A. minutissima exploits the solid substratum when biofilms are relatively thin but persists in thick biofilms because of its ability to tolerate low light conditions (Johnson *et al.* 1997; Peterson 1996). Small-celled species, like *A. minutissima*, are tightly attached (Blenkinsopp and Lock 1994, Korte and Blinn 1983) and predominate in thin biofilms and disturbed assemblages (Johnson *et al.* 1997, Peterson 1996). Tolerance to cadmium in isolates of *A. minutissima* has been demonstrated (Takamura *et al.* 1989) and several authors have reported a correlation between high numbers of this species in streams with increased concentrations of a variety of metals (Deniseger *et al.* 1986; Leland and Carter 1984). Hence, the growth form, and possibly, also the inherent metal tolerance of *A. minutissima* might explain why this species proliferated after zinc and cadmium exposure.

Development of filamentous green algae on old biofilms contributed probably to the overall increase in chlorophyll observed at the end of the second laboratory experiment. Normal maturation and species succession can lead to growth of chain forming diatoms and loosely attached green algae such as *Ulothrix* sp. in older biofilms (Johnson *et al.* 1997). Hence, shifts from free-living diatoms to filamentous diatoms and green algae occur in stream assemblages. The growth of green algae can be potenciated by a Zn-rich environment (Genter *et al.* 1987). Thus, development of *Stigeoclonium* sp. in our old, Zn-exposed communities could, according to Harding and Whitton (1976), be interpreted as resulting from a genuine metal resistance of this green algae.
Similar studies on the specific adaptation mechanisms of benthic diatoms like *G. parvulum* that uniformly occur under all conditions are currently underway based on indications on metal adaptation in planktonic diatoms.

Laboratory metal exposure of biofilms originating from the metal polluted stream was assumed unlikely to be a stress, because the experimental metal concentrations were derived from the *in situ* levels. Indeed, EP biofilms were not inhibited and algal growth was even potentiated, during metal exposure in the laboratory, as indicated by the algal fluorescence (and Chl. *a*) increase in either young or old biofilms. Combined

observations of Admiraal *et al.* (1999) and Lehmann *et al.* (1999) on the inhibition of bacterial metabolism and photosynthetic activity of benthic assemblages at the same river stations in the River Dommel indicate tolerance of the assemblages in the polluted stream. Blanck *et al.* (1988) demonstrated that pollution can induce community tolerance (PICT) through genetic adaptation or species succession. Such responses of biofilms to metal constraint were also indicated in the present study by the recovery of the photon yield of the young exposed biofilms during 14 days of exposure. In particular for Zn but also for a metal such as Cd subjected to precipitation in the biofilm, long term exposure may be needed in experiments to allow succession in favour of more tolerant algae (Say and Whitton 1981; Foster 1982) and bacteria (Lehmann *et al.* 1999). The present experimental observations on the photon yield of microalgal communities confirm the field observations on metal induced community tolerance in the same river (Admiraal *et al.* 1999; Lehmann *et al.* 1999). However, some caution is needed in interpreting the present observations on the photon yield obtained with the PAM, because little experience has been gained yet with the application of this technique to biofilms.

In conclusion, this study demonstrated that biofilms from a clean environment were more severely affected by the introduction of metals during an early developmental stage than during maturity. Such mature biofilms were highly resistant to metals even without a pre-exposure history. Still, the communities exposed in the field to very high metal concentrations contained species with known metal tolerance. Metal exposure of clean communities in experiments induced changes in the species composition and photon yield that indicated selection for metal tolerance. However, metal binding in the biofilm, the biofilm architecture and the capacity of species to adapt to metals are strongly interacting determinants of such a process of selection.

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