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Functional recovery of biofilm bacterial communities after copper exposure

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Abstract

Potential of bacterial communities in biofilms to recover after copper exposure was investigated. Biofilms grown outdoor in shallow water on glass dishes were exposed in the laboratory to 0.6, 2.1, 6.8 μmol/l copper amended surface water and a reference and subsequently to un-amended surface water. Transitions of bacterial communities were characterised with denaturing gradient gel electrophoresis (DGGE) and community-level physiological profiles (CLPP). Exposure to 6.8 μmol/l copper provoked distinct changes in DGGE profiles of bacterial consortia, which did not reverse upon copper depuration. Exposure to 2.1 and 6.8 μmol/l copper was found to induce marked changes in CLPP of bacterial communities that proved to be reversible during copper depuration. Furthermore, copper exposure induced the development of copper-tolerance, which was partially lost during depuration. It is concluded that bacterial communities exposed to copper contaminated water for a period of 26 days are capable to restore their metabolic attributes after introduction of unpolluted water in aquaria for 28 days.

1. Introduction

Bacterial communities are susceptible to anthropogenic disturbances like changes in oxygen concentrations and exposure to toxicants. For example, copper has been shown to modify the structure and physiology of bacterial communities. Copper exposure was also shown to change metabolism and induced the development of tolerance to copper in freshwater biofilms (Vymazal, 1984, Barranguet et al., 2002, Barranguet et al., 2003 and Massieux et al., 2004). However, the capability of bacterial communities to recover i.e. to return to the reference situation after toxicant exposure has rarely been investigated (Kelly and Tate, 1998, Griffiths et al., 2001, Kostov and Van Cleemput, 2001 and Kiikkila et al., 2001).

Species diversity has sometimes been correlated to the capability of complex communities to recover after disturbance. Tilman and Dowing (1994), for instance, observed that the productivity of plant communities resisted disturbances when numerous plant species were present. Similar results were obtained for bacterial communities by Griffiths et al. (2000). They found that physiological recovery from chemical stress was more efficient at high bacterial diversity. Bacterial communities are generally very diverse and a high functional redundancy has been presumed so that communities may recover from a disturbance without losing any attributes (Finlay et al., 1997). However, to functionally recover from a disturbance does not necessarily mean that the community returns to its original species composition.

The present study aimed to test the potential of bacterial communities in freshwater biofilms to recover after copper exposure. Since no single method is available to completely characterize a bacterial community, a combination of methods was applied to obtain a more comprehensive view of community responses to copper stress (Westergaard et al., 2001). Genetic analysis based on denaturing gradient gel electrophoresis (DGGE) and physiological analysis based on community-level physiological profiling (CLPP) were combined with changes in community copper-tolerance, according to the concept of pollution-induced community tolerance (PICT; Blanck et al., 1988).

2. Materials and methods

2.1. Experimental set-up

Biofilms were grown on glass discs suspended in a ditch of the polder Demmerik in Vinkeveen (The Netherlands: 52°13′ North 4°56′ East) during 4 weeks in April 2002. In total, about 1600 glass discs (each $\sim$1.5 cm^2) on polyethylene racks (Ivorra et al., 1999) were colonised as well as 16 rectangular glass plates (each $\sim$360 cm^2). The glass discs were used for abiotic analyses whereas the rectangular glass plates were used for the analysis of community-level physiological profiles (CLPP), denaturing gradient gel electrophoresis (DGGE) and pollution-induced community tolerance (PICT). The colonised discs were transferred to acid pre-rinsed aquaria in the laboratory. Aquaria were filled with filtered water (pore size of 15 μm) from the same ditch of the polder Demmerik to avoid depletion or accumulation of nutrients. The water in the aquaria was renewed every week with water from the ditch. The phosphate concentration was measured once a week (Murphy and Riley, 1962). The phosphate levels was 0.28 ± 0.29 μmol/l, the phosphate concentrations were similar between the copper treatments, but was reduced in time, therefore, weekly water refreshments was needed and performed. The light regime was kept constant during the experiment (light 16 h: dark 8 h: light-intensity of 100 μmol m^{-2} s^{-1}, measured with Li Cor LI-1400 equipped with a Li Cor PAR Sensor) sustaining microbenthic algae in the biofilm. The aquaria were continuously aerated and the temperature was controlled to 20 °C.

Each aquarium contained 42 l of filtered field water, refreshed weekly, two racks, each rack carrying ca. 200 colonised glass discs, and three rectangular glass plates (total biofilm area per aquarium: $\sim$1680 cm^2). Four aquaria were used, one reference and three copper treated aquaria (nominal concentrations 1, 3 and 10 μmol/l: $CuCl_2$ Tritisol, Merck, Darmstadt, Germany, from a stock solution of 1 g/l). This period will be further referred to as "the exposure period". After the exposure period, which lasted 26 days, two rectangular plates colonised with biofilms were removed from the copper containing aquaria and the reference aquarium and transferred to acid pre-rinsed aquaria (21 l). The biofilms were

incubated for another 28 days in the new non-copper amended water from the field site. This period will be further referred to as "the recovery period".

2.2. Biofilm harvesting

The biofilm was harvested from several places on the glass plate using a sterile sharp razor blade (ca. 60 cm^2, ca. 4% of the total biofilm biomass was removed keeping the probability of sampling the same bacterial community low). The samples from each aquarium were pooled in two tubes, one for DGGE and one for CLPP and PICT. For practical reasons, the treatments were not replicated. However, temporal pseudo-replications were made. The biofilms were harvested on several occasions along the experiment: days 0, 3, 5, 12, 19, 26, 33, 41 and 54. Hurlbert (1984) stated that this procedure is proper when the successive dates are not taken as independent replicates of a treatment, which was avoided here.

2.3. Denaturing gradient gel electrophoresis (DGGE)

The genetic structure of the bacterial communities of every aquarium was characterised using DGGE. Bacterial specific polymerase chain reaction (PCR)-DGGE was performed according to the method described by Muyzer et al. (1993) and further adapted by Massieux et al. (2004). Bacterial specific primers were used, targeting one specific region of the 16S rRNA gene (Muyzer et al., 1993). The DGGE gradient of the denaturing agents, urea and formamide, was ranging from 35% at the top to 60% at the bottom of the gel. The gel was submerged in a Tris–acetate–EDTA buffer (0.5 × TAE) containing 40 mM Tris, 40 mM acetic acid, 1 mM EDTA, pH 7.6 and submitted to 75 V for 16 h. The DGGE gel was analysed using the relative band intensity (Muylaert et al., 2002) in Phoretix 1D as described in Massieux et al. (2004).

2.4. Community-level physiological profiles (CLPP)

Prior to the determination of CLPP and PICT, the harvested biofilm was mixed with 5 ml BisTris buffer (10 mM; pH 7; 4 °C), mixed on a tissue homogeniser (25,000 rpm) and sonicated, 1 pulse of 5 s (68 watts). The samples were maintained in melting ice. The biofilm suspensions were then centrifuged to remove most algae (10 min at 500 *g*). The optical density of the resulting bacterial suspensions was measured at 750 nm for every aquarium. This optical density was used to standardise the inoculum size in CLPP determination (ECO-plates: BIOLOG Inc. Hayward, USA). Each ECO-plate contains a mineral medium, the tetrazolium redox dye and a set of 31 different carbon substrates plus a control, in triplicates (Garland, 1997). The homogeneous suspensions were transferred to 2 ml plastic tubes that were rapidly frozen in liquid nitrogen, and maintained at −70 °C until analysis. These frozen extracts were used for CLPP analysis and PICT determination.

Bacterial dilution series (3^{-1} until 3^{-9}) were made to inoculate the ECO-plates. The plates were measured with a spectrophotometer at a wavelength of 590 nm every 8 h for 7 days. The plates were incubated in the dark at 20 °C and a humidity of ≈85%. The CLPPs were calculated based on the response in the 31 individual substrates that was corrected for the response of the average colour in the plate (AWCD: average well colour development) according to the procedure described by Van Elsas et al. (in press) and Boivin et al. (2005).

The number of substrates being utilised per heterotrophic bacterial community was calculated by counting the wells with a higher colour formation than 0.3 (absorbance at 590 nm after 7 days of incubation).

To survey the activity of the bacterial community in the ECO-plate, the AWCD was calculated after 3 days incubation. This AWCD was given per biofilm surface (AWCD abs/cm^2).

Simultaneously with CLPP determination the number of colony forming units (CFUs) in the standardised sample was determined by plating on Tryptone Soya Broth agar (0.3 g/l) and calculated per unit of biofilm surface (cm^2). The agar plates were incubated in the dark at 25 °C for a period of 8 days.

2.5. Determination of bacterial PICT

The frozen bacterial suspensions were thawed and diluted in buffer (BisTris; pH 7; 100 mM) to an activity equivalent to an AWCD of 0.8 abs at 590 nm after 7 days of incubation in ECO-plates at 20 °C. Precise inoculum standardization is necessary because the PICT response is dependent on inoculum density (Van Beelen et al., 2004). These diluted bacterial suspensions were exposed to $CuCl_2$ (0, 0.2, 0.5, 2, 5, 20, 50, 160, 470, 1570 μmol/l) for 4 h at 20 °C. Subsequently, the copper-exposed suspensions were inoculated in BIOLOG® GN2 plates (100 μl per well). These plates contain a mineral medium, the tetrazolium redox dye and a set of 95 different carbon substrates plus a control (no carbon).

The highest colour development after 7 days of incubation was used for quantification of the microbial response and subsequently for calculation of the EC_{50}-values. The EC_{50}-values were calculated by fitting the data with a log normal sigmoidal curve, using a non-linear regression (GraphPad Prism, version 2.01, June 1996) (Boivin et al., 2005). Based on previous investigations at our laboratory, EC_{50}-values with a standard error lower than 2 and a correlation coefficient higher than 0.5 were found appropriate limits for the calculation of PICT.

Cumulative sensitivity distributions of the bacterial communities, so-called substrates-utilization sensitivity distributions (SuSDs), were constructed using the $EC_{50,\ well\ fitted}$. From these SuSDs, a median was calculated using GraphPad Prism and given as *a* SuSD (median ($EC_{50,\ well\ fitted}$)). PICT is present if the *a* SuSD significantly increases when incubated in the presence of increased toxicant concentrations.

2.6. Determination of copper concentrations

An aliquot of 1 ml of water (filtered and unfiltered) was taken in triplicates from every aquarium before and after Cu was added during the exposure period and before and after the water was refreshed during the recovery period. An aliquot of 1 ml of water was acidified with 30 μl HNO_3 (65% p.a. Merck). Three glass discs per treatment were sampled every week for metal analysis. Biofilm samples were freeze-dried over night and 1 ml of 65% HNO_3 was added to the samples, which were kept at room temperature for 2 days. The samples were then centrifuged at 10,000 *g* for 5 min to remove suspended particles. Water and biofilm samples were measured on a flame atomic absorption spectrophotometer (AAS; Perkin Elmer 110B, Boston, USA; detection limit 50 μg/l). Samples with a concentration lower than 80 μg/l were determined on an AAS graphite furnace (Perking Elmer, 5100 PC, Boston, USA; with background correction, detection limit 3 μg/l).

2.7. Statistics

The shifts between bacterial communities determined with DGGE profiling were graphically represented in a non-parametric multi-dimensional scaling ordination (NMDS), which represents the matching similarities calculated in a triangular matrix of similarity coefficients computed between every pair of samples (Clarke and Warwick, 1998). The shifts observed in the NMDS representation were assessed using the analysis of the similarity functions (ANOSIM) within the Primer 5 software package.

Multivariate analysis was applied to analyse the changes in CLPPs between the different bacterial communities using the software package Canoco V.4.0 (CPRO-DLO, Wageningen, The Netherlands). The CLPPs were first analysed with a detrended component analysis (DCA). The length of gradient (based on DCA) was lower than 3 (Van Wijngaarden et al., 1995) and therefore the different samples were collated in a redundancy analysis (RDA) using sampling day in the aquaria as a covariable. Subsequently, principal response curves (PRC) were made (Van den Brink et al., 1999). For each time point the effect of copper treatments on the bacterial communities was calculated relative to its reference (0 μmol/l Cu). Therewith the variation of copper-exposed bacterial communities in relation to the reference (the vertical axis) is plotted versus time (horizontal axis). The explained variation in the PRC is given by the eigenvalue. The influence of copper on differences in CLPPs of the different bacterial communities was tested taking the biofilm copper concentrations as a continuous variable using a Monte Carlo permutation test in an RDA. To test for significance of copper: 9999 permutations were performed and a significance level of 0.05 was chosen. Unfortunately, the sample from 1 μmol/l treatment sampled on day 54 is not shown. Due to a manipulation error the sample was discarded from the analysis.

A one-way ANOVA was used to statistically compare the AWCD and the number of CFUs between the different sampling dates and between the different treatments (copper and temperature).

To test the significance of the *α* SuSD shifts, one-way ANOVA was performed (data were assumed to be normally distributed and the variance independent). Because more than two samples were compared, a Bonferroni correction was applied. In a Bonferroni correction, the *α* level is adjusted downward and to consider chance capitalization more comparisons are made.

A one-way ANOVA was used to statistically compare the copper concentration in the water and in the biofilm between the different sampling days and between the different copper treatments.

3. Results

3.1. Copper accumulation in the biofilm

Ditch water and biofilms grown in the ditch contained, respectively, 0.11 ± 0.09 μmol/l and 0.05 ± 0.04 μg copper/mg per dry weight biofilm. Copper concentration in biofilms on the glass plates stabilized at levels of 98.5 ± 22.5, 15.6 ± 1.9 and 3.8 ± 1.2 μmol/g DW during exposure to actual copper concentrations of, respectively, 6.8 ± 2.0, 2.1 ± 0.7 and 0.6 ± 0.2 μmol/l in the water (Fig. 1). This accumulation of copper in the biofilms was roughly proportional to the concentration in the water. The copper concentration in the biofilm exposed to 6.8 μmol/l copper decreased from 120 ± 0.01 μmol/g DW at the beginning of the recovery period (day 26) to 25.5 ± 3.8 μmol/g DW at the

end of the period (day 54). Therewith, the copper concentration in the copper-exposed biofilm at the end of the recovery period was still 25 times higher than the copper concentration in the reference biofilm, indicating that four water refreshments were not sufficient to allow for a complete biofilm copper depuration. Similar trends were observed in the other treatments. To exclude any release of copper from the aquaria walls, clean aquaria were used during the recovery period.

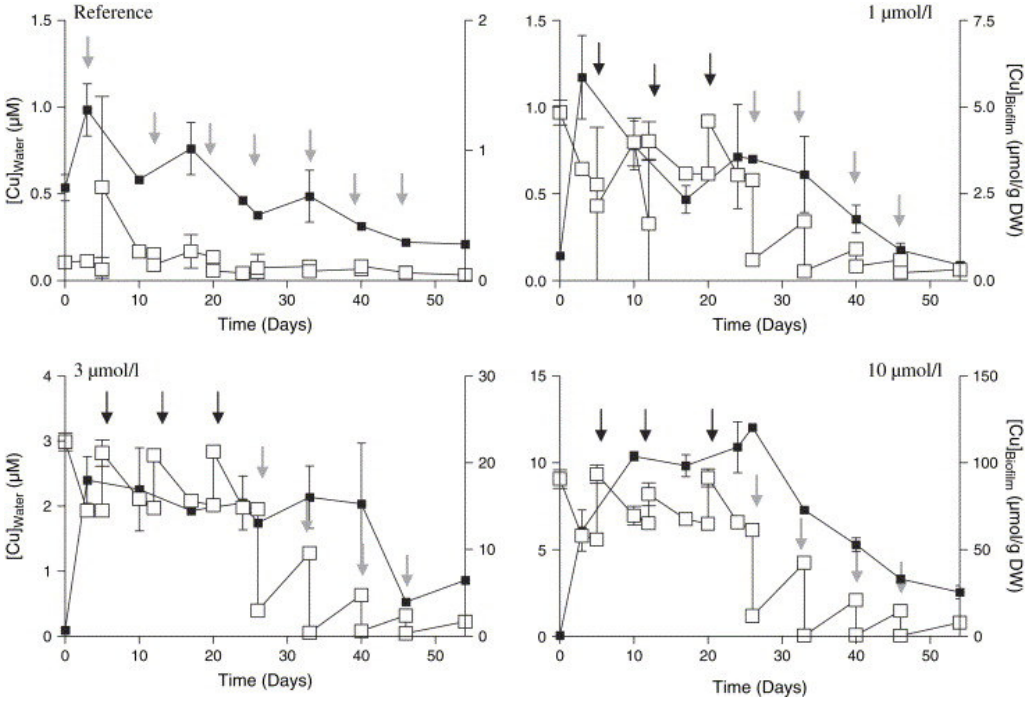

Fig. 1. Actual copper concentrations in four aquaria following regular additions of copper (total nominal concentrations: 1, 3 and 10 µmol/l, and a reference). Open symbols: copper concentrations in water (µmol/l). Closed symbols: copper concentrations in the biofilms (µmol/g DW). Black arrows indicate copper containing water refreshments. Grey arrows indicate water refreshment without copper. Error bars indicate standard deviations.

3.2. Denaturing gradient gel electrophoresis (DGGE)

Fig. 2 shows the results of the analysis of similarities between DGGE patterns observed for the different biofilms expressed in a non-parametric multi-dimensional analysis (NMDS: stress factor = 0.08). The composition of bacterial communities changed over time in all aquaria. In addition, during the exposure period, the bacterial community from the most polluted biofilm (exposed to 6.8 µmol/l Cu) had a significantly different banding pattern (p = 0.03) from the bacterial communities in the reference, 0.6 and 2.1 µmol/l copper-exposed biofilms. Fewer and different bands were present in the community exposed to the highest copper concentration. These differences were more obvious during prolonged incubation. In conclusion, the aquaria treated with 6.8 µmol/l copper showed the largest deviation from the control aquarium with respect to the structure of the microbial community.

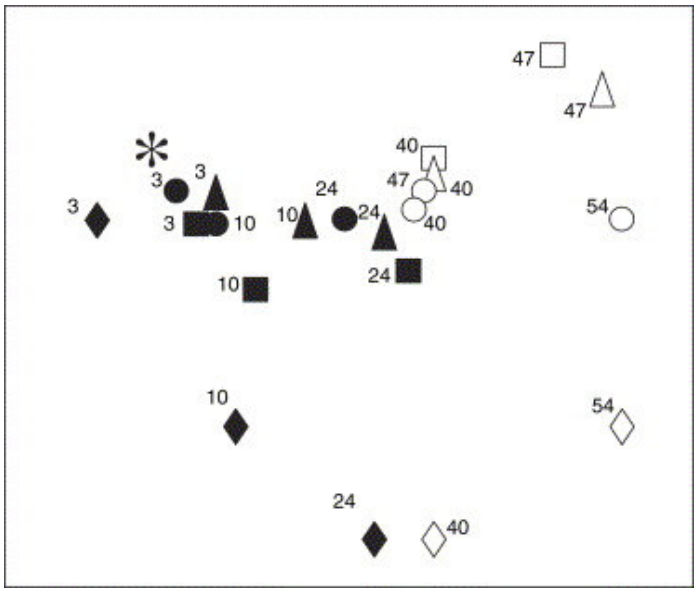

Fig. 2. Non-metric multi-dimensional scaling (MDS) of denaturing gradient gel electrophoresis (DGGE) of biofilm samples from different aquaria. The asterisk represents the biofilm collected from glass plates in the ditch (field situation). Copper treatments: reference (●), 1 μmol/l Cu (▲), 3 μmol/l Cu (■) and 10 μmol/l Cu (♦), respectively. The sampling day is written beside each symbol. Closed symbols represent the exposure period. Open symbols represent the recovery period.

Fewer and different bands were still present in the community exposed to 6.8 μmol/l copper compared to the other treatments. During the recovery period the structure of the bacterial community from the 6.8 μmol/l copper biofilm was still significantly different (ANOSIM: $p > 0.5$) from that in the other three aquaria that were treated with 0.6, 2.1 μmol/l copper and the reference. Assuming that the differences between aquaria are caused by the different treatment levels of copper it is concluded that exposure to 6.8 μmol/l copper provoked distinct changes in DGGE profiles of bacterial consortia, which under the given experimental conditions did not reverse upon weekly addition of unpolluted water.

3.3. Community-level physiological profiles (CLPP)

Fig. 3 represents CLPP shifts expressed in principal response curves (PRC). The first part of the PRC represents the copper exposure period (days 0–26) whereas the second part represents the recovery period (days 26–54). A decrease in the canonical coefficient corresponds to fewer substrates used or to limited carbon-source utilization than used in the reference. After 12 days of exposure to 2.1 and 6.8 μmol/l copper, bacterial communities differed the strongest from the reference. These differences were maintained until day 33 ($p = 0.008$).

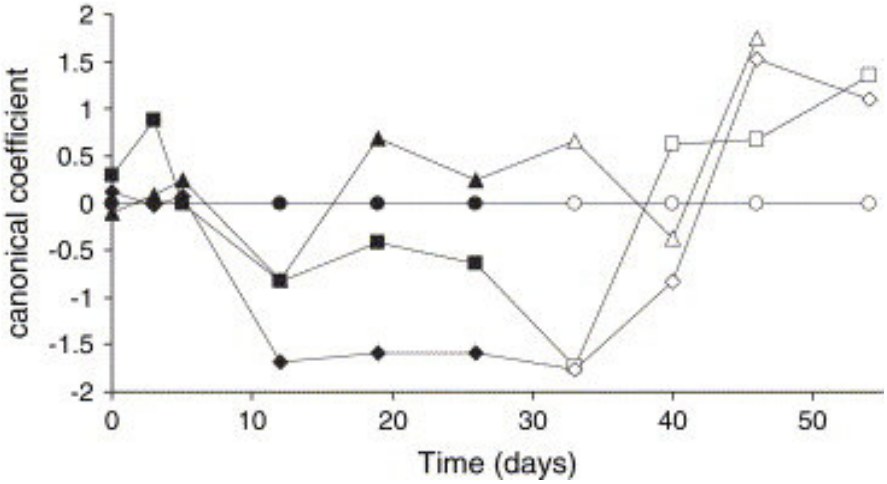

Fig. 3. Principal response curves (PRC) of community-level physiological profiles (CLPP) of aquatic bacterial communities from several biofilms. From days 0 until 26, the biofilms were exposed to different copper concentrations and from days 26 until 54 all the aquaria were refreshed with ditch water without copper addition. Closed symbols represent the exposure period. Open symbols represent the recovery period. Copper treatments: 0 μmol/l Cu (●), 1 μmol/l Cu (▲), 3 μmol/l Cu (■) and 10 μmol/l Cu (♦), respectively.

Based on Monte Carlo permutation test, the first axis of the PRC explains 11% of the variation between the different bacterial communities and the differences between the bacterial communities explained in the PRC were significant (p = 0.006). The decrease in the canonical coefficient associated with different metabolic profiles were primarily due to lower utilization of β-methyl-D-glucoside, D-xylose, α-cyclodextrin, glycyl-L-glutamic acid, glucose-1-phosphate, D,L-α-glycerol phosphate, and a higher utilization of I-erythritol, 2-hydroxy benzoic acid, 4-hydroxy benzoic acid, glycogen and α-keto butyric acid by the bacterial community exposed to copper. During the recovery period the metabolic profiles of the bacterial communities from 2.1 to 6.8 μmol/l copper tended to converge towards the reference. At the end of the recovery period, differences in the metabolic profiles between the individual aquaria were not statistically significant (Monte Carlo permutation: p = 0.66). A more uniform substrate utilization profile among the bacterial communities of the different treatments was the cause of this similarity. Assuming that the differences between aquaria are primarily caused by the different exposure concentrations of copper, it seems that copper had a significant effect on the metabolic profiles up to 6.8 μmol/l, but that during a recovery period, these effects disappeared, and finally the metabolic profiles of the communities were not statistically different any more from every copper treatment, and bacterial communities could utilize more similar carbon sources at the end of the recovery period than at the end of the exposure period.

During incubation of the biofilms on glass plates in the aquaria, the number of colony forming units (CFUs) stayed constant (6.5 ± 0.5 log CFUs/cm^2; mean of all aquaria on every sampling points; n = 36) and the average well colour development (AWCD) in the ECO-plates decreased significantly in all bacterial communities (AWCD abs/cm^2 decreased from 1.1 ± 0.3 to 0.5 ± 0.3; p < 0.01; mean of all aquaria on every sampling points; n = 36). The numbers of CFUs and the AWCD in the ECO-plates, respectively, 6.3 ± 0.5 log CFUs/cm^2 (mean of the reference, 0.6 and 2.1 μmol/l on every sampling points; n = 27) and 0.8 ± 0.3 abs/cm^2 (mean of the reference, 0.6 and 2.1 μmol/l on every sampling points; n = 27) did not show significant differences under the given experimental

conditions between bacterial communities exposed to the reference, 0.6 and 2.1 μmol/l copper. However, the sub-samples (n = 9) of the bacterial communities exposed to 6.8 μmol/l copper had a lower AWCD (AWCD abs/cm^2 was 0.4 ± 0.1; p = 0.03), and a higher number of CFUs (7.4 ± 0.3; p = 0.02) than the sub-samples taken from the aquaria that were treated with other Cu concentrations. During the recovery period, the number of CFUs of the bacterial communities exposed to 6.8 μmol/l copper stayed higher and the AWCD stayed lower than the other treatments. Therefore, while the number of CFUs increased in the presence of 6.8 μmol/l copper, the activity of the bacteria, based on AWCD measurements, decreased. Those differences in bacterial biomass and activity were maintained until the end of the experiment (day 54).

3.4. Pollution-induced community tolerance (PICT)

Tolerance characteristics for copper were determined using multi-well plates, allowing the construction of substrates-utilization sensitivity distributions (SuSDs) based on numerous EC_{50}-values. In Fig. 4, the copper-tolerance of the different bacterial communities is shown. The bacterial communities from the reference aquarium showed variable copper-tolerance in time (a SuSD rising from 1.3 to 3.0 mg/l). However, a SuSDs were always higher for copper treated bacterial communities than for the reference. After 12 days exposure to 6.8 μmol/l copper, the bacterial community showed an increase in tolerance compared to the reference a SuSD increased from 1.7 to 6.7 mg/l. Bacteria from the copper treated biofilms (0.6, 2.1 and 6.8 μmol/l Cu) showed a significantly higher a SuSD after 26 days of incubation, indicating an increased copper-tolerance. The highest tolerance for copper was reached after 26 days of incubation for all copper treatments. Copper-tolerance for all copper treatments (0.6, 2.1 and 6.8 μmol/l) had clearly decreased on days 41 and 54, respectively, after 2 and 4 weeks of depuration.

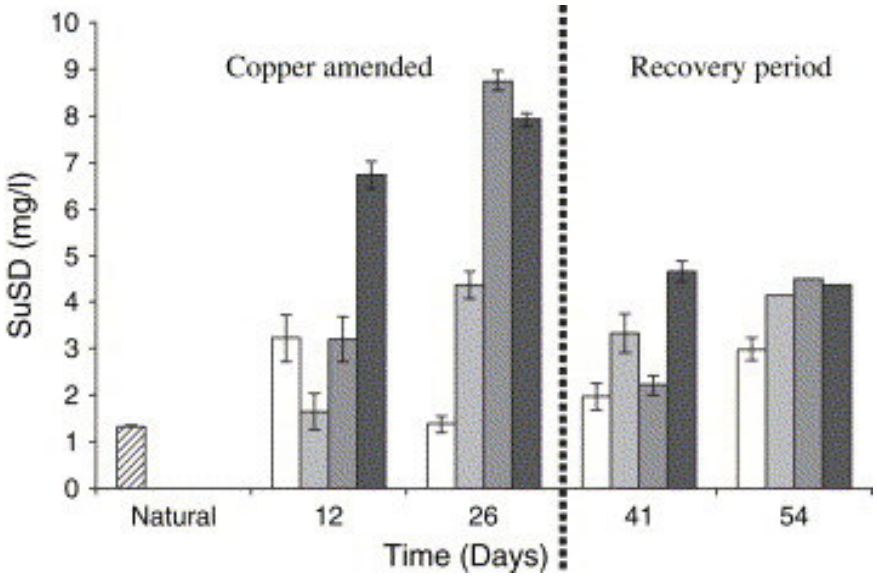

Fig. 4. The median of the substrates-utilization sensitivity distribution (a SuSD mg/l) of aquatic bacterial communities from biofilms exposed to different copper concentrations; 0 μmol/l Cu: white bars, 1 μmol/l Cu: light grey bars, 3 μmol/l Cu: dark grey bars and 10 μmol/l Cu: black bars. The error bars represent 95% confidence intervals.

4. Discussion

In the present study the potential of bacterial communities in freshwater biofilms to recover after copper exposure was investigated. Also, the importance of comparing results with different techniques that described genetic and physiological structure was stressed (Westergaard et al., 2001). DGGE and CLPP shifts in bacterial communities were shown to correlate with copper exposure concentrations in the aquaria. Assuming that these shifts are primarily caused by the different copper exposures in the aquaria it appears that only physiological traits based on the CLPP shifts recovered from copper exposure. The observation is consistent with the conclusions of Finlay et al. (1997) on the diversity of bacterial taxa, demonstrating a high level of redundancy in bacterial communities that allows these communities to adapt to new conditions.

Assuming that changes between aquaria are primarily caused by differences in copper exposure, it seems that the copper-induced successional changes during the exposure period remained present in the recovery period despite bacterial re-inoculation via water refreshments using natural surface water. This regular bacterial re-inoculation did not break-up genetic differences between bacterial communities that developed differently in the various copper treatments. One could expect that bacterial communities from biofilms exposed to the different copper treatments would regain their uniform genetic structure more quickly when bacterial communities are re-inoculated to the system during the recovery period via natural surface water. Several explanations may be brought forward explaining the maintained genetic differences. First, autotrophic microorganisms that dominated the biofilm exposed to copper also stayed visually distinct from the reference situation. Because a close relationship is expected between autotrophic and heterotrophic microorganisms (Romani et al., 2004), persistent differences of bacterial community composition could theoretically be maintained via long lasting effects of copper on the autotrophic microorganisms that dominated the biofilm (Massieux et al., 2004). Second, the so-called founder effect. Indeed it might be arduous to invade an established community than a pioneer community. And third, the copper concentrations in the biofilm remained relatively high after 28 days of recovery. In the highest copper treatment, the copper concentration after 28 days of recovery was 25 μmol/g, therefore the bacterial communities were still exposed to copper despite the weekly water refreshment.

The metabolic profiles of bacterial communities were significantly different upon copper exposure, but these differences were lost after depuration. Genetically different consortia were found to sustain a similar capacity to use carbon substrates. Griffiths et al. (2000) observed functional recovery of bacterial communities after a heat shock while the structure of the bacterial communities, based on DGGE analysis remained different. Griffiths et al. (2000) induced differences in bacterial community diversity by fumigation. Two months after a heat shock, all treated and untreated bacterial communities could utilize grass residue to the same extent. However, in that study, the bacterial communities could not utilize grass residue to the same extent after a persistent copper stress. Kiikkila et al. (2001) made similar conclusions after studying copper-exposed microbial communities using phospholipids fatty acid analysis (PLFA). The PLFA profiles did not change towards the unpolluted site after copper availability was reduced. However, the community that had developed in the presence of copper seemed to have lost its copper-tolerance, tested with [^{3}H]-thymidine incorporation, when copper availability decreased after mulch addition.

The physiological parameters that were determined in the present study are based on the use of BIOLOG® plates. These plates support primarily the fast growing, cultivable bacteria whereas DGGE focuses on dominant genotypes (Verschuere et al., 1997 and Smalla et al., 1998). Therefore, these different techniques highlight different parts of the bacterial community, potentially providing a more complete image than would be obtained using only one technique.

The present experiment did not show a complete loss of tolerance after the recovery period, but a plausible reason for this may be the incomplete release of copper bound to the biofilm, in combination with a restricted time for recovery. Yet, both the induction and the decrease of community tolerance were evident. Also, the *a* SuSD values found for terrestrial bacterial communities exposed to copper were in the same range as the ones found in clean soils (*a* SuSD 3 mg/l) and polluted soils (*a* SuSD 8 mg/l) using the same technique (Van Beelen et al., 2004). During the exposure phase and the depuration phase, Biolog-PICT could not be directly explained by genetic changes. Genetically different communities can have similar tolerance for a contaminant for the same reasons as these may be capable to use the same carbon substrates. Therefore, in view of our results, it seems that the exposure to a contaminant determined the level of tolerance to that contaminant and not the community composition.

5. Conclusion

After copper exposure, heterotrophic bacterial communities functionally recovered after copper exposure when determining the utilization of simple carbon substrates from Biolog plates. However, DGGE banding patterns of bacterial communities of aquatic photosynthesizing biofilms did not recover after copper exposure. This indicates that genetically different bacterial communities can sustain similar primary functions and have similar tolerance to a contaminant.

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