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Short-term response of monospecific and natural algal biofilms to copper exposure

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The effect of copper additions (Cu ranging from 0 to 30 μM) on the photosynthesis of three different microalgal biofilms was studied to identify the factors that cause sensitivity differences between benthic and pelagic algae. The response of biofilms which colonized artificial substrata in the River Meuse was compared with those of two laboratory-grown monospecific biofilms, one consisting of the diatom *Synedra ulna*, and the other composed of a filament-forming cyanobacterium, *Oscillatoria* sp. The photosynthetic yield Φ_{II} (quantum efficiency of photosystem II) was studied with PAM (Pulse Amplitude Modulated) fluorimetry. *S. ulna* biofilms appeared to be the most sensitive to Cu, followed by the cyanobacteria, while natural biofilms, dominated by supposedly very sensitive diatom species such as *Melosira varians* and *Diatoma vulgare*, were the most resistant to Cu. In the highly productive biofilms, pH is suggested to play a role in lowering toxicity by helping the precipitation of cupric ions. Cu accumulation by the biofilms during the exposure period followed a linear relationship with Cu concentration, saturation not being observed; natural biofilms had an accumulation factor of $1–2{\cdot}5 \times 10^3$ relative to the concentrations in the water, while the diatoms growing unattached to the substratum had a higher concentration factor, up to $4{\cdot}9 \times 10^3$. It was concluded that the physical structure of the biofilm (package of cells and thickness), and not the species composition, was the main factor regulating the sensitivity of the biofilm to Cu toxicity during short-term exposures.

Key words: biofilms, Cu, cyanobacteria, diatoms, River Meuse

Introduction

Autotrophic biofilms (or periphyton) colonize every wet sediment or solid surface where light is sufficient to ensure photosynthesis and are responsible for an important accumulation of biomass. The algae involved create key sites for intense metabolic activity, where resulting gradients of pH, oxygen, nutrients and CO_2 become extreme (Revsbech & Jørgensen, 1986; Liehr *et al*., 1994; Woodruff *et al*., 1999).

Algal biofilms are responsible for high oxygen production rates, ensuring oxic conditions at the water bottom interface, and are an important food source for grazing consumers. In shallow freshwater systems they can be sites of accumulation of organic matter and metals; hence biofilms have been proposed as model systems for the study of pollution and indicators of the health of riverine systems (Newman & McIntosh, 1989; Reavie & Smol, 1998) and their use as tools of ecotoxicity tests has become increasingly frequent (Prygiel *et al*., 1999).

Because they are attached, biofilms integrate the ecological variation of the ecosystem over time, and can record changes in water quality (Farag *et al*., 1998). However, biofilms are also quite dynamic structures, subject to sloughing, succession of species due to the establishment of vertical gradients of light and nutrients (Steinman, 1992), seasonal shifts in composition (Stevenson, 1997 and references therein) and eventual shifts of algal species due to changes in water quality (Ivorra *et al*., 1999), which imply a change in the community tolerance of pollutants.

Rivers are influenced by human activities, regularly receiving organic substances, heavy metals and pesticides (among others) from industrial and agricultural activities. The quality of European river waters such as the Meuse decreased alarmingly with industrial development until the 1970s, when measures were taken to restore the rivers and their biodiversity to more pristine conditions. In the Meuse, biodiversity was partly restored, but the benthic communities still indicate polluted conditions. The levels of copper (Cu) and zinc still exceed the current safety standards set provisionally for toxic metals (Stuijfzand, 1999). These standards were selected empirically, not for the field situation. Furthermore these 'safe' levels can affect natural bacterioplankton in the Meuse when added in short-term experiments (Tubbing *et al*., 1993*b*). Tubbing *et al*. (1995) showed that very low concentrations of Cu could trigger a shift in the planktonic population (phyto- and zooplankton) but the effects of these sublethal concentrations of Cu on periphyton communities are not known.

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In this study, we aimed to assess the effects of short-term acute Cu pollution on natural biofilms from the River Meuse for comparison with the more frequently used plankton toxicity tests, and with laboratory-grown monospecific biofilms composed of a diatom (*Synedra ulna*) and a cyanobacterium (*Oscillatoria* sp.). Both species were isolated from the natural epilithic population in the Meuse and are known to have different sensitivity to metals. The different responses of the three types of biofilm to Cu and the structural importance of biofilms in the regulation of metal accumulation and tolerance are discussed.

Materials and methods

Sampling site

The Meuse is a European river running from France through Belgium into the Netherlands, and its morphology has been greatly modified for navigation. It is a highly eutrophic river fed by rainwater and influenced by urban and agricultural activities (Admiraal *et al*., 1993). The river was highly polluted with metals until the initiation of the rehabilitation programme; at present, metal levels have dropped considerably (Baggelaar & Baggelaar, 1995). Total dissolved Cu concentrations have been quite low in recent years, ranging from 0·05 to 0·08 μM (Stuijfzand, 1999). In the Dutch part, dissolved Cu and Cu content in the sediments (136 mg kg dry sediment^{-1} in 1998) is higher than in other countries. Nutrient levels are still high and may bind metal ions and hence influence the metal bioavailability to organisms (Tubbing *et al*., 1995).

The sampling site was at Keizersveer (51° 43′ N, 4° 54′ E, NL), close to a major intake point of a drinking water reservoir, 855 km from the river source. Samples were taken in December 1997 and March and April 1998. Physical and chemical characteristics of the waters during this period are shown in Table 1.

Biofilms

Etched glass discs (1·5 cm^2) were used as artificial substrata for biofilm colonization. Polyethylene racks served as a support (as in Ivorra *et al*., 1999); the racks were placed vertically in the river parallel to the current flow and left for periphytic algae to colonize. Biofilm samples were collected after a 3 week colonization period for short-term experiments with intact natural biofilms, and for the isolation of microalgal clones. The colonized glass discs were carried to the laboratory in a coolbox filled with river water. At each time of sampling, water was collected in acid-rinsed bottles for the experiments.

Microscopic observations (Olympus BH microscope, ×40 magnification) to assess the composition of the epilithic community were carried out on fixed samples, and 300 cells were counted per treatment. For the identification of diatom species, the frustules were cleaned according to Barber & Haworth (1981) and permanent slides were mounted with high-refraction resin (Naphrax).

Short-term experiments were performed with the natural and monospecific biofilms. Natural biofilm samples were exposed for 7 and 24 h to four different total Cu concentrations, in filtered (0·45 μm) and non-filtered river water. Cu was added to the river water to concentrations of 0, 7, 15 and 30 μM Cu (0, 445, 953 and 1905 ppb respectively). Water samples were taken at 7 and 24 h for determination of Cu concentration during and after exposure of the biofilms, as described below. The photosynthetic parameters F_s and F'_m (see below) were measured at 2, 5, 7 and 24 h under continuous light (70 μmol m^{-2} s^{-1}) representative of the field irradiance conditions. At the end of the experiment, pH was quite similar in all vials (8·2–8·4).

Monocultures

Some colonized discs were collected and brought to the laboratory for the isolation of single algal cells. The glass

Table 1. Physical and chemical parameters measured at Keizersveer during the sampling period (1997/8) and average metal concentrations measured in the water by the RIZA (Institute for Inland Water Management and Waste Water Treatment) during 1998

Parameter	December 1997	March 1998	April 1998	Average 1998
T (°C)	7·3	9·6 (1·3)	11·7 (1·2)	
pH	7·6	7·7	7·8	
Extinction coefficient (m^{-1})	1·69	2·22 (1·31)	1·76 (0·22)	
Conductivity (μS cm^{-1})	624	436	452	
Dissolved oxygen (mg l^{-1})	13·0	11·8	12·1	
Current velocity (m s^{-1})	0·9	–	0·3	
Cu in water (μM)	0·072	0·066	0·060	0·107
Cd (μM)				0·003
Cr (μM)				0·090
Hg (μM)				0·000
Ni (μM)				0·078
Pb (μM)				0·033
Zn (μM)				0·810

Values are the average (SD).

discs were sonicated in an ultrasonic water bath (Branson 2210, frequency: 47 kHz) for several seconds to suspend the biofilm. The cell suspension obtained was examined with a binocular microscope. Single cells were separated with a capillary tube and placed on agar plates. A portion of agar with the cell was placed in a sterilized tube containing sterile WC medium (Nichols, 1973) and cultured until a small pellet appeared. For growing the cyanobacterial cultures O2 medium was used, which is suitable for cyanobacteria (van Liere & Mur, 1978).

Small portions of the aggregate were distributed among two or three sterilized 100 ml Erlenmeyer flasks, containing sterilized medium and four etched glass discs, to provide the same substratum as for the natural multispecies biofilms. The Erlenmeyer flasks were placed in a cultivation room on a rotary plate and allowed to grow for 2–3 weeks; the medium was renewed regularly.

Glass discs colonized by monospecific cultures of either diatoms or cyanobacteria, were exposed for 6 h to five different concentrations of Cu, approximating to 0, 7, 12, 18, 22 μM (0, 445, 762, 1143 and 1397 ppb respectively). Twenty vials, four replicates for each concentration, were filled with 22 ml of medium without iron and EDTA (Ivorra *et al*., 1995) and the colonized discs were placed in the vial with the added Cu concentration. In the experiment with *S. ulna* the biofilm obtained on the discs was thin and non-cohesive, with a large amount of cells growing loose. Two of the four replicates for each concentration included colonized discs and the other two only a concentrated suspension of 300 μl of algae. The vials were placed in an incubation room under the same conditions as for their growth, at 20 °C and 70 μmol m^{-2} s^{-1} photon irradiance (measured with a LICOR LI-185B), and gently agitated on a rotary table throughout the experiment. At the end of the experiment, pH was measured in every vial and found to be quite stable (pH = 7·3–7·2).

Two replicate discs per concentration were kept deep frozen (−80 °C), one for pigment measurements and a second one for ash-free dry weight (AFDW) and for atomic absorption spectrometry (AAS) measures of Cu concentration in the biofilm (see below). Pigments were extracted from freeze-dried samples with 95% methanol and 5% ammonium acetate and analysed by high-performance liquid chromatography (HPLC) according to Barranguet *et al*. (1997).

Measurements using the variable fluorescence technique

In vivo chlorophyll fluorescence was measured with the PAM (Pulse Amplitude Modulated) technique. Changes in the fluorescence yield can be used to determine changes in photochemical efficiency and to evaluate the physiological status of the biofilm, specially with toxicants such as Cu, which affect photosystem II (PSII) directly (Barranguet *et al*., 1998; Snel *et al*., 1998). Cu appears to block electron transport on the oxidizing side of the reaction centres of PSII (Baszynski *et al*., 1988).

The fluorescence parameters used in the present study were: 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; F_s, fluorescence signal when a part of the reaction centres are closed (reduced); and F'_m, the maximum fluorescence intensity obtained by saturation of the reaction centres under steady-state conditions (Schreiber *et al*., 1986). F_s and F'_m were obtained under actinic light conditions (samples illuminated with a light intensity high enough to allow photosynthesis). These parameters allow the calculation of the photosynthetic efficiency Φ_{II}, which under steady-state illumination reflects the relative electron transport rate of PSII and is calculated from the formula:

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

Further information describing the PAM technique and its principles is available in Genty *et al*. (1989); see Kromkamp *et al*. (1998) for its use on microphytobenthos samples.

A MINI-PAM Fluorometer model 0221 (Walz, Germany) was used for the present experiments. An external white light source (halogen lamp, 2050-H) provided the actinic illumination, set at a constant photon irradiance of 70 μmol m^{-2} s^{-1}. PAM measurements were made with the fluorometer fibre optic placed below the glass vial in the centre of the samples. The PAM settings (gain and measuring light) were adjusted for each sample before the beginning of the dark measurement and changed if necessary to be above the detection limit. Six saturating light pulses at intervals of 20 s were applied for the measurement of the photosynthetic efficiency (Φ_{II}), and an average of the three last pulses (when values were observed to reach a steady state) was used for the calculation. To measure F_0, samples were left in the dark for at least 20 min; F_0 measurement of biomass has the advantage of being non-destructive (unlike pigment extraction) and allows repeated measurements on the same replicates.

EC_{50} values (toxicant concentration causing an end point decrease of 50%) were estimated from the percentage of the decrease in quantum efficiency in the biofilms (Φ_{II}) relative to the controls. For this purpose, a logistic response model (Haanstra *et al*., 1985) calculated with Kaleidagraph software was used. EC_{50} values were calculated for an exposure to Cu of 6 h (monospecific biofilms) or 7 h (natural biofilms).

Cu analysis

The biofilm was sonicated in 1·5 ml double-distilled water to remove it from the glass discs. The suspension with the biofilm was put into Eppendorf vials, centrifuged and stored overnight at −20 °C. Afterwards, the samples were freeze-dried to avoid Cu loss during the drying process and weighed.

Subsamples (0·1–6 mg) were placed in Teflon vessels; blanks and Buffalo River Sediment (NIST 2704) were included in extra vessels as references for the destruction process. Fifty microlitres of nitric acid (Baker Ultrex) was added to all Teflon vessels. The samples were placed in a microwave oven using a programme for a stepwise acid extraction process (Lehman *et al.*, 1999). Double-distilled water (1·95 ml) was added to dilute the acid concentrates. Finally, the samples were analysed either in a graphite furnace or by flame AAS. The lowest Cu concentrations in the water and biofilms were determined with a Graphite Furnace Atomic Absorption Spectrometer (Perkin Elmer 5100, detection limit 3 μg l^{-1}) equipped with Zeeman background correction. For the concentrations higher than 80 μg l^{-1} Cu, a Flame Atomic Absorption Spectrometer (Perkin Elmer 1100B, detection limit 50 μg l^{-1}) was used.

Results

Cu concentration

Cu concentration in the water after the exposure compared with the initial concentration varied between 45% and 77% in the experiments with monocultures, the highest values being for the lower Cu concentrations added. In the natural biofilm experiments, recovery of Cu from the water was 60–70% after 7 h exposure, and 40–50% after 24 h. No significant differences appeared between trials with filtered and non-filtered water, indicating that most of the Cu was accumulated by the biofilm, and not by the particles in suspension in the river water.

The accumulation of Cu by the biofilms (per unit dry weight, DW) followed a linear model (Figs 1, 2). In all cases, there was a general trend that more Cu per gram DW was accumulated with increasing Cu concentrations. Intact natural biofilms and *S. ulna* cells in suspension accumulated the most Cu (more than 5000 μg g^{-1} DW) (Figs 1, 2). Covariance analyses showed that the only

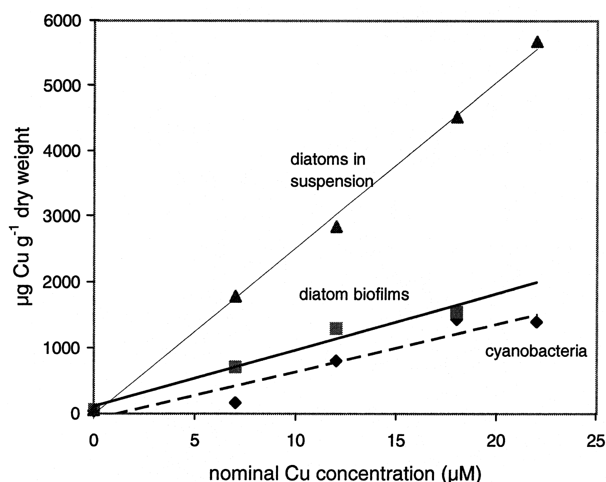

Fig. 1. Cu accumulated in the cultures of *Synedra ulna* (diatom) and *Oscillatoria* sp. (cyanobacteria) per gram dry weight after 6 h of incubation at different nominal Cu concentrations. The data were fitted to a linear model.

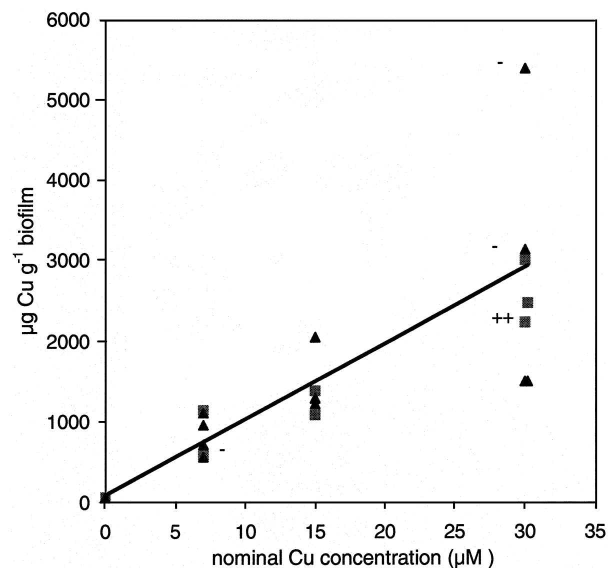

Fig. 2. Cu accumulated in the natural biofilms per gram dry weight after 7 h of incubation at different Cu concentrations, in non-filtered river water (triangles) or filtered river water (squares). The two trials were pooled to obtain the regression line. Biofilms of unusually high or low density are indicated separately: –, biofilms with 1–2 mg cm^{-2} DW; + +, biofilms with 3–4 mg cm^{-2} DW, the rest of the samples were between 2 and 3 mg cm^{-2} DW.

significant difference ($p < 0·001$) was between the slopes of the regression lines of Cu in the suspended *S. ulna* compared with those of the other monospecific and multispecies biofilms. Neither the slope, nor the intersection with the ordinate, was significantly different for any of the attached biofilms, so the transfer rates of Cu from the water to the biofilms are expected to be similar.

The nominal concentration factor in the biofilm recalculated from μg Cu g^{-1},

$$([Cu]_{Cu\,treated\,biofilm} - [Cu]_{control\,biofilm})/[Cu]_{water}$$

which is dimensionless, varied between $0·3 \times 10^3$ and $1·1 \times 10^3$ for *Oscillatoria* sp. For the biofilms formed by *S. ulna* the concentration factor was $1·2–2 \times 10^3$, and for those in suspension, the concentration factor was much higher, $3·6–4·9 \times 10^3$. In natural biofilms, the nominal Cu accumulation factor fluctuated between 1×10^3 and $2·5 \times 10^3$; there was no significant difference between the biofilms exposed for 7 h and for 24 h. However, it appeared that density, measured as dry weight per unit area, could influence the relative Cu absorption per gram of biofilm ($r = -0·54$; $p < 0·05$).

Periphyton biomass and pigments

Monospecific biofilms. DW was lower for the monoculture biofilms than for the natural biofilms (1–4 mg cm^{-2}): 0·7–1·6 mg cm^{-2} for *Oscillatoria* sp, and 0·2–0·4 mg cm^{-2} for *Synedra ulna*.

There was no change in the value of F_0 (minimum dark fluorescence, proportional to chlorophyll content) in the course of the incubations in the control trial of *S. ulna*

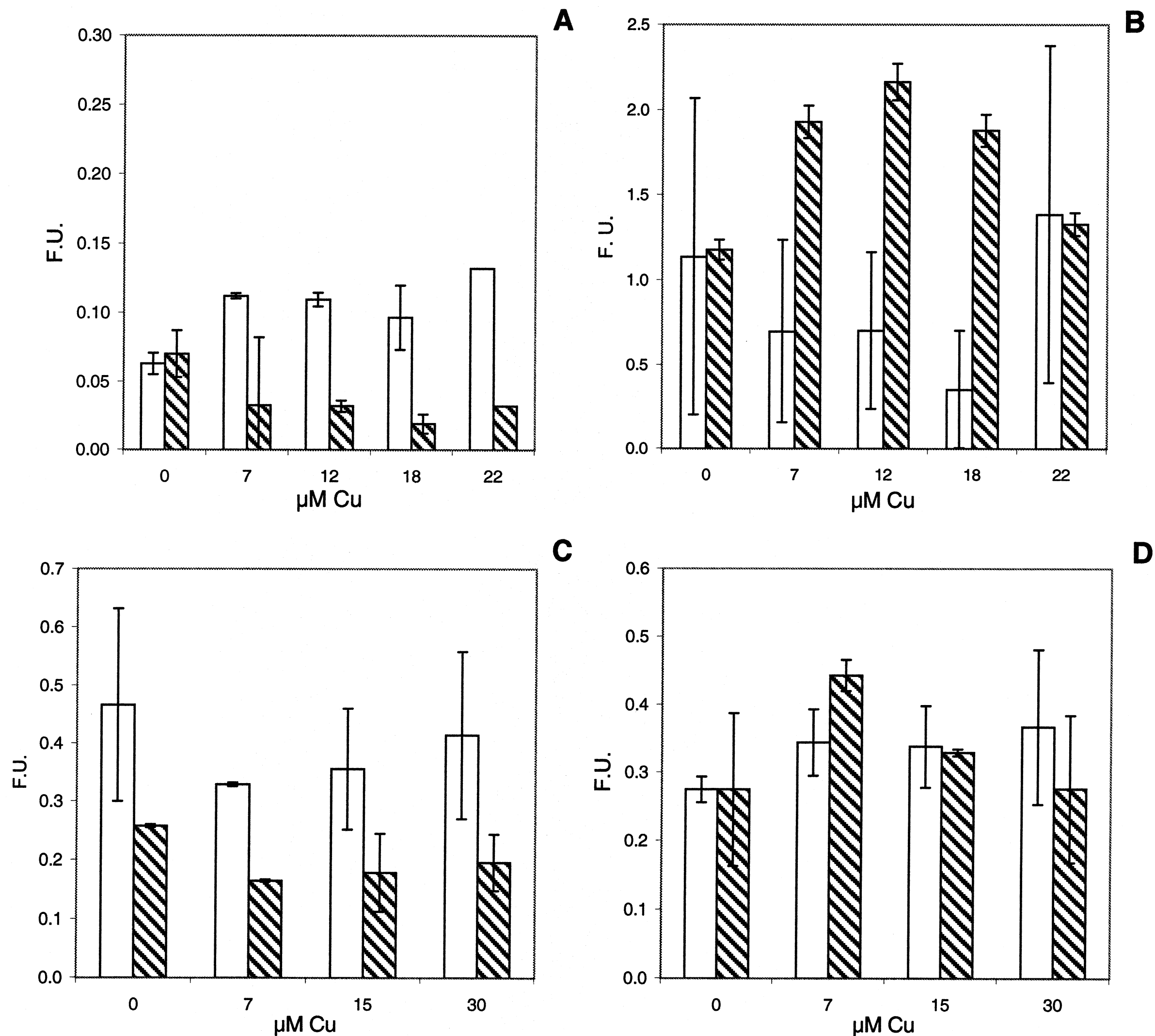

Fig. 3. Effect of Cu on F_0, a measure of algal biomass in fluorescent units (FU). Average F_0 values and SDs at the beginning (open bars) and end of the incubation (striped bars) of the biofilms exposed to different levels of Cu. (*A*): *Synedra ulna* monospecific biofilm, 6 h of incubation. (*B*): *Oscillatoria* sp. monospecific biofilm, 6 h of incubation. (*C*): natural biofilm after 7 h of incubation. (*D*): natural biofilm after 24 h of incubation.

biofilms lacking Cu addition. For all trials with added Cu, there was a steep decrease in F_0, to about 30% of the initial value (Fig. 3*A*). This decrease could have been an artifact of the setup: the *S. ulna* cells formed a very thin non-cohesive biofilm, which tended to fall off the glass discs during the Cu exposure, and as the PAM sensor was located under the glass discs, the fluorescence signal of the detached cells was not detected. In the vials containing the *S. ulna* suspension, the same lack of precision in measuring F_0 also occurred, as the cells were not homogeneously distributed on the bottom of the vial. For the *Oscillatoria* films, formed by a tight network of filaments, an increase in the F_0 values after the incubation occurred for all Cu concentrations except for 22 μM (Fig. 3*B*).

The effect of Cu on the biofilm pigments was noticeable in the experiments with *S. ulna*, but the lack of adequate replication makes drawing conclusions difficult, because part of the variability could be due to biomass heterogeneity between replicates. To avoid this variability, the data were normalized to chlorophyll *a* (Chl *a*). A slight decrease in concentrations of the pigments diadinoxanthin ($r = -0{\cdot}88$; $p < 0{\cdot}05$) and β-carotene ($r = -0{\cdot}74$; $p < 0{\cdot}1$) was seen, for at the highest concentration (22 μM nominal) part of the Chl *a* went into the production of allomers, as seen by the increase of the ratio Chl *a*+allomers/Chl *a* (Fig. 4). Fucoxanthin and Chl *c* decreased in proportion to Chl *a*. In the *Oscillatoria* biofilm, only zeaxanthin decreased ($r = -0{\cdot}84$; $p < 0{\cdot}1$, not shown) and the other pigment concentrations remained unchanged.

Natural biofilms. After 3 weeks of colonization in the river, thick biofilms (1–2 mm) had developed on the discs, with some variation in DW between replicates ($2{\cdot}6 \pm 0{\cdot}7$ mg cm^{-2}). Unlike the cultured biofilms, the natural biofilms appeared to be very resilient to Cu exposure and no effect of Cu exposure on DW was detected after 7 h. AFDW constituted only 17–23% of DW, indicating a high silt and inorganic content in the biofilm, and was not affected by exposure to Cu.

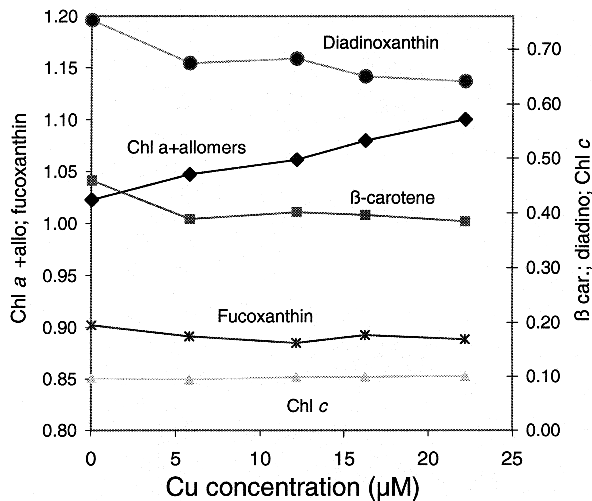

Fig. 4. Pigment concentrations analysed by HPLC (normalized to Chl *a* without its allomers) of *Synedra ulna* in suspension after exposure to a range of Cu concentrations. The primary *Y*-axis is for Chl *a* + allomers and fucoxanthin; the secondary *Y*-axis is for *β*-carotene, diadinoxanthin and Chl *c*.

F_0 values showed high variation between glass discs (coefficient of variation, CV = 30%, $n = 31$). Compared with the F_0 at the beginning of the experiments, after 7 h, there was a decrease in all Cu treatments and the controls (Fig. 3*C*), which was not significantly different between control and Cu-exposed treatments (*t*-tests, $p > 0{\cdot}05$). This was possibly due to the stress of the transfer. However, after 24 h of exposure a recovery of the biomass occurred in the 0 and 7 μM Cu treatments, indicating net growth; no significant change occurred at 15 μM Cu and there was a decrease of 0·1 units at 30 μM Cu (Fig. 3*D*).

Chl *a* concentrations measured by HPLC also showed variation between disc replicates, ranging from 2·4 to 3·9 μg cm^{-2}. Comparing the control discs with the Cu-enriched ones, there was no significant degradation of pigments after the 7 h experiment. Chlorophyll allomerization, and production of phaeophorbides and phaeophytin *a* were not evident after exposure to Cu.

The communities were mainly composed of diatoms, algae containing Chl *b* (such as chlorophytes) being absent. Microscopic counting revealed a mature periphytic community, mainly composed of *Melosira varians* Agardh (almost 50%), followed by *Navicula* sp. (20%), *Diatoma vulgare* Bory and *Fragillaria cappucina* Desmazières (both 6%) (Fig. 5).

Photosynthetic efficiency

After Cu addition, the photosynthetic efficiency (Φ_{II}) of the *S. ulna* biofilm was lowered, EC_{50} being 10·73 μM Cu (95% confidence limits (CL): 8·7 and 13·2 μM). There was no difference between the photosynthetic efficiencies of *S. ulna* on the discs and in suspension, both of which were severely affected after adding 12 μM Cu (Fig. 6). For the cyanobacterial biofilm, even though Φ_{II} showed a decrease with the Cu addition, 22 μM Cu did not cause a reduction of 50% of activity for all replicate samples. The confidence intervals of the EC_{50} thus became very wide (EC_{50}, 29·4 μM Cu; 95% CL: 11·8 and 73·6). Nevertheless, despite these wide confidence limits, it appears that the diatom biofilm was more vulnerable to Cu than the cyanobacterial film (Fig. 6).

Unlike the monocultures, the natural biofilm seemed to be very resilient to (acute) Cu addition, even when the highest concentration added was raised from 22 to 30 μM Cu. An adverse effect of Cu on photosynthesis could be perceived as a decrease in the photosynthetic efficiency

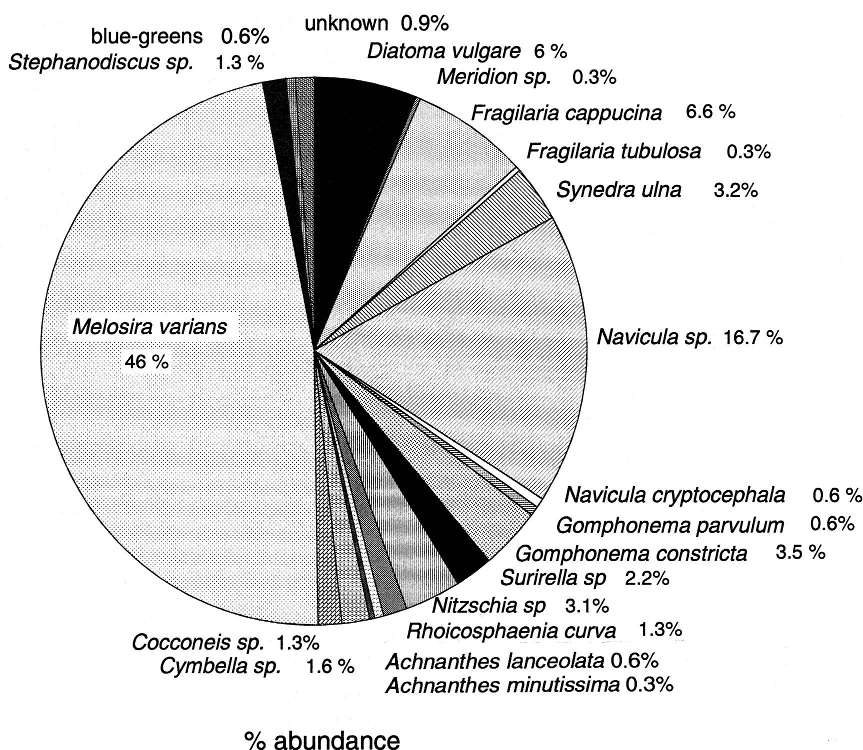

Fig. 5. Microalgal species composing the population of natural biofilms colonized in the River Meuse in March (as a percentage). The examination of the biofilm community was carried out on fixed samples. For diatom identification, the frustules were cleaned and mounted on resin.

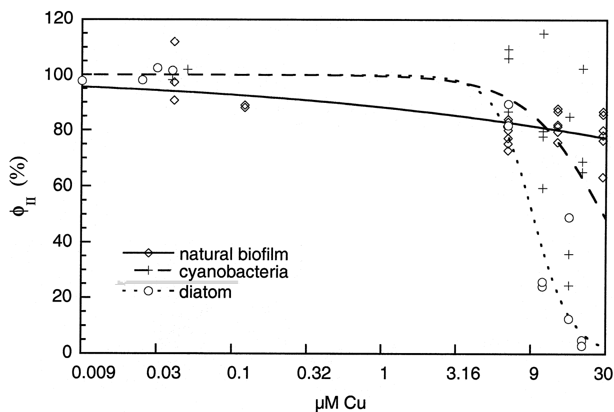

Fig. 6. Dose–response curves of the monospecific biofilms (diatom *S. ulna* and cyanobacteria *Oscillatoria* sp.) and intact natural biofilm from the River Meuse after respectively 6 h (monospecific biofilms) or 7 h (natural biofilms) of exposure to a range of Cu concentrations. The photosynthetic yield is represented as a percentage of the 100% activity in the controls. Photosynthetic yield: $\Phi_{II} = (F'_m - F_s)/F'_m$.

with increasing Cu concentrations (Fig. 6). Whereas the Φ_{II} values in controls tended to be stable or increase during the first 7 h, trials with added Cu showed a slow but constant decrease in their photosynthetic efficiency, which was more pronounced in the 30 μM trials. However, the decrease of Φ_{II} after 7 and 24 h of incubation was not pronounced enough to determine an EC_{50} value, because 50% activity was not reached, but the photosynthetic activity decreased by 20% with respect to the controls at 30 μM Cu (EC_{20}) at 7 h. No significant difference could be seen between Φ_{II} measured in the filtered trials and in non-filtered ones.

Discussion

Biofilm algae have been found to be much more resistant to metal toxicity than planktonic algae (*c.* 10 times), hence their activity is more resilient to environmental changes (Gustavson *et al.*, 1995). Planktonic communities are subject to dilution and export from the contaminated area; moreover, they show a high physiological plasticity. Tubbing *et al.* (1993*a*) observed a relatively low EC_{50} for Cu in short-term exposures (0·6–1·9 μM Cu) of the phytoplankton in the River Rhine and an EC_{10} in the range of the background concentrations (0·05–0·16 μM Cu). It was also suggested that an adaptation or induction of tolerance to Cu occurred for phytoplankton in the Meuse at the background concentrations (similar to those in the Rhine), in functioning (production) as well as in species composition (Tubbing *et al.*, 1995).

In contrast to the high sensitivity of phytoplankton, our results showed a high resistance of the epilithic algae from the Meuse to Cu exposure, especially on the thicker natural biofilms. That would mean a tolerance at least 10–20 times higher for the monospecific biofilms than for the Meuse phytoplankton, and much more than that for the natural biofilms. The structure of the biofilm itself, a tight package of cells in a dense mucous matrix, has been evoked as a reason for lower metal sensitivity (Starodub *et al.*, 1987; Admiraal *et al.*, 1999; Lehman *et al.*, 1999). Natural biofilms also have a significant number of metal binding sites located either in the organic matrix, at the surface of the cells, or in the organic particles trapped by the biofilm. These binding sites can immobilize Cu, lowering Cu availability to the microalgae. The Meuse natural biofilms had an amount of detritus, silt and organic matter that the monospecific biofilms did not have. Also, the increasing thickness of the biofilm made the penetration of Cu slower, as shown by the higher Cu accumulation per unit DW of the thinner biofilms with respect to the thicker ones. A thinner biofilm will have a relatively larger area exposed to Cu and accumulate more Cu per unit of dry weight than a thicker biofilm. This could explain the high levels of Cu per unit DW in thinner biofilms and the low levels in thicker biofilms. Such findings are in agreement with those of Ivorra *et al.* (2000) who showed older (i.e. thicker) biofilms to be more resistant to zinc pollution.

Because of their capacity to accumulate metals, biofilms change the metal availability in the water by active removal (Gray & Hill, 1995). But this accumulation is not constant in time: Vymazal (1984) showed that Cu accumulation by periphyton was most active during the first few hours of exposure. In our results, we observed the highest accumulation in the diatom cell suspension, and the least accumulation was found in the very compact, thin cyanobacterial films. This seems to confirm that the packing of cells is indeed a determining factor for the overall biofilm tolerance of toxicants. However, the accumulation of Cu per unit DW by the three biofilm types (diatom, cyanobacteria and multi-species) followed the same linear model, without showing saturation at the Cu concentrations used. The diatoms in suspension, on the other hand, had a higher Cu accumulation, due to breaking of the biofilm structure. Also, in the natural biofilms the Cu content in the biofilms after 7 h was no different from the accumulation after 24 h of exposure, indicating rapid saturation of the Cu binding sites in the biofilm at a fixed copper concentration.

The diatoms in suspension, or in thin non-cohesive biofilms, were most affected by Cu. In fact, the EC_{50} (10·73 μM Cu) was only slightly higher than that found for the marine planktonic diatom *Phaeodactylum tricornutum* (7·9 μM Cu) by Cid *et al.* (1995) after 4 h exposure to Cu. A decrease in the photosynthetic efficiency of PSII could already be seen at 12 μM Cu and above in *S. ulna*. Consistent with the decrease in photosynthetic efficiency, there was greater structural damage in the suspended diatoms than in all biofilms, revealed by the decrease in photosynthetic pigments relative to Chl *a*, and by the dark fluorescence to a lesser extent, due to the difficulties in measuring a reliable F_0 in suspension. Structural damage has been observed to the chloroplasts (outer membranes destroyed and thylakoids swollen) of algae exposed to copper (Stoyanova & Tchakalova, 1993), and Baszynsky *et al.* (1988) also found a significant decrease in Cpa

(Chlorophyll *a* protein complex of PSII) in copper-exposed spinach and modifications to the structure of chloroplasts and degradation of the grana stacks and lamellae.

Chl *c* and fucoxanthin seemed to be at least as resistant to copper as Chl *a*. On the other hand, diadinoxanthin and β-carotene degraded substantially. In the case of the cyanobacteria, zeaxanthin was the only pigment affected. For both diadinoxanthin and zeaxanthin, the degradation could be the effect of oxidative stress due to the Cu (Porra *et al*., 1999). The allomerization of Chl *a* at 7 μM Cu was noticeable in *S. ulna* even before the decrease in photosynthetic activity. Cid *et al*. (1995) found an increase in the proportion of Chl *a* allomer in *P. tricomutum*, which reached a maximum at the highest Cu concentration used (15·7 μM Cu), and attributed this production to the rise in internal pH in the cells. At that Cu concentration, *P. tricornutum* had stopped growing and photosynthesis was less than 50% of the control value. We found an increase in the proportion of allomers only in the *S. ulna* suspension, which was the most affected by the Cu. Probably, for the biofilms the Cu concentration and the exposure time were not sufficient to cause allomerization. Consistently, the natural biofilms showed no signs of structural damage after 7 h of exposure, but F_0 values did decrease after 24 h exposure to 30 μM Cu, suggesting the importance of long-term exposure to Cu for the growth of the periphytic community.

Besides the fact that the duration of the experiment was restricted to a few hours and the biofilm was thick and compact, hindering Cu penetration, other factors may explain the resistance to Cu. A factor which can play a role in increasing the tolerance of biofilms is the higher pH inside the biofilm matrix in contrast with planktonic algae. Liehr *et al*. (1994) found that high pH increased the precipitation of metals in the biofilm by decreasing their solubility as ions, and that metal speciation and amount of the binding sites in organic components of the biofilm were also affected by pH. In our case, the monocultures were incubated in medium with a neutral pH, which remained constant during the experiment. The Meuse water had a slightly alkaline pH, 7·8 during the time of our measurement, but seasonally it can reach more than 8. During the incubation, the pH in the waters increased up to 8·2–8·4, due to photosynthetic activity, even in the 30 μM Cu trials. This increase in pH was certainly more pronounced inside the biofilm, and could have lowered the concentration of the Cu ions in solution.

In summary, our results indicate that the higher tolerance to Cu of periphyton biofilms compared with phytoplankton is due to the thickness and the density of the cell packing, thus making natural biofilms resilient to Cu toxicity. At the present background Cu concentrations in the River Meuse, biofilms are likely not to need adaptation, as seen by the species composition of the natural biofilms (see below). Biofilm algae in the Meuse proved to be almost 100 times more resistant to Cu than phytoplanktonic algae, for which Cu in very low concentrations acts as an effective algicide. Phytoplankton is more sensitive for detecting small increases in metal concentrations in the water, and biofilms can still be useful indicators of chronic exposure to metals and their influence on the sediments and benthic organic matter composition.

Differences in response between monospecific and natural biofilms

The composition of the periphytic community itself in terms of algal species is a vital element in determining the tolerance to metals (Say & Whitton, 1981). The changes undergone by a periphytic community under metal pollution are very complex, and an evolution to communities dominated by tolerant forms has been pointed out by several authors (Leland & Carter, 1984; Clements & Kiffney 1994; Admiraal *et al*., 1999). Genter *et al*. (1987) observed a shift in the community composition from diatoms, to chlorophytes to cyanobacteria under zinc exposure. The epilithic community may shift to more resistant species because of the metal, eliminating sensitive forms such as *S. ulna*. In our tests, *S. ulna*, even when forming a biofilm, was the most sensitive algae to Cu. This coincides with the results of Leland & Carter (1984) in an oligotrophic stream, and those obtained during a Cu enrichment experiment by Navarro *et al*. (1998), who found a marked decrease in *S. ulna* in Spanish periphyton after 2 weeks of exposure to Cu.

In seasonal terms, metals may be more toxic to biofilm populations in spring and autumn, when the periphyton is mainly composed of diatoms; in summer, cyanobacteria prevail and the population can become less vulnerable to Cu pollution. This has also been pointed out for phytoplankton (Winner & Owen, 1990). Cyanobacteria have been reported as having a high resistance to metals and toxins in general (Izaguirre, 1992; Moffett & Brand, 1996). They have been observed to dominate periphytic populations after bad Cu and ammonia pollution (Cattaneo, 1992).

Among the periphytic algae in the River Meuse, the thin cyanobacterial biofilms were seen to be more resistant to Cu pollution than the diatom biofilm composed exclusively of *S. ulna*, but the natural biofilm was the most resistant. The high resistance of the natural biofilm to Cu during our short-term experiment is certainly not due to the species composition of the biofilm; the film was almost exclusively composed of diatoms, and most of the population was the colony-forming diatom *Melosira varians*, which is known to be very sensitive to metals (Peres *et al*., 1997). The fucoxanthin/Chl *a* ratio ranged between 0·36 and 0·38 in all samples of natural biofilm, which is typical of diatoms (Jeffrey & Vesk, 1977). The biofilm also had a significant amount of *Diatoma vulgare*. This and *M. varians* are late successional species, typical of nutrient-rich streams (Richardson *et al*., 1996), but vulnerable to perturbations (Medley & Clemens, 1998).

The effect of Cu on natural biofilms is bound to be less than the effect on the most resistant of its individual species (in this case *Oscillatoria*). Care should be taken

when assessing the sensitivity of individual periphyton species (as in published phytoplankton unialgal species tests), because the natural resistance of the algae will be reinforced to a different extent by the buffering capacity of the biofilm thickness. However, short-term toxicity tests with monospecific biofilms can provide further insight into the specific tolerance of individual algal species and their defence mechanisms against metal pollution. When immersed in a thick biofilm, metal-sensitive species will not be affected by metal concentrations that are deleterious to young, thin biofilms constituted by a small number of species. The assessment of the longer-term effect of metals on intact biofilms in various stages of development will produce an integrated picture of the adaptation of the biofilm community.

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