Applied and Environmental Microbiology, Aug. 2004, p. 4512–4521 Vol. 70, No. 8
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.8.4512–4521.2004

Analysis of Structural and Physiological Profiles To Assess the Effects of Cu on Biofilm Microbial Communities

B. Massieux,[1]* M. E. Y. Boivin,[2] F. P. van den Ende,[3] J. Langenskiöld,[3] P. Marvan,[4] C. Barranguet,[3] W. Admiraal,[3] H. J. Laanbroek,[1] and G. Zwart[1]

Netherlands Institute of Ecology, Centre for Limnology, Nieuwersluis,[1] Rijksinstituut voor Volksgezondheid en Milieu, Bilthoven,[2] and Department of Aquatic Ecology and Ecotoxicology (AEE), University of Amsterdam, Amsterdam,[3] The Netherlands, and Institute of Botany, Czech Academy of Sciences, Brno, Czech Republic[4]

Received 25 November 2003/Accepted 14 April 2004

We investigated the effects of copper on the structure and physiology of freshwater biofilm microbial communities. For this purpose, biofilms that were grown during 4 weeks in a shallow, slightly polluted ditch were exposed, in aquaria in our laboratory, to a range of copper concentrations (0, 1, 3, and 10 μM). Denaturing gradient gel electrophoresis (DGGE) revealed changes in the bacterial community in all aquaria. The extent of change was related to the concentration of copper applied, indicating that copper directly or indirectly caused the effects. Concomitantly with these changes in structure, changes in the metabolic potential of the heterotrophic bacterial community were apparent from changes in substrate use profiles as assessed on Biolog plates. The structure of the phototrophic community also changed during the experiment, as observed by microscopic analysis in combination with DGGE analysis of eukaryotic microorganisms and cyanobacteria. However, the extent of community change, as observed by DGGE, was not significantly greater in the copper treatments than in the control. Yet microscopic analysis showed a development toward a greater proportion of cyanobacteria in the treatments with the highest copper concentrations. Furthermore, copper did affect the physiology of the phototrophic community, as evidenced by the fact that a decrease in photosynthetic capacity was detected in the treatment with the highest copper concentration. Therefore, we conclude that copper affected the physiology of the biofilm and had an effect on the structure of the communities composing this biofilm.

Copper has been applied as an algaecide for many years, e.g., in antifouling ship paints, and has been used in the composition of many materials, such as porcelains and bricks, which for a long time were simply deposited in dumping grounds when no longer in use, increasing the load of cupric compounds in soils and waters, and leading to concerns about their toxicity. However, our knowledge of the effects of this metal on the structure and function of microbial communities in freshwater is still limited. Several detrimental effects of Cu have been reported to occur in pure cultures of phototrophic species. These effects include depression of photosynthesis and respiration, inhibition of cell division, and cell death (15). Aquatic microorganisms, phytoplankton and bacteria, often live in communities, forming organized assemblages at the surfaces of rocks, sediment, and submerged plants. These epilithic, epiphytic, or epipelon assemblages are generally described as biofilms (30). Barranguet et al., studying heavy metal effects on aquatic biofilms, showed that Cu affects the physiology of phototrophic organisms (1), that it accumulates in phototrophic biofilms proportionally to the concentration of exposure, and that some algal species react morphologically to an increased Cu concentration (3). However, the measured Cu effects on the photosystem were not related to changes occurring in the composition of the phototrophic community as observed by microscopy. Nevertheless, as suggested by Barranguet et al. (4), changes in phototrophic community structure could lead to a modification in the type of organic compounds released by the phototrophic organisms, which in turn could induce a change in the heterotrophic community structure, as Watson and Bollen (37) demonstrated.

In this study, we investigated both the phototrophic and the heterotrophic microorganisms in a biofilm exposed to copper. The questions we posed were whether physiological and structural changes would occur within a biofilm exposed to an increasing range of Cu concentrations and whether there was a clear relation between the effects on phototrophs and those on heterotrophs. In order to assess changes in species composition, denaturing gradient gel electrophoresis (DGGE) (29) was used to generate genetic community structure fingerprints of the eukaryotic, cyanobacterial, and bacterial biofilm compartments. We chose the community-level physiological profile (CLPP) technique (31), to assess variations in the metabolic capacity of the heterotrophic bacterial community in relation to Cu exposure. The pulse amplitude modulation (PAM) fluorescence technique is based on the measurement of the fluorescent emission of light-stimulated pigments (21). The PAM technique allowed a direct assessment of the toxicant effect on the photosynthetic capacity of the algal community, where Cu interacts directly with photosystem II (PSII). Combining these techniques, we found significant differences at the genetic and physiological levels among bacterial communities and differences between unexposed and exposed phototrophic communities at the physiological level.

MATERIALS AND METHODS

Sampling site. The Demmerik polder (52°13′N, 4°56′E) in The Netherlands is characterized by patchy pollution due to loading of urban wastes from the city of Amsterdam. From the beginning of the 17th century until 1977, peat was exca-

* Corresponding author. Mailing address: Netherlands Institute of Ecology, Centre for Limnology, Rijkstraatweg 6, 3631 AC Nieuwersluis, The Netherlands. Phone: 31 (0) 294 239 309. Fax: 31 (0) 294 232 224. E-mail: b.massieux@nioo.knaw.nl.

TABLE 1. Primer sequences and target sites

Primer[a]	Sequence (5′ to 3′)	Target site	Characteristic
GC-EUK1427F	TCTGTGATGCCCTTAGATGTTCTGGG[b]	1427–1453[d]	Eukaryotic primer
EUK1616R	GCGGTGTGTACAAAGGGCAGGG	1616–1637[d]	Eukaryotic primer
GC-CYA371F	CAGCAGTGGGGAATTTTCC[c]	371–390[e]	Cyanobacterial primer
CYA783R	GACTACWGGGGTATCTAATCCCW	738–761[e]	Cyanobacterial primer
GC-357F	CCTACGGGAGGCAGCAG[b]	357–374[e]	Universal bacterial primer
R518	ATTACCGCGGCTGCTGG	518–525[e]	Universal bacterial primer

[a] R (reverse) and F (forward) designations refer to primer orientation in relation to the rRNA.
[b] A 40-nucleotide GC-rich clamp (5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC-3′) is attached to the 5′ end of the primer.
[c] A 40-nucleotide GC-rich clamp (5′-CGCCCGCCGCGCCCCGCGCCGGTCCCGCCGCCCCGCCCG-3′) is attached to the 5′ end of the cyanobacterial primer.
[d] *Saccharomyces cerevisiae* numbering of 18S rRNA nucleotides.
[e] *Escherichia coli* numbering of 16S rRNA nucleotides.

vated and used as fuel. Mud was then mixed with sand, manure, and city waste from Amsterdam to fill the cavities. City wastes were composed of pipe heads, porcelains, and coins, but also of ceramics containing heavy metals and other industrial by-products. In a study of pollution levels in this area, Cu was found to be exceeding the intervention limit (4 μg/liter) in all surface water samples and in most of the soil samples (16).

Transplant experiment setup. Polyethylene racks containing approximately 200 smooth glass disks (1.5 cm^2) and smooth rectangular glass plates (~360 cm^2) were suspended vertically under a floater in a ditch (approximately 1.20 m deep), perpendicular to the water flow at a depth of 20 cm, and left to be colonized by epiphytic microorganisms (for technical details, see reference 19). The small glass disks were used to collect suitable samples for PAM analysis, while the large glass plates were used to collect enough material for CLPP and DGGE fingerprints. Four aquaria were prepared to receive biofilms by an acid rinsing treatment and were preloaded for 24 h with tap water to which Cu ($CuCl_2$, Tritisol [Merck, Darmstadt, Germany] Cu standard) at the treatment concentration (0, 1, 3, or 10 μM) was added. After a 4-week colonization in April 2002, the biofilms were removed from the ditch, transported to the laboratory in a cooler filled with ditch water to prevent desiccation, and placed in aquaria (three large glass plates and three racks of disks per aquarium) containing 28 liters of filtered (pore size, 15 μm), freshly added ditch water. No significant difference between biofilms from the glass disks and those from the plates could be detected by eye. Cu was added to the aquaria at the expected concentrations. Aquaria are referred to below as control (0 μM) and 1, 3, and 10 μM aquaria. The temperature of all four aquaria was established at 21 ± 1°C and was verified daily directly in the aquarium water. Light was set at an intensity of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at the water surface during a light-dark cycle of 16 and 8 h, respectively, by using a table light system consisting of one row of 10 30-W fluorescent light tubes. The water was constantly bubbled with air to avoid stratification and CO_2 limitation and was totally replaced every 6 to 7 days with filtered site water. Cu concentrations were measured and corrected upon each renewal of the water. The pH increased slightly, on average, from 8.3 to 8.6 in all aquaria, without any significant differences between treatments. The phosphate concentration was measured once a week by following the protocol described by Murphy and Riley (27). Total phosphorus concentrations were consistently higher than 0.1 μM. Therefore, phosphate was probably not a limiting factor.

Copper analyses. The copper concentration was monitored both in the water and in the biofilm by using atomic absorption spectrometry (AAS) according to the method of Barranguet et al. (1). Briefly, on every sampling day, water samples were taken from each aquarium before and after water renewal. The impor-

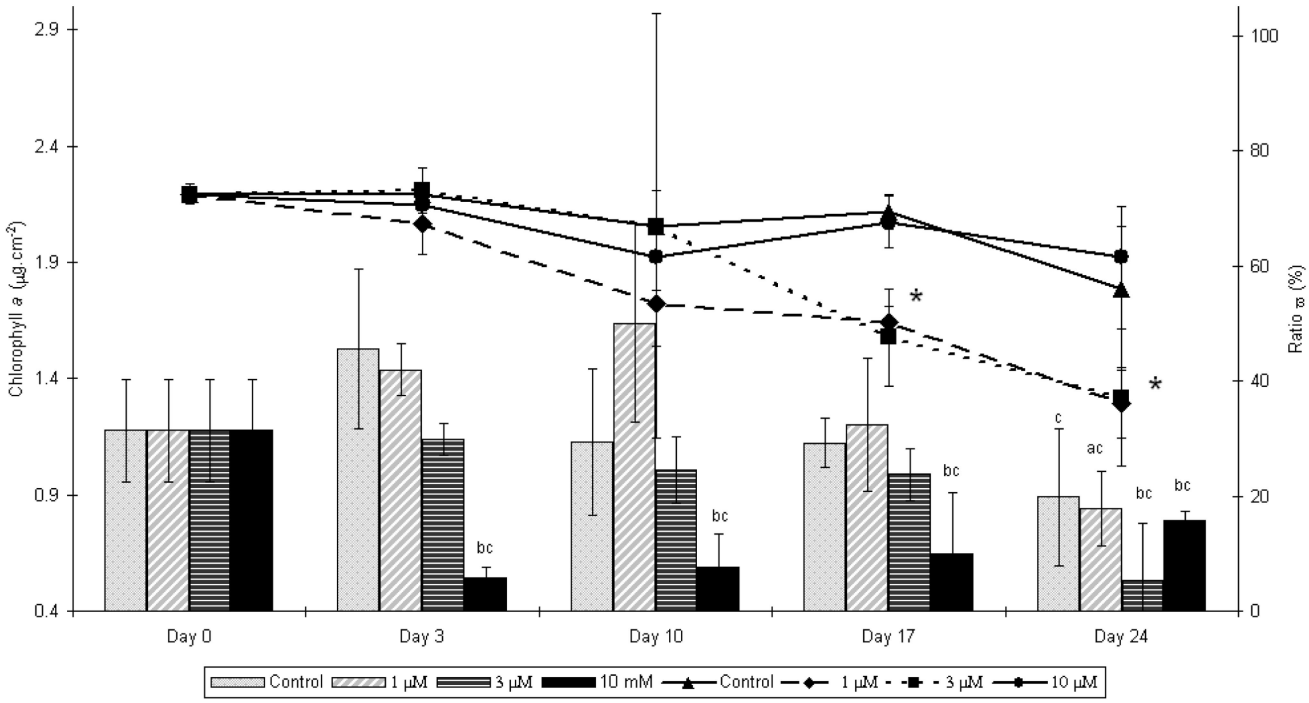

FIG. 1. Chlorophyll *a* content (bars) and organic matter content ($\bar{\omega}$) (lines) of the biofilms from the four aquaria. A letter or an asterisk (for chlorophyll *a* content or $\bar{\omega}$, respectively) indicates a significant difference. [a], significantly different from day-3 control; [b], significantly different from day-3 control and day-3 1 μM treatment; [c], significantly different from day-10 1 μM treatment; *, significantly different from the control and the 10 μM treatment.

TABLE 2. Cu concentrations and Cu effects on phototrophic parameters[a]

Day	Cu concn (μg/mg [dry wt] ± SD)							
	In biofilm				In water			
	0 μM	1 μM	3 μM	10 μM	0 μM	1 μM	3 μM	10 μM
Day 0	0.045 ± 0.005	0.045 ± 0.005	0.045 ± 0.005	0.045 ± 0.005	4.706 ± 4.946	7.600 ± 0.427	25.130 ± 0.710	75.310 ± 3.470
Day 3	0.083 ± 0.010	0.372 ± 0.076	1.144 ± 0.173	3.876 ± 0.752	5.180 ± 0.389	21.730 ± 1.648	81.279 ± 3.907	269.716 ± 17.149
Day 7								
Day 10	0.049 ± 0.001	0.247 ± 0.044	1.076 ± 0.303	6.598 ± 0.165	4.203 ± 1.433	11.537 ± 0.149	40.307 ± 0.752	133.145 ± 4.484
Day 17	0.064 ± 0.016	0.149 ± 0.025	0.921 ± 0.009	6.252 ± 0.403	2.962 ± 1.952	7.590 ± 1.521	20.362 ± 1.199	93.189 ± 7.763
Day 24	0.039 ± 0.003	0.227 ± 0.096	0.978 ± 0.198	6.928 ± 0.933	2.072 ± 1.150	3.994 ± 1.142	14.285 ± 1.776	50.098 ± 3.291

[a] Cu concentrations were measured in the biofilms and the water during the experiment. Φ_0 and Φ_{PSII}, both indicators of the biofilm response to the treatment, were measured by the PAM fluorescent technique. All results are shown for aquaria to which the indicated concentrations of Cu have been added (0, 1, 3, or 10 μM).

tance of particles in copper speciation in the water, e.g., in case of complex formation with humic substances (26), was evaluated by comparing a 1-ml unfiltered water sample to a filtered sample. Weekly, three biofilm samples per treatment were scraped off by using a sterile razor blade and freeze-dried at −20°C prior to AAS or Flame AAS analysis (for concentrations above 0.1 mg of Cu/liter).

Chlorophyll *a* content analysis. Four small glass disks per aquarium were harvested for chlorophyll *a* content analysis. Chlorophyll *a* was extracted overnight by using 90% acetone and was measured with a UV-1601 UV-visible spectrophotometer (Shimadzu, Duisburg, Germany) at a wavelength of 665 nm before and after acidification with 0.4 N HCl (25).

PAM fluorescent technique measurements. In order to assess the response of the phototrophic community to the toxic stress, we used the PAM fluorescence technique. Two chlorophyll fluorescence parameters were investigated: the maximum quantum yield (Φ_0) and photosynthetic efficiency (Φ_{PSII}) (for further details, see reference 32). The parameter Φ_0 represents the yield of dark-adapted cells, while Φ_{PSII} reflects the relative electron transport rate of PSII (2). To measure Φ_0, samples were left in the dark for at least 20 min.

Determination of phototrophic community structure by microscopy. The relative abundance of the phototrophic organisms was estimated by microscopy. Each sampling day, six disks per aquarium were harvested and fixed with formaldehyde for further microscopic investigations. The percentage of a defined area covered by the algal species was used to estimate the proportion of individual algal species in the biofilms. Counting of cells proved impossible, since the algae grew mainly in colonies. Proportions of diatoms could not be estimated due to the fixation method and the biofilms' thickness. Biofilm thickness was not measured per se, but differences could easily be noticed by eye.

Physiological structures of heterotrophic communities. Bacterial communities were harvested from the biofilms on days 0, 3, 5, 12, 19, and 26 in order to create CLPPs according to a method described elsewhere (M. E. Y. Boivin, B. Massieux, A. M. Breure, F. P. van den Ende, G. Greve, M. Rutgers, and W. Admiraal, submitted for publication). For this purpose, bacterial communities were incubated and monitored in Biolog ECO-plates. The Biolog ECO-plates contain a set of 31 different carbon compounds, and the degradation rates of these compounds are used as substrate utilization capacity measurements of the microorganism or microbial community studied. The inoculated biomass was standardized to an optical density of 0.068 at 750 nm. Plates were incubated in the dark (to prevent any phototrophic growth) at 20°C and 85% ± 5% air humidity. CLPPs were calculated according to the method of Boivin et al. (submitted) from absorbance data measured every 8 h for each well at 590 nm by using a microplate Expert 96 reader spectrophotometer (Asys Hitech, Eugendorf, Austria).

DGGE profiles. Eukaryotic, cyanobacterial, and bacterial community samples, corresponding approximately to 1/10 of the surface of a large plate, were scraped off by using sterile razor blades and collected on days 0, 3, 5, 10, 17, and 24. Communities were monitored by DGGE between day 3 and day 24 of the Cu exposure period. Separately, bacterial communities were compared in duplicate between days 0 and 3 to determine the natural heterogeneity. All profiles were

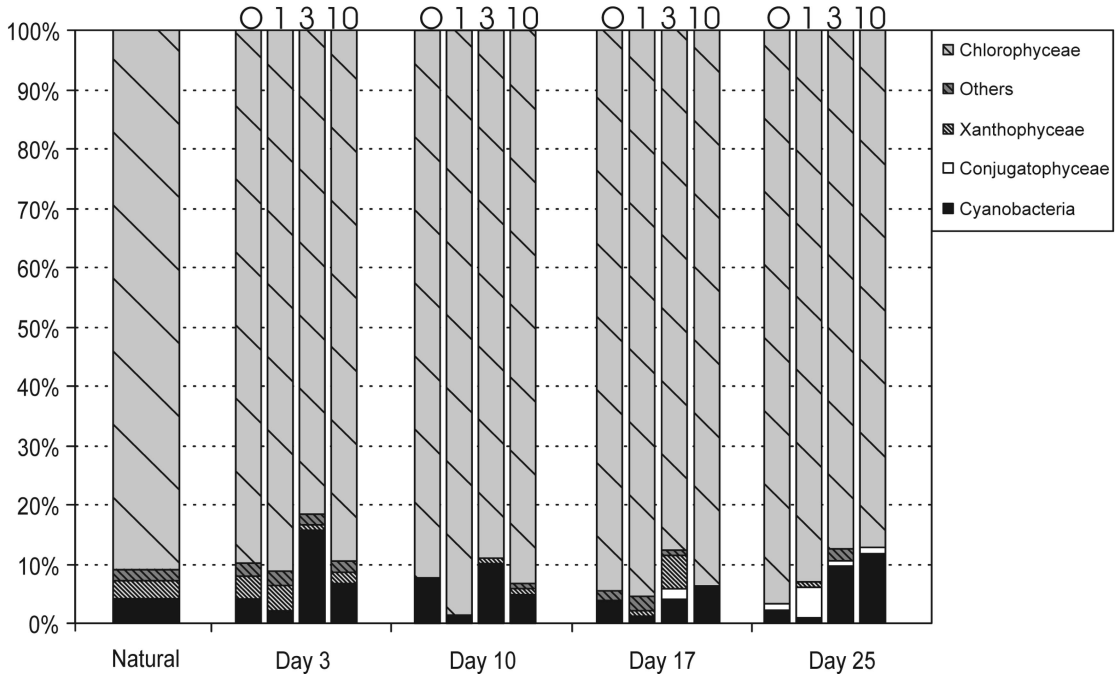

FIG. 2. Area covered (expressed as a percentage of a defined area) by *Chlorophyceae*, *Xanthophyceae*, *Conjugatophyceae*, *Cyanobacteria*, and others in the biofilms, determined by microscopy. Numbers above columns indicate the initial micromolar concentration of Cu in the aquarium.

TABLE 2—*Continued*

$\Phi_0 \pm$ SD				$\Phi_{PSII} \pm$ SD			
0 μM	1 μM	3 μM	10 μM	0 μM	1 μM	3 μM	10 μM
0.625 ± 0.007	0.659 ± 0.006	0.654 ± 0.008	0.639 ± 0.006	0.489 ± 0.313	0.536 ± 0.025	0.496 ± 0.029	0.455 ± 0.012
0.599 ± 0.004	0.637 ± 0.008	0.629 ± 0.009	0.590 ± 0.008	0.438 ± 0.229	0.483 ± 0.029	0.467 ± 0.015	0.431 ± 0.027
0.549 ± 0.010	0.562 ± 0.015	0.545 ± 0.019	0.518 ± 0.006	0.411 ± 0.033	0.440 ± 0.032	0.451 ± 0.019	0.366 ± 0.014
0.566 ± 0.010	0.596 ± 0.020	0.567 ± 0.030	0.493 ± 0.006	0.407 ± 0.029	0.504 ± 0.025	0.433 ± 0.067	0.339 ± 0.020

obtained after DNA extraction using the protocol described by van Hannen et al. (35). PCR was performed using eukaryotic 18S rRNA gene primers (35), cyanobacterial 16S rRNA gene primers (20), and bacterial 16S rRNA gene primers for DGGE (29) (Table 1). PCR amplifications were performed in 50-μl volumes containing template DNA, *Taq* DNA polymerase buffer (Invitrogen, Carlsbad, Calif.), 0.5 μM each primer, 200 μM each deoxynucleotide, 400 ng of bovine serum albumin, 1.5 mM $MgCl_2$, and 1.3 U of *Taq* DNA polymerase. PCR cycling was performed using a Thermo Hybaid thermocycler (Hybaid, Ashford, United Kingdom). The temperature program of the eukaryotic PCR was as follows: 5 min at 94°C; 30 cycles with an annealing temperature of 60°C (30 s) for the first 5 cycles and a touchdown step (in which the annealing temperature was decreased from 60 to 55°C) for 25 cycles, and extension at 68°C for 90 s. The final extension step lasted 10 min. PCR product concentrations were estimated by separation on 1% agarose gels stained with ethidium bromide and digital gel image analysis using Phoretix 1D software (Nonlinear Dynamics, Newcastle, United Kingdom).

Eukaryotic DGGE was performed as described by van Hannen et al. (35). Equally sized PCR products were separated on a 1-mm-thick vertical gel containing 8% (wt/vol) polyacrylamide (acrylamide to bisacrylamide, 37.5:1) and a linear gradient of the denaturing agents urea and formamide increasing from 25% at the top to 55% at the bottom (100% denaturant corresponds to 7 M urea and 40% [vol/vol] formamide). Cyanobacterial PCR amplification was performed using cyanobacterium-specific primers (Table 1) and the protocol described by Janse et al. (20). Bacterial PCR amplification was performed using the protocol described by Muyzer et al. (29). Cyanobacterial and bacterial PCR products were quantified as described above and separated on a 1-mm-thick DGGE gel with a linear gradient of the denaturing agents urea and formamide increasing from 35% on top to 60% at the bottom. All DGGE were performed in a buffer containing 40 mM Tris, 40 mM acetic acid, and 1 mM EDTA (pH 7.6) (0.5× TAE) at 75 V for 16 h, and gels were stained with ethidium bromide (1 μg/μl).

DGGE gel picture analysis. DGGE gel pictures were analyzed by using Phoretix 1D as follows. Lanes were created manually, with a fixed width of 5% of the standard lane width. Each lane represents one sample. Background noise was subtracted by using the rolling ball algorithm with a radius of 50 pixels. First, automatic band detection was performed with a minimum slope of 75 to 100 and a noise reduction of 5, and peaks smaller than 2% of the maximum peak were discarded. The edge detection method was fixed to a width of 1. Then bands were assessed and corrected by eye, one by one. Bands were then matched to create a matrix containing band percentage values of each sample. The band percentage matrix was subjected to nonmetric multidimensional scaling (NMDS) analysis using Primer software (version 5; Primer-E, Ltd., Plymouth, United Kingdom) (12).

Statistical analyses. Differences in Cu accumulation in the biofilms, the ash-free dry weight/dry weight ratio ($\bar{\omega}$), chlorophyll *a* content, Φ_0, and Φ_{PSII} were statistically tested by using a parametric factorial ANOVA (analysis of variance) design. This design allows the analysis of two factors and their interaction term. In our case, one factor is copper (Cu), the second is the incubation time (T), and their interaction term ($Cu \cdot T$) corresponds to the duration of exposure to Cu. Prior to assessment by the factorial ANOVA, estimated values for the parameters listed above were tested for normality (Kolmogorov-Smirnov test) and homoscedasticity (F_{max} test). All data followed a normal distribution, and based on the F_{max} test ($P < 0.05$), all variances were homogeneous, except for the chlorophyll *a* measurements. If the factorial ANOVA revealed significant effects, a Tukey honestly significant difference post hoc test ($P < 0.05$) was performed to group homogeneous means (13). CLPP fingerprint data were compared in a nonparametric multivariate analysis, principal component analysis, to investigate a possible correlation between toxic stress and changes in the substrate utilization capacity of the heterotrophic compartment. The significant influences of the variables (Cu and days of incubation) were tested in a redundancy analysis using a Monte Carlo permutation test (with 9,999 permutations). The NMDS ordination analysis endeavors to position data on a plot of 2 dimensions representing the matching similarities calculated in a triangular matrix of similarity coefficients computed between every pair of samples (8). In this study, the triangular similarity matrix calculation was based on band percentage data retrieved from the gel image analysis using Phoretix 1D and was performed using a Bray-Curtis model, with square root transformation, in the Primer package. NMDS representations were assessed by using the analysis of similarities (ANOSIM) function within the Primer 5 software package.

RESULTS

Copper analysis. The Cu concentration in the water remained relatively stable and close to the nominal concentrations during the whole experiment, except for the 10 μM treatment, in which it dropped slowly after each renewal of the water, from approximately 9.5 to 6 μM. For biofilms from 1 and 3 μM treatments, the Cu content increased within the first 3 days of exposure and remained stable until the end of the experiment (3.9 ± 1 and 16.2 ± 2 μmol/g [dry weight] of biofilm, respectively). The Cu content increased continuously in biofilms in the 10 μM aquarium until day 10, when it stabilized around 100 μmol/g (dry weight) of biofilm. The Cu content of biofilms from the control never reached more than 1.3 μmol/g (dry weight) of biofilm. Cu, T, and $Cu \cdot T$ all correlated positively with the Cu concentration in the biofilm ($P \leq 0.0001$ for all). This indicates a synergic effect of T and Cu; the longer a biofilm was exposed to Cu, the more the biofilm accumulated Cu.

Biofilm biomass. The equilibrium between the organic and inorganic matter contents of the biofilm was estimated as the ratio between the ash-free dry weight (estimate of the organic-matter content) and the dry weight (estimate of total [organic plus inorganic matter] content) of the samples ($\bar{\omega}$). The ratio $\bar{\omega}$ correlated negatively with Cu, T, and $Cu \cdot T$ ($P < 0.001$, $P < 0.001$, and $P < 0.004$, respectively) (Fig. 1). Most of the variance was supported by results from the last 2 sampling days of the 1 and 3 μM treatments, indicating a decrease in their biofilms' organic matter production. Cu, T, and $Cu \cdot T$ all correlated negatively with the biofilms' chlorophyll *a* content ($P < 0.001$, $P < 0.001$, and $P < 0.003$, respectively). The chlorophyll *a* content of the control did not change significantly over time (Fig. 1). The chlorophyll *a* content of the biofilms from the 10 μM aquarium generated most of the variance, indicating a decrease in the photosynthetic potential of these biofilms.

Algal biofilm compartment. Photosynthetic capacity was negatively correlated with the factors tested ($P < 0.001$ for all factors) (Table 2). The largest decrease in Φ_0 was observed in

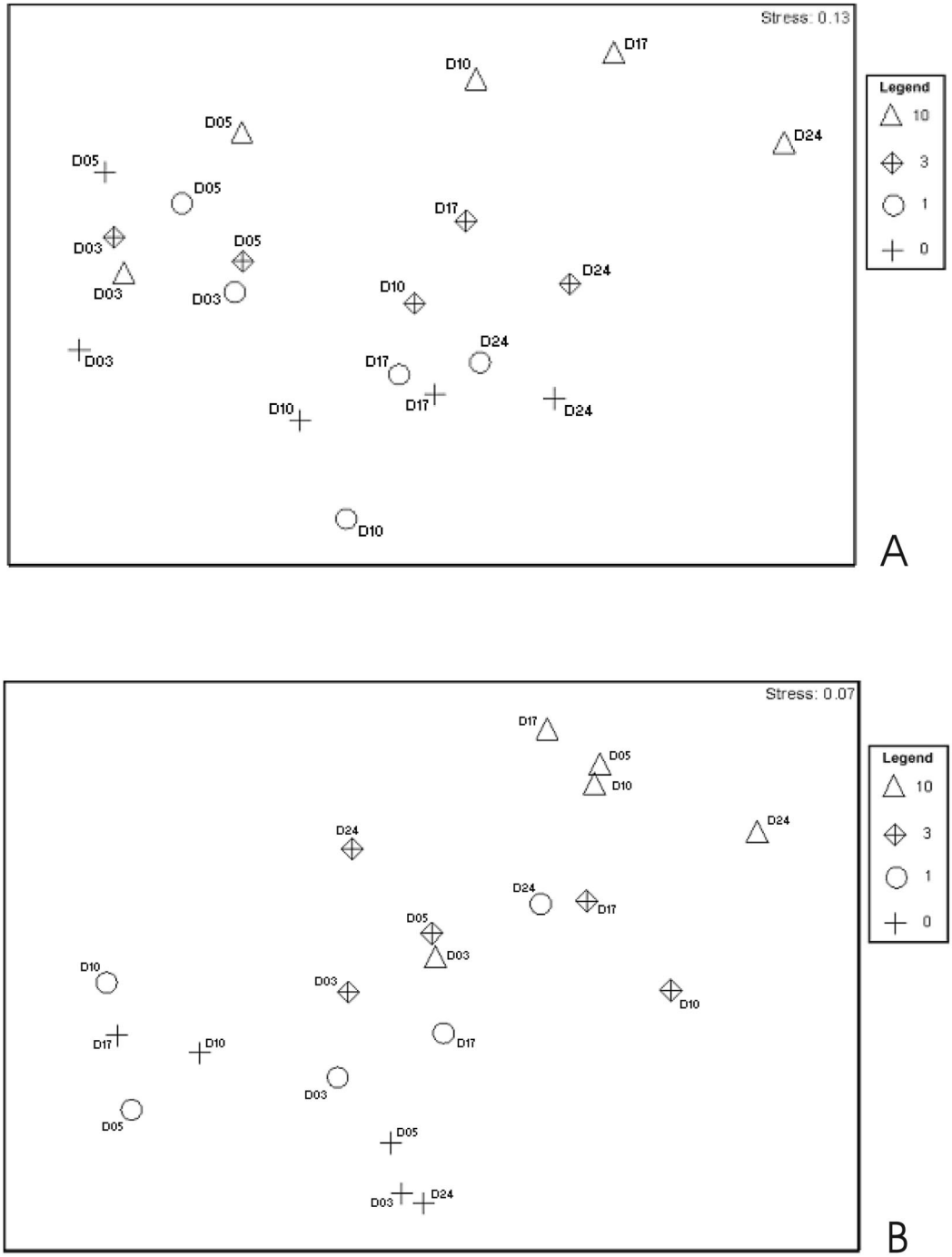

FIG. 3. Two-dimensional representations of NMDS of a time series of genetic profiles of the phototrophic communities exposed to a Cu gradient ranging from 0 to 10 μM. NMDS analyses of DGGE profiles of the eukaryotic community (A) and the cyanobacterial community (B) are shown. Days are given next to symbols.

the 10 μM aquarium: 22% between day 7 and day 24. In 3 weeks' time, the decrease was approximately 10% in control, 1 μM, and 3 μM aquaria, indicating a relatively stable metabolic capacity of their algal consortia.

The three factors also correlated negatively with the biofilms' photosynthetic efficiency, Φ_{PSII}, in all aquaria ($P < 0.001$) (Table 2). The control and 3 μM aquaria showed linear decreases in Φ_{PSII} between day 7 and day 24 of the experiment (17 and 13%, respectively). The Φ_{PSII} of 10 μM biofilms was most strongly affected, with a value on day 24 representing only 25.5% of the value on day 7. The pattern of 1 μM Φ_{PSII} values revealed first a decrease (18% between day 7 and day 17) and then an increase, such that the Φ_{PSII} on day 24 was only 6% lower than that on day 7.

At the beginning of the experiment (day 0), all aquaria were dominated by *Chlorophyceae* (90% of the total). In the control

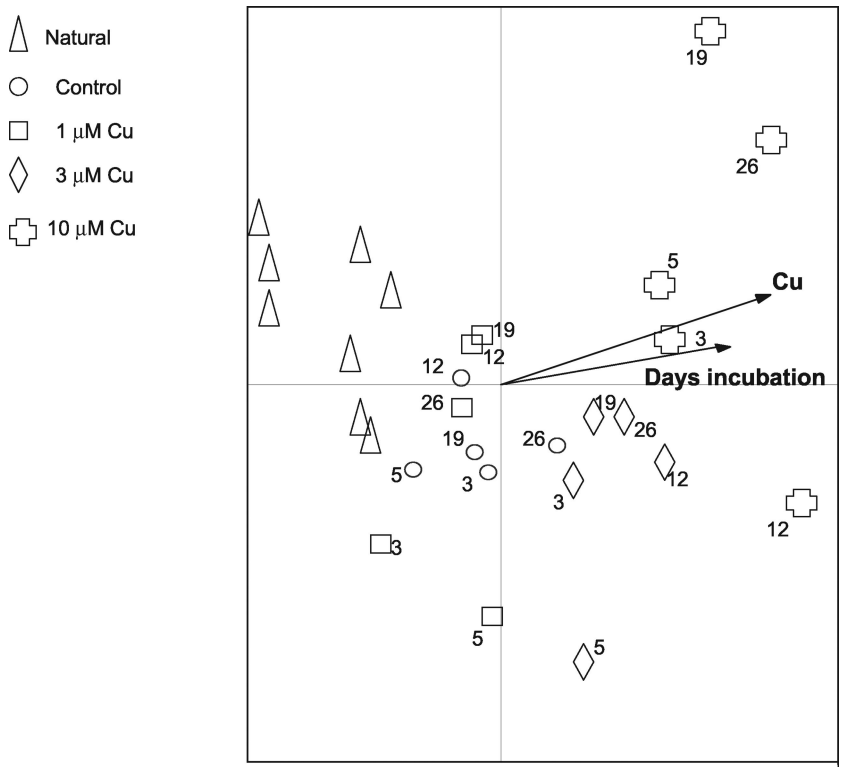

FIG. 4. Principal-component analysis of the CLPPs of the four aquaria on days 3, 5, 12, 19, and 24. Triangles, natural communities; circles, control; squares, 1 μM Cu; diamonds, 3 μM Cu; crosses, 10 μM Cu. The horizontal axis explains 19.4% of the variance observed, while 15.1% can be explained by the vertical axis. Redundancy analysis was performed by using a Monte Carlo permutation test (9,999 permutations). The first axis was highly significant ($P = 0.0001$), and the variables, Cu concentration in the biofilm and days of incubation, significantly influenced the observed variance ($P = 0.0013$ and $P = 0.0016$, respectively).

and 1 μM aquaria, the area covered by cyanobacteria did not represent more than 2% on day 25 (Fig. 2). In contrast, the cyanobacterial area represented 11% of the biofilms, at the end of the experiment, in 3 and 10 μM aquaria. The physiognomy of the biofilms was also affected by the growth of the chain-forming diatom *Melosira varians*, changing from long filaments to short tufts.

The genetic analyses of phototrophs included eukaryotes and cyanobacteria. The eukaryotic NMDS representation of the DGGE data indicates that there was a community shift in all the aquaria, including the control (Fig. 3A). The community of the 10 μM treatment shifted in a different direction from those for the three other treatments (with a larger amplitude). A global test analysis with Cu as the factor explaining the similarity distribution by using the global ANOSIM method gave a significance level above the usual acceptance level of 5% (6.6%; $R = 0.159$). Only the DGGE profiles for the 1 and 10 μM treatments could be significantly separated from each other in the pairwise analysis. The NMDS analysis of the cyanobacterial community did not reveal any consistent trend in the changes observed (Fig. 3B), although the global ANOSIM analysis of data revealed that cyanobacterial profiles were significantly different from one another with respect to Cu treatment.

Bacterial compartment. Eight different samples taken from the natural community at day 0 showed differences in CLPPs (Fig. 4). However, exposure to high Cu concentrations (3 and 10 μM) did affect the substrate utilization capacity of the community. Both Cu concentrations measured in the biofilm and the incubation time were correlated with changes in the substrate utilization capacity of the communities ($P = 0.0013$ and $P = 0.0016$, respectively, by redundancy analysis with Monte Carlo permutation).

In the bacterial-community DGGE profiles, 66 different sequence types could be detected (Fig. 5). NMDS analysis of the DGGE bacterial profiles showed that a clear change occurred in the 10 μM Cu treatment after 10 days of exposure (Fig. 6). ANOSIM analysis revealed a global test significance level of 0.1%, considering Cu as an ordination factor ($R = 0.352$). All possible pairs of samples were significantly different from each other when tested by pairwise analysis. The NMDS representation also suggested that there was a gradual effect of Cu on the communities (Fig. 6). The similarity values between samples taken after 3 and 24 days of Cu exposure from the control, 1 μM, 3 μM, and 10 μM aquaria were 61, 43, 23, and 21%, respectively. A closer look at the bacterial DGGE profiles, excluding sampling days 10, 17, and 24 for the 10 μM aquarium, showed that the control data points all clustered in one area of the NMDS plot, indicating that the bacterial community of the control showed relatively little change during the 4 weeks of the experiment (NMDS representation not shown). From day 0 to day 3, all communities showed relatively high similarity, except for those of the 10 μM aquarium, and the control communities were closest to the natural situation (Fig.

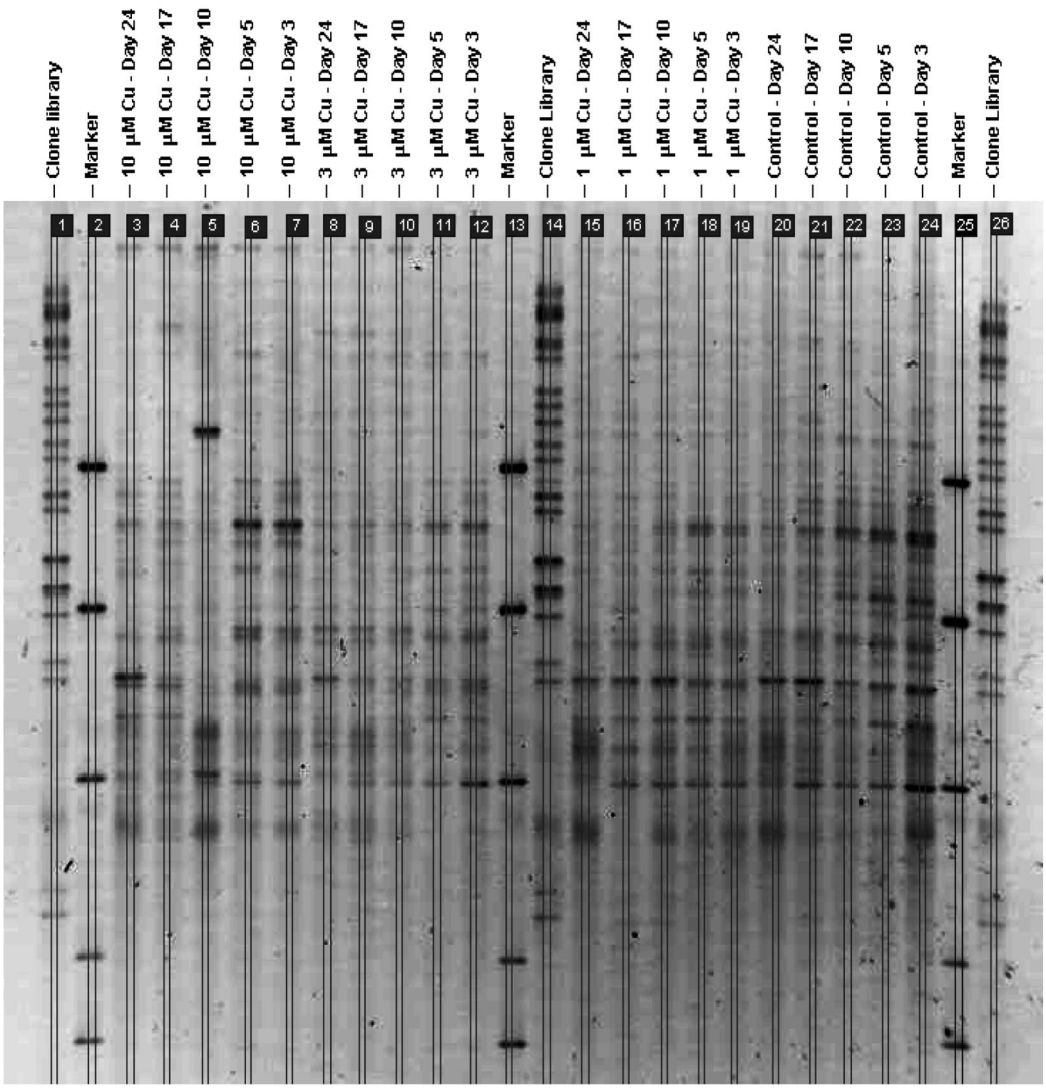

FIG. 5. Bacterial DGGE gel picture of biofilm samples exposed to 0, 1, 3, and 10 μM Cu and sampled on days 3, 5, 10, 17, and 24 of the exposure period. Gel image analysis was performed by using the Phoretix 1D software package. Background was subtracted, and bands were automatically detected and manually corrected. A band percentage matrix was generated, and sample similarities were analyzed by NMDS.

7). The average similarity value of the bacterial communities at day 3 compared to day 0 was 78% for the control aquarium, 65% for the 1 μM aquarium, 65% for the 3 μM aquarium, and 39% for the 10 μM aquarium.

DISCUSSION

We focused our study on the development of communities, derived from a single natural community, exposed to a range of copper concentrations. We chose the DGGE technique for profiling the community as one of the better options for a time series study (7, 9, 11). DGGE data have been used mainly as qualitative data in previous statistical analyses (14, 36). However, more information can be obtained if the data are treated quantitatively (28). Therefore, we used band percentages as estimators of the relative sequence abundances in a sample.

The phototrophic compartment of the biofilms was dominated by chlorophytes and cyanobacteria. Heterotrophic eukaryotes were present at low abundance throughout the experiment, suggesting that changes in the eukaryotic DGGE profiles were mainly due to changes in the eukaryotic phototrophic community. The eukaryotic community exposed to 10 μM Cu changed over time, but the extent of this change did not exceed the change over time in the control aquarium. Consequently, no correlation between change and Cu treatment can be supported by analysis of DGGE patterns. The changes in cyanobacterial DGGE profiles did not show a consistent direction related to the Cu treatment. The organic matter contents of biofilms exposed to intermediate Cu concentrations decreased significantly. In addition, the chlorophyll *a* content decreased, indicating a possible decay of the biofilm as a whole. Intriguingly, at first glance, the ratio between the organic and inorganic matter contents of the biofilms from the 10 μM aquarium did not vary significantly during the experiment, indicating that the microorganisms were continuously producing biomass. However, the chlorophyll *a* contents of the biofilms from

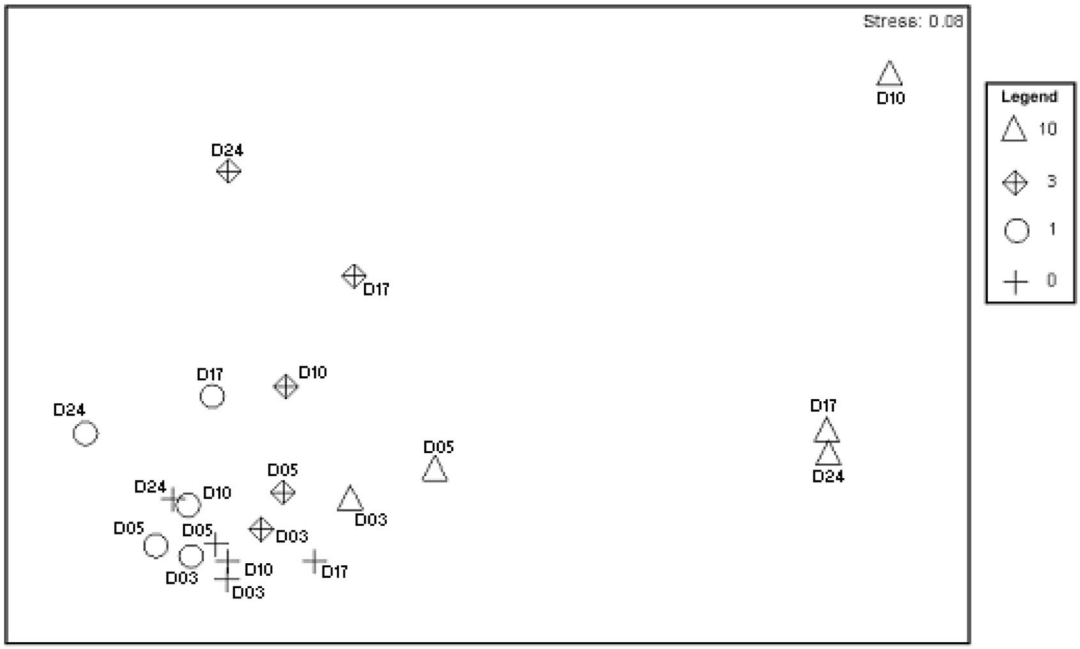

FIG. 6. Two-dimensional representation of NMDS of the bacterial communities of biofilms exposed to Cu concentrations ranging from 0 to 10 μM and sampled on days 3, 5, 10, 17, and 24 of the Cu exposure period.

the 10 μM aquarium decreased significantly, and the corresponding decrease in photosynthetic efficiency, Φ_{PSII}, indicates that phototrophic organisms were affected by the Cu stress. This could be explained in two ways. One explanation may be that the phototrophic community was initially dominated by green algae rich in chlorophyll *a* and later by phototrophic organisms with low chlorophyll *a* content. Another explanation may be that it was mainly the heterotrophic microorganisms that produced the biomass.

The DGGE analysis makes clear that bacterial communities in all aquaria changed over time. The extent of the change was proportional to the copper concentration, with dramatic changes occurring in biofilms exposed to the highest concentration after 10 days of exposure, when the Cu concentration in

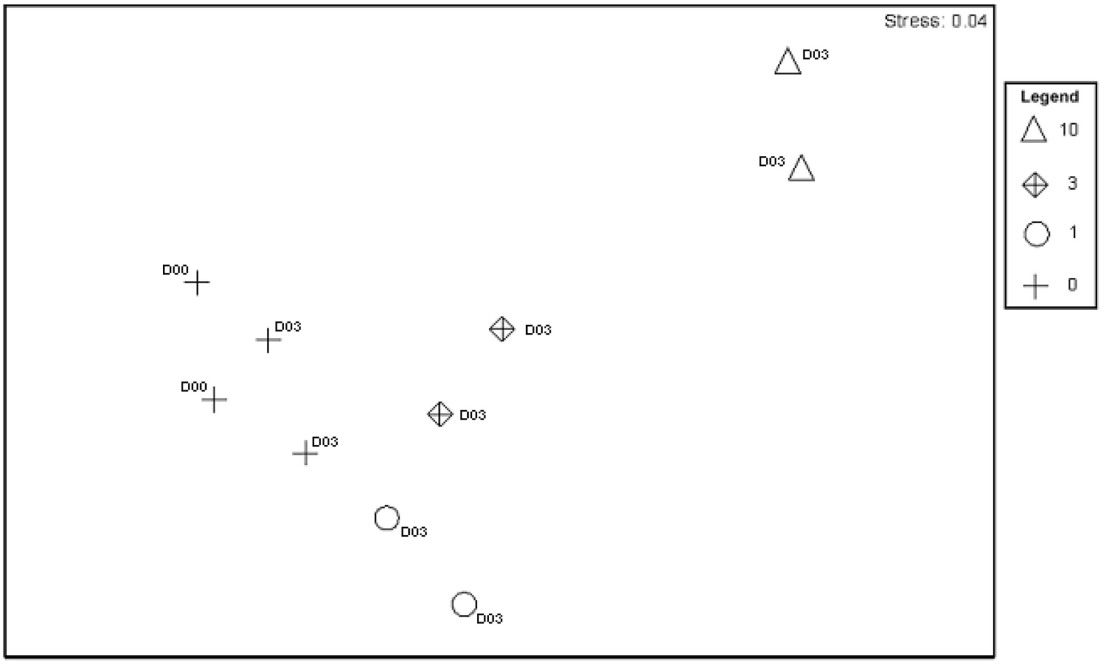

FIG. 7. Two-dimensional representation of NMDS of the bacterial communities of biofilms exposed to Cu concentrations ranging from 0 to 10 μM and sampled on days 0 and 3.

these 10 μM biofilms reached a plateau at 100 μmol/g. The proportionality of the change demonstrates that copper is indeed a strong driver of structural changes. The increasing divergence of the bacterial-community structures in the different treatments coincided with the development of differences in metabolic capacities as evidenced by CLPP analysis. The main explanatory variable for the metabolic capacity differences was the copper concentration in the biofilm. Thus, it appears that copper directly or indirectly causes both structural and physiological changes in the bacterial community. The proportional effect of Cu as observed here is in contrast to observations by Konstantinidis et al. (22), who used terminal restriction fragment length polymorphism to study bacteria along a depth profile in two copper-contaminated lake sediments. These authors did not detect large differences between bacterial communities growing at Cu concentrations ranging from 3 to 87 μM. Our results suggest that the complex and organized structure of the biofilm (10, 18, 30) would not protect the bacterial community in the same way that sediments do, even through the formation of extracellular polymeric substances was found to increase several resistance capacities of each encased organism (24) by reducing the bioavailability of heavy metals (33).

Short- and long-term toxicity tests of heavy metals on aquatic biofilms have focused either on the response of the phototrophic compartment (1, 2, 5, 17) or on that of the heterotrophic compartment (34), but toxic effect assessments based on physiological tests (3, 19) or studies considering the biofilm as a whole have not investigated the effect of Cu (6, 23). Possible interactions between phototrophic and heterotrophic compartments of a biofilm may be disturbed when one compartment is severely affected by a stress factor, e.g., an increase in toxicant concentration. In a recent publication, Barranguet et al. (4) reported preliminary results providing insights on the disturbance by Cu of the relationship between algae and bacteria of a biofilm. We extend the knowledge of this relationship by showing that Cu can affect the bacterial community both physiologically and structurally without an apparent link to disturbance of the phototrophic community. Further studies are needed both on the metabolic relationship between phototrophic and heterotrophic organisms and on the relationship between physiological community changes and structural changes.

ACKNOWLEDGMENTS

We thank Gerdit Greve for technical assistance.

This work is part of the Stimulation Programme System-Oriented Ecotoxicological Research (SSEO) project "Changing consortia of algae and bacteria in polluted rivers and lakes: discriminating between the roles of toxicants and natural variables" and is supported by the Netherlands Organisation for Scientific Research (NWO).

REFERENCES

1. **Barranguet, C., E. Charantoni, M. Plans, and W. Admiraal.** 2000. Short-term response of monospecific and natural algal biofilms to copper exposure. Eur. J. Phycol. **35:**397–406.
2. **Barranguet, C., M. Jonker, J. Sinke, and W. Admiraal.** 2000. Effects of acute copper contamination on photosynthesis and biomass of periphyton determined with the pulse amplitude modulated fluorescence technique. Verhandl. Int. Vereinig. Limnol. **27:**3195–3198.
3. **Barranguet, C., M. Plans, E. Van der Grinten, J. J. Sinke, and W. Admiraal.** 2002. Development of photosynthesis biofilms affected by dissolved and sorbed copper in a eutrophic river. Environ. Toxicol. Chem. **21:**1955–1965.
4. **Barranguet, C., F. P. Van den Ende, M. Rutgers, A. M. Breure, M. Greijdanus, J. J. Sinke, and W. Admiraal.** 2003. Copper-induced modifications of the trophic relations in riverine algal-bacterial biofilms. Envir. Toxicol. Chem. **22:**1340–1349.
5. **Blanck, H., S. Å. Wängberg, and S. Molander.** 1988. Pollution-induced community tolerance—a new ecotoxicological tool, p. 219–230. *In* J. Cairns, Jr., and R. Pratt (ed.), Functional testing of aquatic biota for estimating hazards of chemicals. ASTM STP 988. American Society for Testing and Materials, Philadelphia, Pa.
6. **Blanck, H., W. Admiraal, R. F. M. J. Cleven, H. Guash, M. A. G. T. van den Hoop, N. Ivorra, B. Nyström, M. Paulsson, R. P. Petterson, S. Sabater, and G. M. J. Tubbing.** 2003. Variability in zinc tolerance, measured as incorporation of radio-labeled carbon dioxide and thymidine, in periphyton communities sampled from 15 European river stretches. Arch. Environ. Contam. Toxicol. **44:**17–29.
7. **Casamayor, E. O., C. Pedró-Alió, G. Muyzer, and R. Amann.** 2002. Microheterogeneity in 16S rDNA-defined bacterial populations from a stratified planktonic environment is related to temporal changes and to ecological adaptations. Appl. Environ. Microbiol. **68:**1706–1714.
8. **Clarke K. R., and R. M. Warwick.** 2001. Change in marine communities: an approach to statistical analysis and interpretation, 2nd ed. PRIMER-E, Plymouth, United Kingdom.
9. **Crosby, L. D., and C. S. Criddle.** 2003. Understanding bias in microbial community analysis techniques due to *rrn* operon copy number heterogeneity. BioTechniques **34:**790–802.
10. **Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costeron, and E. P. Greenberg.** 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280:**295–298.
11. **Ferris, M. J., and D. M. Ward.** 1997. Seasonal distributions of dominant 16S rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. **63:**1375–1381.
12. **Field, J. G., K. R. Clarke, and R. M. Warwick.** 1982. A pratical strategy for analysing multispecies distribution patterns. Mar. Ecol. Prog. Ser. **8:**37–52.
13. **Fowler, J., L. Cohen, and P. Jarvis.** 1998. Practical statistics for field biology. John Wiley & Sons Ltd., Chichester, United Kingdom.
14. **Fromin, N., J. Hamelin, S. Tarnawski, D. Roesti, K. Jourdain-Miserez, N. Forestier, S. Teyssier-Cuvelle, F. Gillet, M. Aragno, and P. Rossi.** 2002. Statistical analysis of denaturing gel electrophoresis (DGE) fingerprint patterns. Environ. Microbiol. **4:**634–643.
15. **Gibson, C. E.** 1972. The algicidal effect of copper on a green and a blue-green alga and some ecological implications. J. Appl. Ecol. **9:**513–518.
16. **Grant, J., L. van Hulst, and L. van Zon.** 1999. Puin, pijpekoppen en potscherven. M.S. thesis. Free University, Amsterdam, The Netherlands.
17. **Gustavson, K., S. Pertersen, B. Pedersen, F. Stuer-Lauridsen, S. Pedersen, and S. Å. Wänberg.** 1999. Pollution-induced community tolerance (PICT) in coastal phytoplankton communities exposure to copper. Hydrobiologia **416:** 125–138.
18. **Hammer, B. K., and B. L. Bassler.** 2003. Quorum sensing controls biofilm formation in *Vibrio cholerae.* Mol. Microbiol. **50:**101–114.
19. **Ivorra, N., J. Hettelaar, G. M. J. Tubbing, M. H. S. Kraak, S. Sabater, and W. Admiraal.** 1999. Translocation of microbenthic algal assemblages used for in situ analysis of metal pollution in rivers. Arch. Environ. Contam. Toxicol. **37:**19–28.
20. **Janse, I., M. Meima, W. E. A. Kardinaal, and G. Zwart.** 2003. High-resolution differentiation of cyanobacteria using rRNA-internal transcribed spacer denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. **69:**6634–6643.
21. **Kolbowski, J., and U. Schreiber.** 1995. Computer-controlled phytoplankton analyzer based on 4-wavelengths PAM chlorophyll fluorometer, p. 825–828. *In* P. Mathis (ed.), Photosynthesis: from light to biosphere, vol. V. Proceedings of the Xth International Photosynthesis Congress. Kluwer Academic Publishers, Dordrecht, The Netherlands.
22. **Konstantinidis, K. T., N. Isaacs, J. Fett, S. Simpson, D. T. Long, and T. L. Marsh.** 2003. Microbial diversity and resistance to copper in metal-contaminated lake sediment. Microb. Ecol. **45:**191–202.
23. **Lehmann, V., G. M. J. Tubbing, and W. Admiraal.** 1999. Induced metal tolerance in microbenthic communities from three lowland rivers with different metal loads. Arch. Environ. Contam. Toxicol. **36:**384–391.
24. **Lewis, K.** 2001. Riddle of biofilm resistance. Antimicrob. Agents Chemother. **45:**999–1007.
25. **Lorenzen, C. J.** 1967. Determination of chlorophyll and pheo-pigments: spectrophotometric equations. Limnol. Oceanogr. **12:**343–346.
26. **McKnight, D.** 1981. Chemical and biological processes controlling the response of a freshwater ecosystem to copper stress: a field study of the $CuSO_4$ treatment of Mill Pond reservoir, Burlington, Massachusetts. Limnol. Oceanogr. **26:**518–531.
27. **Murphy, J., and J. P. Riley.** 1962. A modified single solution method for the determination of phosphate in natural waters. Anal. Chim. Acta **27:**31–36.
28. **Muylaert, K., K. Van der Gucht, N. Vloemans, L. De Meester, M. Gillis, and W. Vyverman.** 2002. Relationship between bacterial community composition and bottom-up versus top-down variables in four eutrophic shallow lakes. Appl. Environ. Microbiol. **68:**4740–4750.
29. **Muyzer, G., E. C. De Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis

analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:**695–700.

30. **O'Toole, G., H. B. Kaplan, and R. Kolter.** 2000. Biofilm formation as microbial development. Annu. Rev. Microbiol. **54:**49–79.
31. **Preston-Mafham, J., L. Boddy, and P. F. Randerson.** 2002. Analysis of microbial community functional diversity using sole-carbon-source utilization profiles—a critique. FEMS Microbiol. Ecol. **42:**1–14.
32. **Rohácek, K., and M. Barták.** 1999. Technique of the modulated chlorophyll fluorescence: basic concepts, useful parameters, and some applications. Photosynthetica **37:**339–363.
33. **Teitzel, G. M., and M. R. Parsek.** 2003. Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa.* Appl. Environ. Microbiol. **69:**2313–2320.
34. **Tubbing, D. M. J., W. Admiraal, and A. Katako.** 1995. Successive changes in bacterioplankton communities in the river Rhine after copper additions. Environ. Toxicol. Chem. **14:**1507–1512.
35. **Van Hannen, E. J., W. Mooij, M. P. van Agterveld, H. J. Gons, and H. J. Laanbroek.** 1999. Detritus-dependent development of the microbial community in an experimental system: qualitative analysis by denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. **65:**2478–2484.
36. **Van Hannen, E. J., G. Zwart, M. P. van Agterveld, H. J. Gons, J. Ebert, and H. J. Laanbroek.** 1999. Changes in bacterial and eukaryotic community structure after mass lysis of filamentous cyanobacteria associated with viruses. Appl. Environ. Microbiol. **65:**795–801.
37. **Watson, G. H., and W. B. Bollen.** 1952. Effect of copper sulphate weed treatment on bacteria in lake bottoms. Ecology **33:**522–529.