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DEVELOPMENT OF PHOTOSYNTHETIC BIOFILMS AFFECTED BY DISSOLVED AND SORBED COPPER IN A EUTROPHIC RIVER

CHRISTIANE BARRANGUET,*† MARC PLANS,†§ ESTHER VAN DER GRINTEN,† JAN J. SINKE,‡ and WIM ADMIRAAL†

†Department of Aquatic Ecology and Ecotoxicology, University of Amsterdam, Kruislaan 320, 1098 SM Amsterdam, The Netherlands
‡Netherlands Institute of Ecology, Centre for Coastal and Estuarine Ecology (NIOO-CEMO), P.O. Box 140, 4400 AC Yerseke, The Netherlands
§Department of Ecology, University of Barcelona, Avenida Diagonal 645, 08028 Barcelona, Spain

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Abstract—Photosynthetic biofilms are capable of immobilizing important concentrations of metals, therefore reducing bioavailability to organisms. But also metal pollution is believed to produce changes in the microalgal species composition of biofilms. We investigated the changes undergone by natural photosynthetic biofilms from the River Meuse, The Netherlands, under chronic copper (Cu) exposure. The suspended particles in the river water had only a minor effect on reduction of sorption and toxicity of Cu to algae. Biofilms accumulated Cu proportionally to the added concentration, also at the highest concentration used (9 μM Cu). The physiognomy of the biofilms was affected through the growth of the chain-forming diatom *Melosira varians*, changing from long filaments to short tufts, although species composition was not affected by the Cu exposure. The Cu decreased phosphate uptake and algal biomass measured as chl *a*, which degraded exponentially in time. Photosynthetic activity was always less sensitive than algal biomass; the photon yield decreased linearly in time. The protective and insulating role of the biofilm, supported by ongoing autotrophic activity, was indicated as essential in resisting metal toxicity. We discuss the hypothesis that the toxic effects of Cu progress almost independently of the species composition, counteracting ongoing growth, and conclude that autotrophic biofilms act as vertical heterogeneous units. Effective feedback mechanisms and density dependence explain several discrepancies observed earlier.

Keywords—Biofilms Copper Diatoms Fluorescence River Meuse

INTRODUCTION

The diversity in the environmental variables in rivers has been shown to reflect in a wide range of tolerance to toxicants found for benthic microalgal consortia [1]. Pollution history could be one of these variables. We concentrate here on the River Meuse, which, like many other European rivers, was highly polluted until the 1970s. Since then, measures have been taken to improve the water quality, mainly reducing the input of organic substances and metals [2]; however, concentrations of metals and other toxicants in the sediments remained high [3,4] and were shown to affect benthic organisms [5].

The microhabitat can be a decisive variable. On hard substrates in shallow freshwater ecosystems, benthic microalgae and bacteria are associated in a mucopolysaccharide matrix, forming biofilms. Autotrophic biofilms constitute a considerable amount of biomass that can act as a filter for organic particles and can accumulate metals, thereby reducing toxicity [6]. Conversely, metals have been pointed out as a factor determining the microalgal composition of biofilms, originating consortia of metal-tolerant species in polluted aquatic systems [1].

On the other hand, short-term experiments have shown that natural photosynthetic biofilms from the River Meuse were highly resistant to copper contamination in concentrations as high as 30 μM Cu, despite the high representation of metal-sensitive microalgae. Monospecific biofilms proved to be more sensitive, especially the noncohesive ones formed by the diatom *Synedra ulna* [7]. Suspended particles in the river water were shown to play a role in lowering acute toxicity of Cu to biofilms in a branch of the River Rhine, The Netherlands [8], in agreement with current viewpoints on the sequestration of metals in river water. However, such sequestration of metals was less evident in the Meuse.

The present study analyzes the effects of chronic Cu toxicity to natural photosynthetic biofilms and proposes the hypothesis that at low temperatures, which do not favor cyanobacterial proliferation, Cu toxicity progresses in biofilms in an indiscriminant way, opposing growth. Such a hypothesis is supported by the simultaneous assessments of Cu effects on biofilms, both in structural and in functional parameters, which are seldom integrated in environmental studies.

We investigated the temporal changes of the capacity of biofilms to accumulate Cu, the biofilm biomass, the photosynthetic activity, and the species composition and compared them to the development of these consortia under pristine conditions. The variations of development, as affected by the metal exposure, are evaluated in order to develop sensitive endpoints for these complex consortia.

MATERIALS AND METHODS

Sampling site

The Meuse is a European river running from France through Belgium into The Netherlands, with a morphology strongly modified for navigation. It is a highly eutrophic river fed by rainwater only, with a distinct minimal flow during the summer. Urban and agricultural activities influence the Meuse wa-

* To whom correspondence may be addressed (barranguet@science.uva.nl).

ter quality [2]. Dissolved Cu concentrations in the water have been quite low during the past years, ranging from 0.05 to 0.08 μM [5].

Nutrient levels (phosphate and ammonium) are to date still high, exceeding the standards set for the River Rhine. Nutrients and organic particles have been suggested to influence the metal bioavailability to organisms by altering metal complexation [9].

The sampling site was chosen at Keizersveer (51°43′N, 4°54′E, NL), near a major intake point of a drinking-water reservoir, 855 km from the river source. The Ministry of Public Works monitors the water quality weekly; a detailed description of the physic and chemical characteristics of the water can be found in [4,5].

Experimental setup

Etched glass disks (1.5 cm^2) were used as artificial substrata for biofilm colonization. Polyethylene racks served as a support: Six racks containing 170 disks each were placed vertically under a floating device in the river at 8 m from the bank, parallel to the current flow, and left to colonize by benthic algae. Biofilm samples were collected after a three-week colonization period in April 1998 under normal river flow conditions. The colonized glass disks were transported to the laboratory in a cooler filled with river water.

Glass disks containing biofilms were placed in eight aquaria (50/aquarium) containing 6 L river water either filtered (0.45 μm) or unfiltered. Light was set at a constant intensity of 50 μmol/m^2/s during a 16:8-h light:dark cycle. Temperature was controlled with a water bath connected to a thermostat and maintained similar to that of the river water, 11.5 ± 1°C. The water was constantly mixed with paddles and replaced every 3 to 4 d (days 3, 7, and 10 of the experiment). The pH was measured in each aquarium at the end of the day.

The biofilms were exposed to Cu ($CuCl_2$, Tritrisol [Merck, Darmstadt, Germany] Cu standard) for two weeks, and the final nominal concentration was 0 (no addition: controls) 1, 3, and 9 μM Cu (64, 191, and 572 μg/L, respectively). Before the experiment started, the aquaria were acid rinsed and preexposed to the nominal Cu concentration for one night.

After each water replacement, new Cu was added. Water samples for Cu determination were taken before and a half hour after the water renewal. Total and dissolved phosphates in the water were analyzed by spectrophotometry according to Murphy and Riley [10].

Two glass disks per treatment (16 in total) were collected daily for plant pigment analyzes and stored frozen (−80°C). Pigments were extracted from freeze-dried samples with 95% methanol and 5% ammonium acetate and analyzed by high-performance liquid chromatography (HPLC) according to Barranguet et al. [11]. Glass disks were also frozen for dry weight (DW) and ash-free dry weight (AFDW) determination and Cu analysis in the biofilm at the end of the first and second weeks of the experiment.

Measurements using the pulse fluorescence technique

The pulse amplitude modulated (PAM) fluorescence technique was used in the present study to measure in vivo chlorophyll fluorescence of intact microalgae consortia. Changes in the fluorescence yield were used to determine alterations in photochemical efficiency and to evaluate the physiological status of biofilm algae, especially in relation Cu exposure, which attacks PSII (photosystem II) directly [8,12].

The photosynthetic parameters F_s and F'_m (see the following) were measured every day around noon under actinic light in light-adapted samples. The photosynthetic parameters of dark-adapted samples were also measured at noon on days 0, 6, and 14 of the Cu exposure.

The fluorescence parameters used in the present study were as follows: F_0, the minimal fluorescence signal of dark-adapted cells, which is used as an indirect indicator of biomass; F_m, the maximal signal of dark-adapted cells, obtained with a saturating light pulse; under actinic light conditions F_s, the fluorescence signal when part of reaction centers are closed and have the photochemical pathway quenched; and F'_m, the maximum fluorescence intensity obtained by saturation of the reaction centers under steady-state conditions. Such parameters allow the calculation of the photosynthetic efficiency ϕ_{II}, which, in steady-state illumination, reflects the relative electron transport rate of PSII and is calculated with the formula

$$\phi_{II} = (F'_m - F_s)/F'_m$$

Further information describing the PAM technique is available in Genty et al. [13], and the application to microphytobenthos samples is described in Kromkamp et al. [14].

A MINI-PAM fluorometer model 0221 (Walz, Germany) was used for the present experiments. An external white-light source (Halogen lamp, 2050-H, GE, Cleveland, OH, USA) provided the actinic illumination, set at a constant intensity of 50 μmol/m^2/s. Six saturating light pulses at intervals of 20 s were applied for the measurement of the photosynthetic efficiency (ϕ_{II}); an average of the three last pulses was used for the calculation. To measure F_0, samples were dark adapted for at least 20 min.

Two replicates per Cu concentration at days 7 and 14 were used to estimate the corresponding effect concentration 50 (EC50) from the percentage of the decrease in quantum efficiency in the biofilms (ϕ_{II}) relative to the control trials. For this purpose, a logistic model (Haanstra et al. [15] calculated with Kaleidagraph® software (Synergy, Reading, PA, USA) was used. The same model was used to calculate EC50s using F_0 and chl *a* as endpoints.

Cu analysis

The concentration of Cu in the water and biofilms was analyzed by atomic absorption spectrometry (AAS) as in Barranguet et al. [7].

Biofilm samples were freeze-dried to avoid Cu loss during the drying. Blanks and Buffalo River Sediment (National Institute of Standards and Technology, Gaithersburg, MD, USA, 2704) were included as references for the destruction process. The samples were placed in a microwave oven using a program for a stepwise acid extraction process [16]; the acid concentrates were diluted with 1.95 ml double-distilled water.

The lowest total Cu concentrations were determined with a graphite furnace atomic absorption spectrometer (Perkin-Elmer 5100 [Oosterhout, The Netherlands] detection limit 3 μg/L) equipped with Zeeman background correction. For the concentrations higher than 80 μg/L Cu, a flame atomic absorption spectrometer (Perkin-Elmer 1100B, detection limit 50 μg/L) was used.

Microalgae identification

Biofilm samples were preserved in formol (three disks per treatments in formol 5%) to study the algal species composition. Microscopic observations were carried at ×40 mag-

nification, using epifluorescence to check the vitality of the algae.

The composition of the epilithic community was assessed by counting at least 300 cells per treatment. To corroborate the identification of diatom species, the frustules were cleaned according to Barber and Haworth [17], and permanent slides were mounted with high-refraction resin.

Statistical analysis

A redundancy analysis (RDA) [18] was performed using the CANOCO™ computer program (Microcomputer Power, Ithaca, NY, USA) to assess the effect of the independent variables Cu (0, 1, 3 and 9 μM), time (initial, week 1, and week 2), and filtration (filtered and unfiltered) on the microalgal species composition. The first step was to determine the length of the gradient within the data set by performing an indirect unimodal detrended correspondence analysis. The gradient was 1.5, implying that the species occurrence increases or decreases within the data set without an optimum. Therefore, a linear model was used on RDA to test the influence of the three factors on the species composition.

RESULTS

Water

The river water was slightly alkaline with an average pH of 7.8. After 3 d in the aquaria (after which the water and Cu were renewed), an increase in pH was measured in all treatments, probably due to microphytobenthos photosynthesis. In the aquaria with the highest Cu concentrations and the lowest photosynthesis rates, pH increased the least (0.2–0.5 units with 9 μM Cu); an increase in pH of more than 1.0 unit occurred in the controls.

High phosphate concentrations were found in the river water used for replacing the water in the aquaria. In the filtered water, total phosphate had a lower concentration than in unfiltered water, appearing mainly as dissolved phosphate (Fig. 1).

After 3 d in the aquaria, phosphate concentrations (dissolved and total) were similar or only slightly lower than the river water values at the high Cu concentrations (3 and 9 μM Cu), while an important decrease of PO_4 concentration was observed in the trials with the lower Cu concentrations (controls and 1 μM). Thus, on day 10 and day 14, PO_4 was almost totally depleted in the control aquaria (Fig. 1).

From the measurements of Cu concentration before and after the water renewals, actual Cu concentrations were calculated on the basis of the averaged exponential decrease estimated from the initial and final measured Cu concentrations at each water replacement (Table 1). Total Cu concentrations were slightly higher in filtered trials than in unfiltered ones. Water recoveries (concentration in the water after the exposure compared to the initial concentration) ranging from 45 to 65% were found for the trials where Cu was added (Table 1); the highest recoveries were observed for the filtered trials, indicating the capacity of the suspended particles <45 μm to sorb part of the Cu in solution.

Biofilms

Cu accumulation. The rates of Cu accumulation in the biofilms increased as the aqueous concentration of Cu increased. During the first week, 60% of the Cu accumulation was completed (Fig. 2).

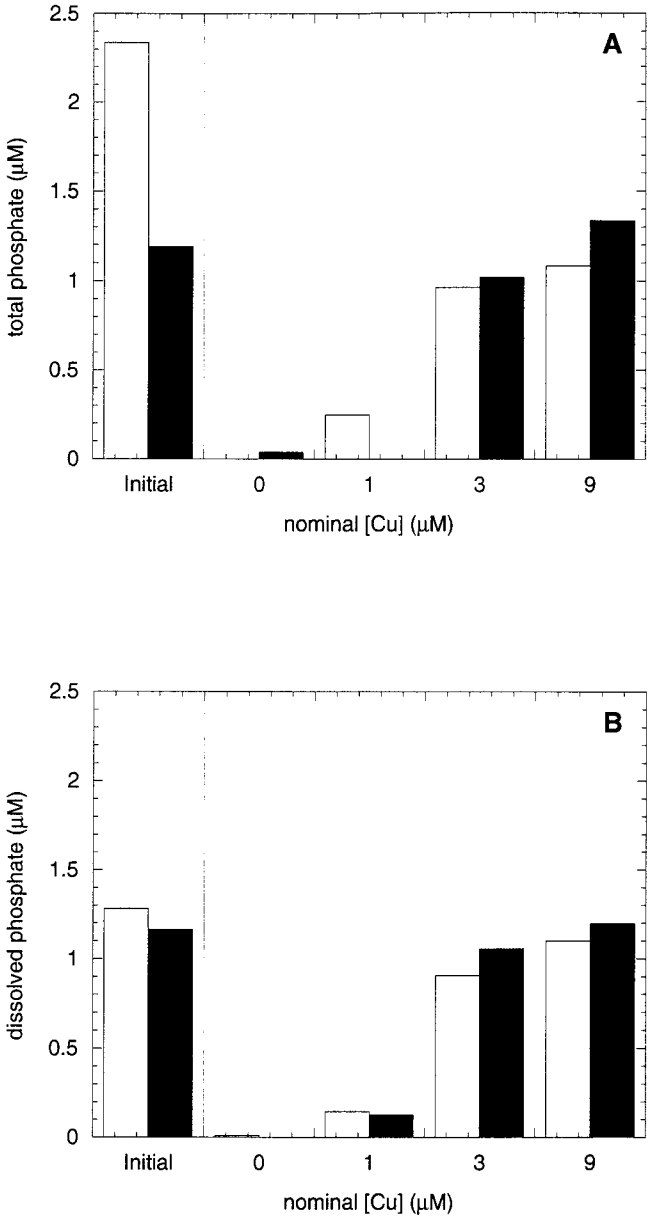


Fig. 1. Phosphate concentrations (μM) in the water enriched with a range of Cu concentrations, at day 10 of the 14-d experiment, before replacing the water in the aquaria. Initial phosphate concentration in the river water is also shown. Open bars: unfiltered water; closed bars: filtered water. (**A**) Total phosphate. (**B**) Dissolved phosphate.

Table 1. Nominal Cu concentrations in filtered (F) and nonfiltered River Meuse (SW, The Netherlands) water compared with the actual water concentration (μM) and water recoveries (%) calculated according to an exponential model for the two-week experiments. Averages and standard deviation

Nominal [Cu] μM	Actual [Cu] μM	Water recoveries (%)
0	0.07 (0.014)	81 (35)
0F	0.09 (0.014)	93 (75)
1	0.66 (0.073)	55 (12)
1F	0.79 (0.088)	66 (20)
3	2.01 (0.155)	45 (12)
3F	2.19 (1.105)	58 (11)
9	5.59 (0.762)	43 (13)
9F	6.70 (1.018)	58 (23)

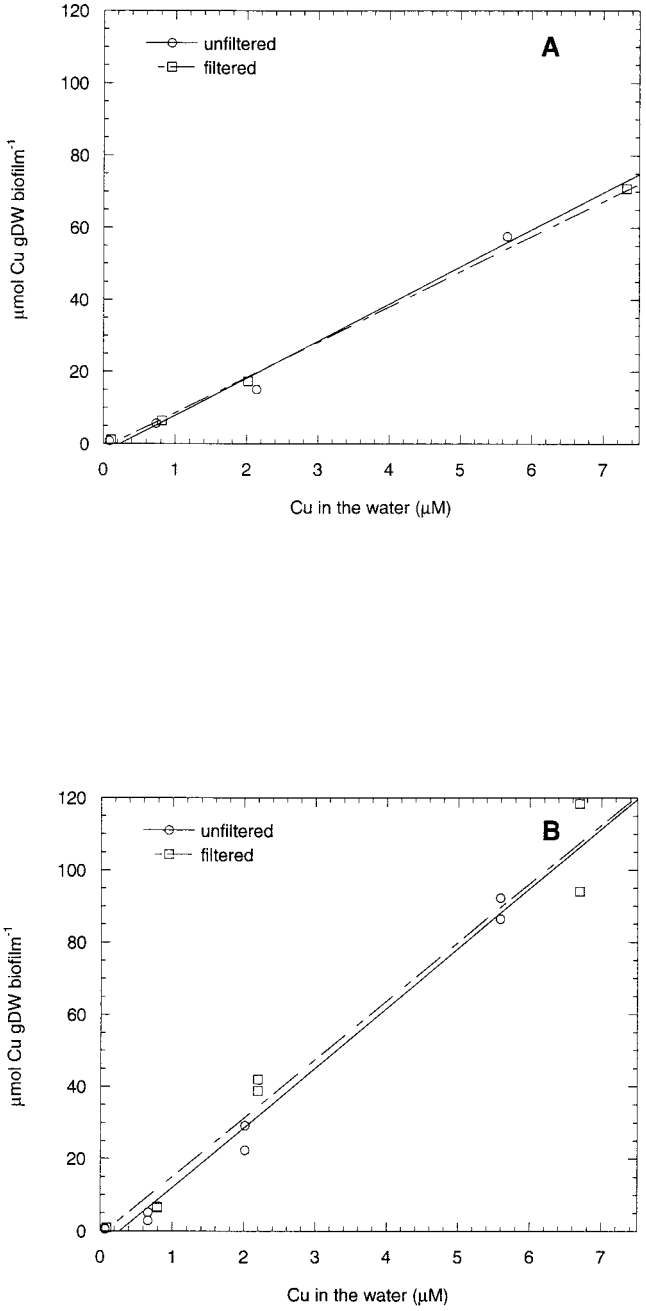


Fig. 2. Copper accumulated in the biofilms per unit dry weight (DW), respective to the Cu concentration in the water (in µM) in filtered and unfiltered trials, after one week of exposure (**A**) and two weeks of exposure (**B**).

The nominal concentration factor in the biofilm was recalculated from µg Cu/g to µg Cu/kg (dimensionless) as

$$[Cu]_{\text{Cu-treated biofilm}}/[Cu]_{\text{water}}$$

After two weeks of exposure, the nominal concentration factor in the biofilms varied between 16.2 and 16.5×10^3.

The Cu uptake by the biofilm relative to actual Cu concentration in the water appeared to be very similar in filtered and unfiltered trials; in both treatments, a similar linear increase of Cu accumulation in the biofilm was observed (Fig. 2).

Algal biomass and pigments. In order to assess the changes in organic matter content of the biofilm during the Cu exposure, the percentage of organic matter in the biofilm was calculated from the AFDW:DW ratio. The initial 14% organic matter content increased to 28% after the two-week experiment in the 0- and 1-µM Cu treatments; in the biofilms exposed to 3 and 9 µM Cu, the levels of organic matter after two weeks remained almost unchanged, between 14 and 17%.

The analysis of photosynthetic pigments reflected the diatom dominance: The chl *c*:chl *a* and fucoxanthin:chl *a* ratios were 0.2 ± 0.03 and 0.46 ± 0.04, respectively, in the controls, typical of diatoms [19].

Small amounts of zeaxanthin and violaxanthin were present throughout the experiment, indicating that cyanobacteria were also present.

For pigments, a clear division was observed between the 0- and 1-µM Cu treatments and the highest concentrations (3 and 9 µM). The *t* tests revealed no difference between pigments in filtered and unfiltered trials; therefore, both data sets were pooled for the calculations. The chl *a* concentration at the beginning of the experiment was 8.1 ± 3.6 µg chl *a*/cm^2; after one week of incubation, controls increased to 11.8 ± 5.1 µg chl *a*/cm^2 and 1 µM to 11.9 ± 2.7 µg chl *a*/cm^2, remaining unchanged during the second week.

In the 3- and 9-µM Cu treatments, microalgal biomass decreased considerably in time. After the first week, the absolute values were 5.6 ± 0.55 µg chl *a*/cm^2 in the 3-µM Cu treatment and 3.0 ± 0.38 µg chl *a*/cm^2 in the 9-µM Cu treatment. The second week showed a further decrease in chl *a* concentrations with average values of 2.7 ± 0.82 µg chl *a*/cm^2 in the 3-µM Cu treatment and 0.75 ± 0.36 µg chl *a*/cm^2 in the 9-µM Cu treatment.

Most pigments degraded with the exposure to Cu following an exponential decrease with time, which allowed for the calculation of degradation rates for both 3- and 9-µM on natural logarithm (LN) transformed data (Table 2). Violaxanthin and zeaxanthin, present in very small concentrations, showed irregular patterns during the two-week experiment, so that a rate of degradation could not be calculated. This is probably due to the small representation and irregular presence of cyanobacteria in the samples. It appeared that chl *a*, fucoxanthin,

Table 2. Plant pigment degradation rates log transformed [LN(mg pigment/cm^{-2})/d] for biofilms exposed to 3 and 9 mM Cu, with their correlation coefficient *R* and level of significance. ** $p < 0.01$; * $p < 0.05$; NS = nonsignificant

		Chl *a*	Chl *a* + allomeres	Chl *c*	Fucoxanthin	Diadinoxanthin	Violaxanthin	Zeaxanthin
3 µM Cu	*R*	0.799**	0.773**	0.800**	0.764**	0.770**	0.131 NS	0.326 NS
	Degradation rate	0.109 (0.017)	0.099 (0.017)	0.119 (0.019)	0.100 (0.0179)	0.112 (0.020)	0.132 (0.212)	0.302 (0.1867)
9 µM Cu	*R*	0.931**	0.911**	0.937**	0.912**	0.870**	0.259 NS	0.448*
	Degradation rate	0.192 (0.015)	0.150 (0.014)	0.233 (0.018)	0.154 (0.014)	0.153 (0.018)	0.354 (0.269)	0.655 (0.2671)

and diadinoxanthin had comparable degradation rates, while chl *c* was more sensitive to Cu contamination.

The chl *a* allomers were produced during the experiment. The chl *a*+allomers:chl *a* ratio increased from 1.1 in controls to 2.5, after exposure to 9 μM Cu, indicating that the degradation rate of the sum of chl *a* plus its allomers was lower than that of chl *a* alone.

The only colored chlorophyll degradation product present throughout the experiments was pheophytin *a*. The pheophytin:chl *a* ratio increased the most at the highest Cu concentration, increasing from 0.09 in controls to 0.4 to 0.9. In terms of concentration per unit surface area, pheophytin *a* was the most abundant at 1 μM Cu concentration, indicating that Cu had an effect on the algal biomass at such low concentration.

The F_0 (proportional to chl *a*) in filtered trials was slightly lower than in unfiltered ones but not significantly different (*t* tests), so the data were pooled for the calculations. Comparing the F_0 with chl *a* (HPLC) on day 14, after transformation of data to LN, both parameters presented the same slope of degradation with increasing Cu concentration (LN chl *a* vs [Cu] slope = −0.317; r^2 = 0.877; LN F_0 vs [Cu] slope = −0.315; r^2 = 0.899). When F_0 was compared with chl *a* plus its allomers (measured as chl *a* in traditional spectrophotometry), the agreement was slightly lower (LN chl *a*+allomers slope = −0.265; r^2 = 0.836).

Because F_0 and chl *a* were so well correlated, the EC50 values by both methods were also in good agreement. Both methods showed a strong effect of Cu at the chosen concentrations after two weeks of exposure, even at the lowest Cu concentrations (Fig. 3).

Microalgal composition. Algal density was high in the biofilms at the beginning of the experiment. The community was dominated by diatoms; the filament-forming diatom *Melosira varians* was the dominant species, representing 50% of the total taxa, followed by the genus *Navicula*, mainly *Navicula lanceolata* (Fig. 4).

After one week of exposure to Cu, the percentage of *M. varians* remained constant or even increased in all treatments (average 57%, standard deviation [SD]: 8.6). In the 0- and 1-μM Cu treatments, the biofilms conserved their filamentous colonies, while the appearance of the biofilms exposed to 3 and 9 μM Cu drastically changed. Despite being mainly composed by *M. varians*, the biofilms hardly protruded from the glass disks, having lost the long hairlike filaments observed at low Cu concentrations.

After two weeks, the difference between biofilms exposed to high and low Cu concentrations (incrusting and filamentous, respectively) became even more obvious. Concerning the species composition, no pattern was easily derived from filtered and unfiltered trials. *Achnantes minutissima*, an opportunistic diatom species considered metal tolerant, did not show any particular increase in the biofilms exposed to Cu.

The representation of *M. varians* decreased slightly in all treatments (except the unfiltered control trials) with varied percentages; at 9 μM Cu, the average amount was as high as 31 ± 17% (Fig. 4). However, some diatoms, like *Surirella* sp. and small *Fragillaria* species, were more abundant after two weeks than at the beginning of the experiments at the intermediate Cu concentrations (1 and 3 μM Cu).

The results of the RDA (direct linear analyses) showed a significant effect of time on the microalgal community caused by week 1. The factors Cu and filtration did not explain the variability of the species composition (Table 3). The significance of week 1 was probably due to the transfer from the river to the aquaria. After extracting the effect of time on the analysis (using weeks 1 and 2 as covariables, Cu and filtration as variables), the effect of Cu and filtration on the algal species composition was yet not significant (p >5 %, Table 3).

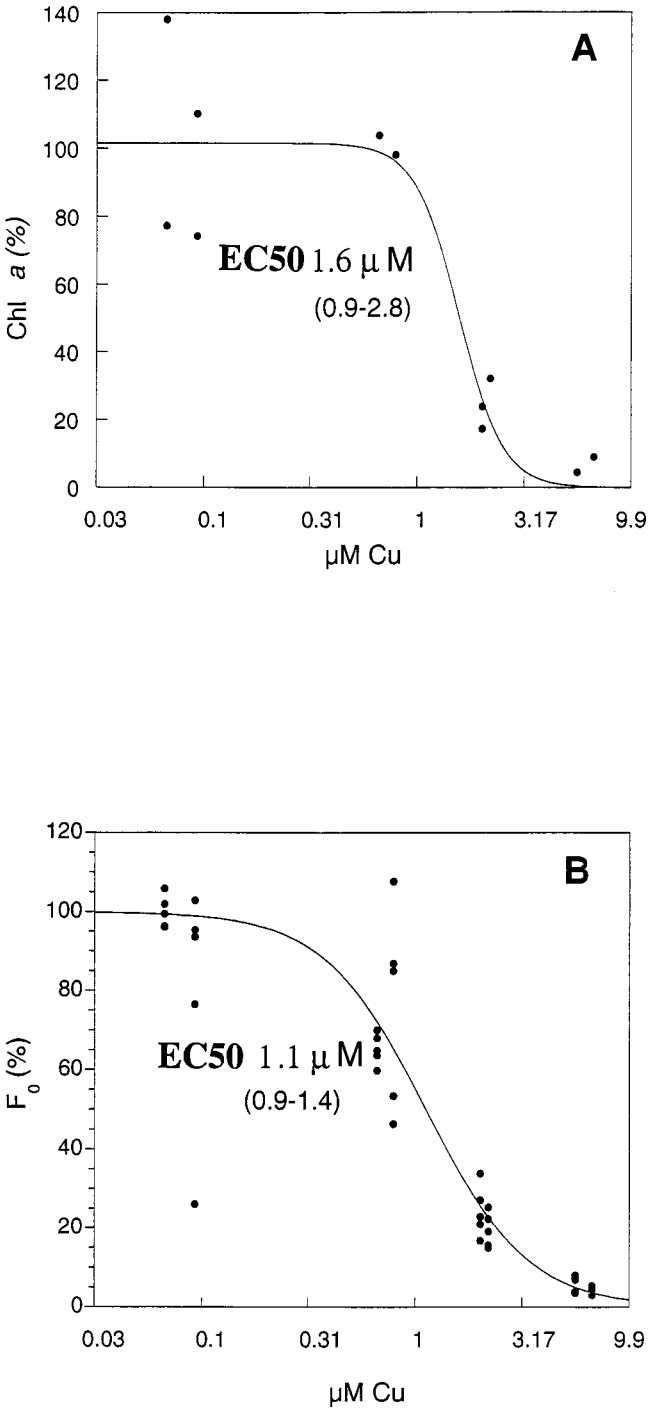


Fig. 3. Dose–response curves of biofilms from the River Meuse exposed to a range of Cu concentrations for two weeks; algal biomass as a percentage relative to controls (100%) was used as endpoint. Effect concentration 50 (EC50) values determined by the logistic model are given, together with the 95% confidence limits (between brackets). (**A**) Biomass measured as chl *a*. (**B**) Biomass measured as dark-adapted fluorescence (F_0).

Photosynthetic efficiency (ϕ_{II}). The effect of Cu on the ϕ_{II} was much less pronounced than on the biomass. The decrease

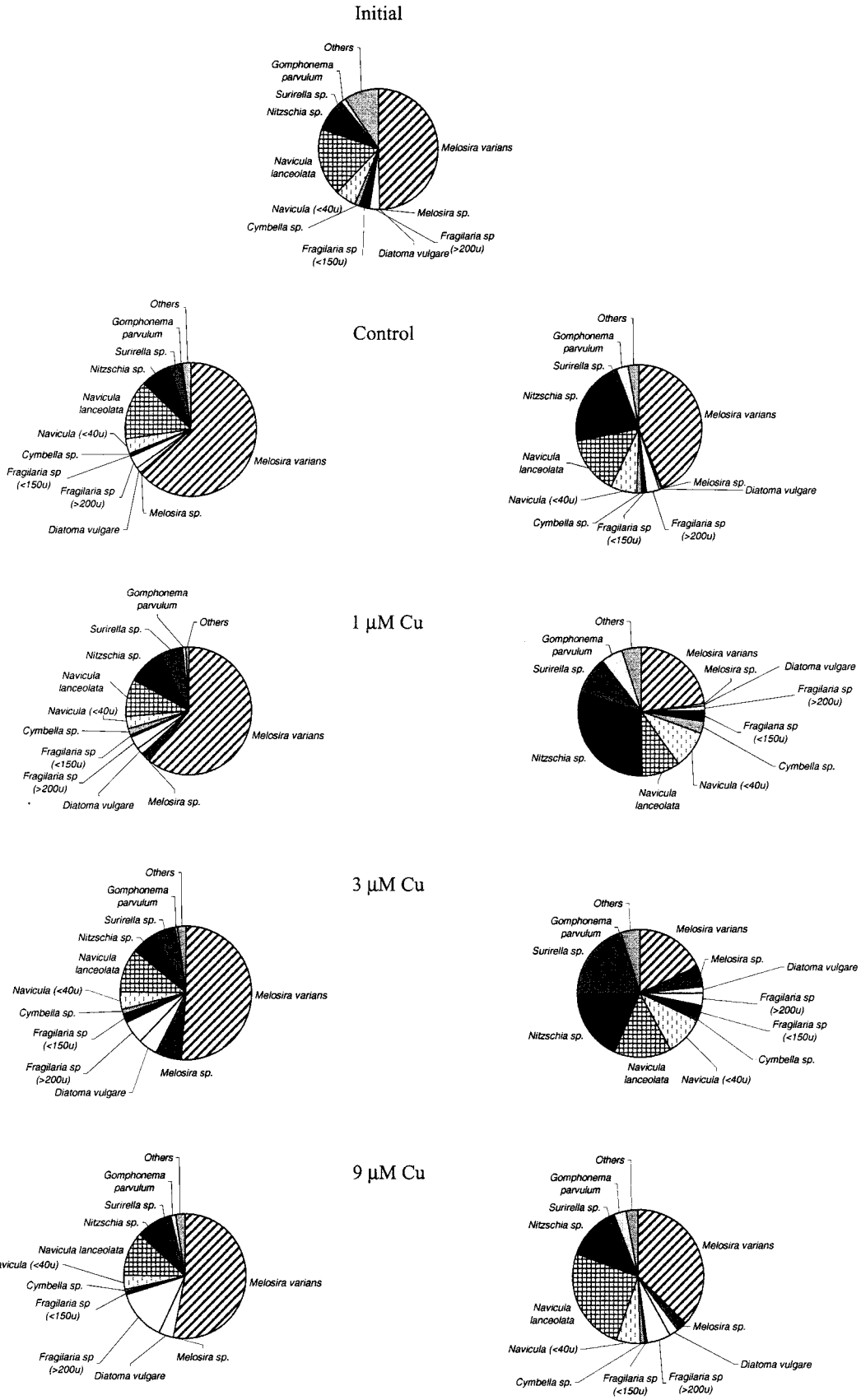


Fig. 4. Microalgal species composition (%) in the biofilms of the River Meuse at the beginning of the experiment, one week and two weeks of exposure to a range of Cu concentrations. Species consistently representing less than 2% of the total were grouped as others. Data from filtered and unfiltered treatments were pooled together because they were not significantly different. The graphs on the left show the composition after one week of Cu exposure, the ones on the right after two weeks of exposure.

Table 3. Output of the redundancy analyses performed with the microalgal species composing the biofilms from the River Meuse. Prior to the analysis, the length of the gradient within the data set was determined, according to this length (1.5 detrended correspondence analysis [DCA]): A direct linear model redundancy analysis (RDA) was applied. The RDA was performed first using time (initial, week 1, week 2), Cu (0-, 1-, 3-, and 9-μM treatment) and filtration (filtered and nonfiltered) as variables. Only week 1 was significant; therefore, a second analysis extracting the effect of time was performed, where Cu and filtration were the variables included and week 1 and 2 were used as covariables. Neither Cu nor filtration was significant.

	Ordination						Testing variables			
	Length of gradient	Total inertia	Total inertia after covariance	Canonical inertia	Eigenvalue, axis 1	Eigenvalue, axis 2	Covariable	Variable	*p*	Lambda-1[a]
Indirect unimodal, DCA	1.5	0.483	—	—	0.198	0.057				
Direct linear, RDA	—	1	—	0.498	0.431	0.05	None	Week 1	0.005	0.37
								Week 2	0.595	0.34
								Cu	0.35	0.04
								Filter	0.225	0.04
	—	1	—	0.419	0.366	0.043	None	Week 2	0.005	0.34
								Cu	0.2	0.04
								Filter	0.745	0.04
		1	—	0.087	0.053	0.034	None	Cu	0.505	0.04
								Filter	0.51	0.04
		1	0.591	0.088	0.058	0.03	Week 1	Cu	0.34	0.05
							Week 2	Filter	0.415	0.04

[a] Explained variance of variable, when considered as the only variable.

of ϕ_{II} could be better fitted to a linear model rather than to an exponential model, with a decrease rate of 0.036 fluorescence units/d in the 9-μM treatments (Fig. 5).

At the beginning of the experiment, the microphytobenthic communities were highly productive; ϕ_{II} values were between 0.6 and 0.7. A decrease of the photosynthetic efficiency during the last measurements also was occasionally detected at 0- and 1-μM Cu concentrations (Fig. 5). Such decreases in activity were possibly due to nutrient limitation at 0 and 1 μM Cu (Fig. 1). The frequency of the water renewals, mainly during the second week, when biomass considerably increased, was not high enough to compensate for the nutrient depletion caused by the fast growth of the microalgae.

In 3- but mainly in 9-μM Cu treatments, the effect of Cu on the photon yield was significant (Fig. 5).

The particles in suspension, as shown in Table 1, lowered the availability of Cu to the biofilm. As the accumulation of Cu by the biofilm was directly proportional to the Cu present in the water, the samples of filtered and unfiltered trials were pooled together for the calculations of the EC50s, using the actual Cu concentration of each aquaria.

During the first week, the calculated EC50 was 18.3 μM Cu; however, a reduction of the activity by 50% was not achieved, resulting in very broad confidence limits (CL = 6.7–50.1). After the second week of exposure, the EC50 decreased to 5.3 μM Cu (CL = 4.6–6.1), indicating the cumulative effect of Cu on the photosynthetic activity (Fig. 6).

DISCUSSION

Role of the river water composition

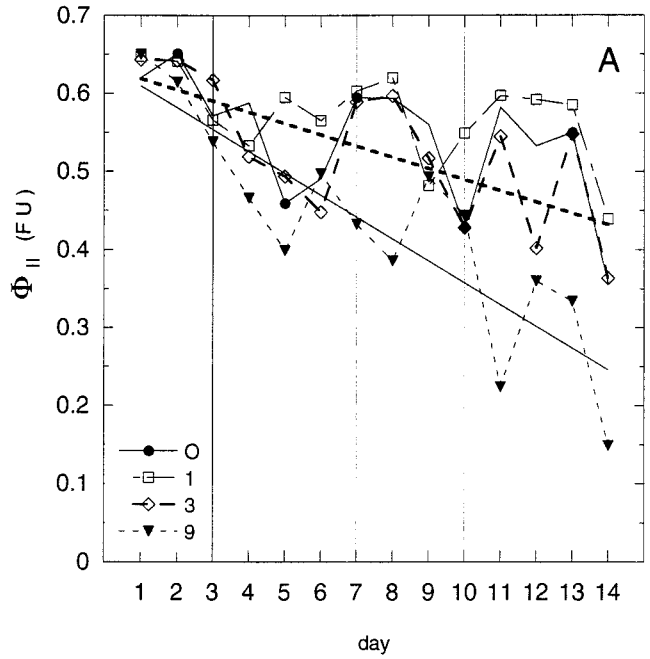
Levels of sensitivity to toxicants in periphyton have been related to the species composition of the community [20] but also to abiotic environmental factors, such as light, nutrients [1], and suspended organic matter [21].

Particles suspended in the water, mainly those rich in organic matter, are among the abiotic factors that regulate Cu availability to organisms because they sorb an important part of the metal ions present in the water [22]. Indeed, amelioration of Cu toxicity to bacteria by natural particulate organic matter has been reported [23]. Suspended particles highly loaded in organic matter lowered acute Cu toxicity to autotrophic biofilms from a branch of the River Rhine [8].

In the case of the River Meuse, short-term toxicity tests demonstrated that although suspended particles slightly reduced the concentration of Cu in suspension, they did not greatly modify the acute effect of Cu to microalgae [7]. The same can be concluded from the present data set. After a chronic exposure, particles lowered the Cu concentration in the water only to a very limited extent. The particles in the Meuse have a low organic content (5–6% DW), and possibly the metal-binding sites are already occupied through the diffuse pollution of the water by metals (e.g., Cu content, 1.5 mM/kg; Zn, 13.9 mM/kg).

However, the Meuse waters may present features for low Cu toxicity, having a high dissolved phosphate content and high pH values, both factors found in combination to decrease metal availability [24,25]. Phosphate concentrations decreased considerably in the controls and at 1 μM Cu, where photosynthetic activity was not affected by Cu. These aquaria also showed the highest pH values; PO_4 uptake rates are reported to increase in freshwater algae with pH to a maximum at 8.5 [26].

In the biofilms exposed to 3 and 9 μM Cu, the phosphate



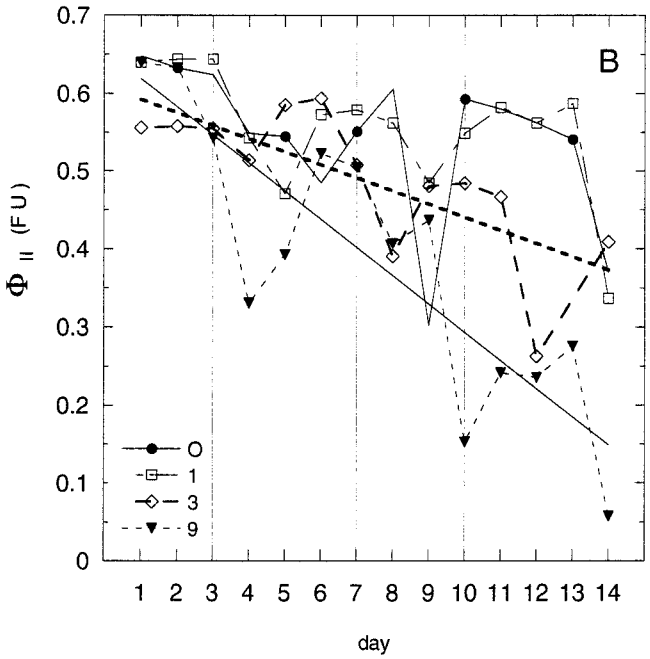
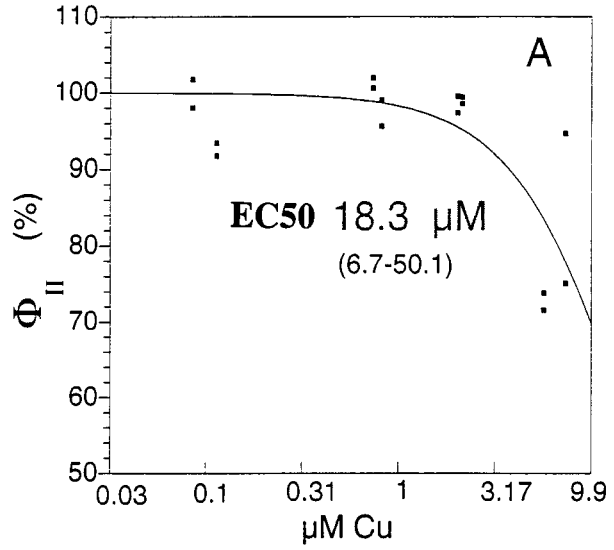


Fig. 5. Changes in the photon yield (ϕ_{II}) of biofilms from the River Meuse, measured daily, at a range of Cu concentrations (0, 1, 3, and 9 μM Cu). The vertical lines mark the days when the water of the aquaria was replaced by fresh river water with a new Cu addition. (**A**) Unfiltered trials. The higher concentrations were fitted to a linear model (dotted line for 3 μM: $y = 0.63 - 0.01x$, $R = 0.67$; solid line for 9 μM: $y = 0.64 - 0.03x$, $R = 0.86$). (**B**) Filtered trials. The higher concentrations were fitted to a linear model (3 μM: dotted line: $y = 0.61 - 0.02x$, $R = 0.74$; 9 μM: solid line: $y = 0.66 - 0.04x$, $R = 0.86$). FU = fluorescence units.



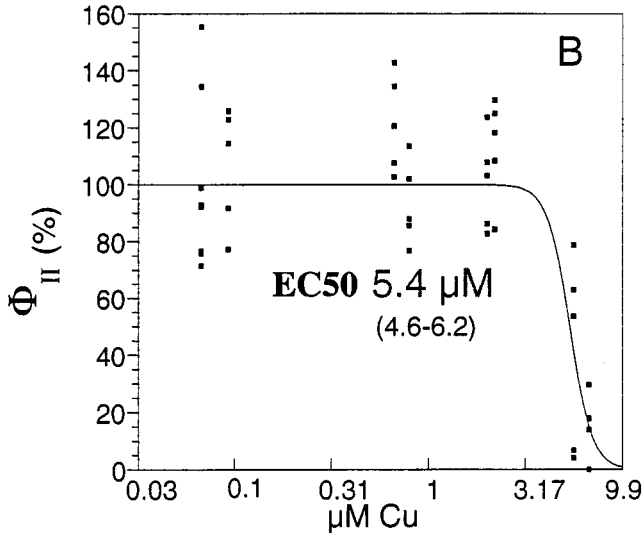


Fig. 6. Dose–response curves of biofilms from the River Meuse exposed to a range of Cu concentrations; photon yield (ϕ_{II}) as a percentage relative to controls (100%) was used as endpoint. Effect concentration 50 (EC50) values determined by the logistic model are given, together with the 95% confidence limits (between brackets). (**A**) One-week exposure. (**B**) Two-week exposure.

concentrations remained high, indicating no additional stress by nutrient starvation that could have increased Cu toxicity. Limiting PO_4 concentrations can increase cellular uptake and toxicity of Cu to microalgae [27]. The uptake of nutrients appeared to have been slowed by the lower rates of photosynthetic activity of the biofilms affected by Cu; in addition to the lowered production rates, Cu interferes with phosphate uptake at pH >5 [26].

It can be concluded that despite that suspended particles did not play a decisive role reducing metal availability in these experiments, the River Meuse water presents physicochemical conditions that do not promote Cu toxicity to biofilms because of the combination of high pH and high nutrient concentration [24,25].

Response of algal consortia in biofilms

Copper is a very effective algaecide [27], considered to be very specific because its action blocks electron transport in PSII [28]. Nevertheless, our results showed that when acting on natural biofilms, Cu seemed to have a slow, progressive but indiscriminate action. We will discuss the changes undergone by the Meuse biofilms in metal accumulation, biomass, biofilm shape, species composition, and activity and develop a working hypothesis covering the wide range of changes observed in biofilm structure and functioning.

We observed that the toxic effect of Cu on biofilms progressed slowly by indiscriminately damaging the algae at or protruding from the biofilm surface. The continuation of the toxic effect in time caused the death of the algae in protruding filaments, the decay of pigments, and biomass. Simultaneously, photosynthetic activity and even some degree of growth of compact nonfilamentous forms are likely to continue in deeper biofilm layers.

We propose that the ratio of toxicant concentration (Cu concentration in the overlying water) to the biofilms' growth potential (the nutrient-stimulated biomass accumulation) determines the long-term effect. At high toxicant concentrations, the toxic action progressing with time erases the biofilm consortium. At relatively low toxicant concentration, we observed that the biofilm can outgrow the downwelling metal stress. Total erasure took place at concentrations of Cu >3 μM, only fractionally higher than those allowing almost uninterrupted development (at 1 μM Cu).

The mode of action described in our working hypothesis explains why the pollution-induced community tolerance (PICT) concept is difficult to apply to metal toxicity to benthic microalgae [29]. The PICT implies an increase in tolerance under exposure to toxicants provoked by a shift of the population by an increasing abundance of tolerant species. However, in biofilms under metal exposure, both metal-sensitive and metal-tolerant diatom species have been observed to decrease in number in equal proportion [30]. It should be stated, however, that the temperature at which our experiments were carried out (11°C) did not favor the shift from diatoms to cyanobacteria, which are more resistant to Cu [7] but need higher temperatures to proliferate [31].

The Meuse biofilms adsorbed Cu (per dry-wt unit) continuously and strictly proportionally to the concentration of metal added, independently of widely diverging conditions of pH, photosynthetic activity, and biomass.

Vymazal [32] indicated that most of the metal accumulation by periphyton took place during the first hours of the exposure, which is not the case in our experiments. After repetitive additions of Cu at high concentrations, Cu accumulation proceeded at an almost constant rate during the two-week exposure period, even when the amount of organic matter and biomass in the biofilms did not increase.

The mechanisms by which biofilms can accumulate metals in such high concentrations are not fully understood. Gray and Hill [33] found that Ni sorption by periphyton was directly dependent on photosynthetic activity, which may explain the continued sorption of Cu by our biofilms. High pH [34] and the precipitation with ferric ions [16] have been suggested, as has the abundance of metal-binding sites in the biofilm, [35] as factors responsible for the metal accumulation. Moreover, diatoms are capable of increasing their polysaccharide production when exposed to Cu, reducing intracellular binding [36]. Several such mechanisms can be used simultaneously by biofilms to immobilize the excess of Cu in the overlying water.

The first observed sign of damage to the biofilm after the Cu exposure was the degradation of photosynthetic pigments and the production of pheophytin *a* and chl *a* allomers, that is, a structural damage to the photosynthetic apparatus. Such production was already noticeable at the lowest Cu concentration (1 μM) and lends support to our assumption of the different fates of superficial and deeper layers of the biofilm. Our results also showed that chl *c* was even more sensitive to Cu than chl *a*, so caution should be taken when using chl *a* alone in the evaluation of the structural damage to chl *c*–containing algae (like diatoms) in pollution studies. Only quantifying chl *a* degradation will underestimate the onset of Cu stress.

The exponential decrease of pigments was not reflected either in a change of microalgal species or in the disappearance of metal sensitive ones but in the change of the biofilm physiognomy. Nevertheless, the modification of the biofilm morphology was noticeable after the first week already, when the abundant filaments of *M. varians* had been replaced by a more incrusting and thinner growth form. Such biofilm structure could act as a barrier to Cu in the biofilm matrix, reducing the exposure surface. McFarland et al. [37] reported frustule anomalies in benthic diatoms exposed to metals, but different growth form on the substrate was never reported earlier.

The redundancy analysis showed that only week 1 was a significant factor affecting the microalgal species composition of the biofilms, probably because of the confinement of the biofilms in the aquaria under conditions more stable than in the river and in the absence of grazers [38]. Copper was not a significant factor determining species composition, confirming that the effect of Cu on the biofilms was more pronounced on the biofilm morphology (absence of filament forming) than on the species composition.

Throughout our experiments, the diatom *M. varians* dominated the periphytic community, even at the highest Cu concentrations. This species is known for being metal sensitive and was reported to disappear after only 24 h of exposure to Cu [39]. Thus, a two-week exposure period would be long enough to show any changes in this species abundance in the Meuse biofilms. On the other hand, the absence of a recolonization in Cu-polluted trials by any species from the metal-tolerant genus *Achnantes* could be due to the short duration of our experiments. Soldo and Behra [40] observed the predominance of this genus only after four weeks of Cu contamination. Microphytobenthic species seem to have different lag times until their success or disappearance in a metal-polluted environment.

All the complex changes induced by the Cu exposure indicate that to assess metal effects on biofilms, it is logical to integrate the changes undergone by the autotrophic biomass of biofilms, including changes in growth forms and species shifts, with their respective time spans. Medley and Clements [39] put forward the need to take into account the microalgal growth form when assessing metal toxicity. This is also clear from our results, which show that a specific growth form may actively change under Cu stress conditions, probably as a form of defense.

Although Cu has a specific toxic effect inhibiting photosynthesis, our EC50 values showed that the photosynthetic activity was less affected by Cu than chl *a*. The reason could be that after the initial decrease of biomass and modification

of the biofilm morphology, the remaining cells, though less numerous, could still perform photosynthesis to a certain extent. In this way, chl *a* values would be more affected than photon yield, which is independent of the algal biomass. Effects of Cu on biofilm biomass prior to measurable effects on photosynthesis were described earlier by Soldo and Behra [40] at concentrations higher than 5 μM Cu.

The effect of Cu on the photosynthesis of the Meuse biofilms manifested as a linear decrease of ϕ_{II} in time, showing a steady progress of the effect of Cu on photosynthesis. This could be explained if the protective feature of the biofilms matrix lost efficiency against metal contamination during the long-term exposure. The loss of a part of the biofilm biomass would also imply a loss of binding sites to immobilize Cu. This would make the biofilm more and more sensitive to Cu, as can be seen by the decrease of apparent EC50 values with exposure time from 18 to 5 μM Cu from the first- to the second-week exposure.

A progressive decrease of photosynthetic efficiency with time, implying a decrease of the biofilm capacity to lower Cu toxicity, can also be seen when comparing the present results with an acute (7-h) experiment with natural biofilms from the Meuse with the same microalgal composition [7]: Concentrations as high as 30 μM Cu decreased photosynthetic activity only by 20%. We then concluded that structure and density rather than specific composition contributed to Cu resilience in biofilms. However, in the present work, we see how the biofilm structural change and Cu penetration into the biofilm are time dependent, resulting in a resilience of the consortium decreasing with time.

Our results lead to the conclusion that the River Meuse biofilms acted, independently of their species composition, as a dynamic unit under Cu exposure with effective feedback mechanisms. Changes in physiognomy and biomass degradation occurred as a result of the attack of the biofilm superficial layer of cells, followed eventually by a decrease in photosynthesis. The doses of Cu needed to operate such changes were very close to those to which the biofilm could successfully survive with minor changes, depending not only on the toxicant concentration but also on the exposure time.

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