Metal induced succession in benthic diatom consortia

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Chapter VI
DIFFERENCES IN ZN TOLERANCE IN STRAINS OF THE
FRESHWATER MICROBENTHIC DIATOM GOMPHONEMA
PARVULUM (BACILLARIOPHYCEAE)

Abstract. Some benthic diatom species such as Gomphonema parvulum Kützing are equally abundant in rivers with low and high metal loads. Our aim was therefore to investigate whether the sensitivity to Zn of a G.parvulum strain isolated from a relatively clean stream differed from that of a strain of the same species developing in a Zn enriched stream. The isolates were cultured as mono-specific biofilms to maintain their benthic growth features. The strain from the polluted stream was cultured with and without addition of Zn (and Cd) in a synthetic medium. Short-term (5 h) toxicity experiments with Zn were performed with the strains using pulse amplitude modulated (PAM) fluorometry. Zn lowered the minimal chlorophyll fluorescence ($F_0$) and the photon yield ($\phi_p$) of the exposed strains after 5 h exposure.

The Zn concentrations that caused a 50% reduction (EC$_{50}$'s) of the $\phi_p$ of the strain from the metal polluted stream were significantly higher than those of the isolate from the unpolluted stream, independently of the presence of Zn and Cd in its culture medium. The absence of tolerance to Cu of the “polluted” strain suggested that Zn tolerance was induced specifically by the chronic exposure to Zn in the field. Observations on field biofilms confirmed a higher tolerance of the G. parvulum population from the polluted stream than that of the G. parvulum population from the reference stream. The genetic nature of this metal adaptation was confirmed by the persistence of the Zn tolerance of the polluted strain two years after isolation.
INTRODUCTION

Among other man-made chemical disturbances, metal pollution is an important stressor affecting the structure of algal communities. Metal pollution is known to exert a selection pressure and to drive succession of algal species towards more tolerant species (Gustavson and Wängberg 1995). Such selection at species level may result in an increased tolerance of communities (Blanck et al. 1988), but may often imply also lead to a loss of diversity (Leland and Carter 1984; Medley and Clements 1998).

Among other algal groups, diatoms are used for the detection of changes in water quality due to their species specific preferences for a range of ecological conditions such as, pH, salinity and nutrients (Lange-Bertalot 1979; Ter Braak and Van Dam 1989; Van Dam et al. 1994; Kelly and Whitton 1995; Pan et al. 1996). However, up to now, research has been mainly concentrated on the use of diatoms as ecological indicators of organic pollution (Descy and Coste 1991; Whitton and Kelly 1995). On the other hand, similar information on the sensitivity to metals of single freshwater diatoms species is rather sparse. Metal toxicity studies with single diatoms species have dealt up to now almost exclusively with planktonic marine and freshwater species (e.g. Jensen et al. 1974; Morel et al. 1978; Gavis et al. 1981; Gensemer et al. 1993; Rijstenbil et al. 1994a,b) and more occasionally with freshwater benthic species (Takamura et al. 1989). The scarceness of ecotoxicity studies using benthic diatoms is probably due to the practical problems quantifying cell numbers, because of their attached form of growth.

In previous field and laboratory experiments, using mixed autotrophic and heterotrophic microbenthic consortia, we observed that the relative abundance of some diatom species, like Achnanthes minutissima Kützing increased in a metal rich environment, whereas other species were almost completely suppressed (Ivorra et al. 1999; Ivorra et al. 2000). Some species, like Gomphonema parvulum, were abundant both under polluted and unpolluted conditions (Ivorra et al. 1999; Ivorra et al. 2000). This offered the opportunity to study metal-induced strain differentiation in G. parvulum. Algal acclimation (Wang 1986) and even tolerance differentiation of strains within a species (Li 1979; Devars et al. 1998) might evolve from a long-term metal
exposure. According to Stokes et al. (1973) algae occurring in polluted sites might be either metal-tolerant or metal-resistant; metal-tolerant species might have metal-sensitive populations (Gavis et al. 1981) in the absence of metals while metal-resistant species might still be tolerant. Foster (1982) found that the net effect of metal pollution was to increase the median resistance of the algae (chlorophytes) at polluted sites, but the distribution of metal sensitivity within all populations remained broad.

The aim of this study was to determine whether the sensitivity to Zn of the benthic diatom *G. parvulum* from a relatively clean stream differed from that of the same species developing in a Zn-rich stream in the same river-system. Since both streams sites had similar low (background) levels of copper, tolerance for Cu was checked for comparison.

**MATERIALS AND METHODS**

**Collection of strains from the rivers**

Cultures were isolated from 6 week old micro-algal biofilms from two neighbouring Belgian-Dutch lowland streams from the River Dommel subsystem, a tributary of the River Meuse. Zn and Cd concentrations in these streams are markedly different (Table 1; see also Ivorra et al. 1999). The reference strain (R) came from the Keersop (51° 20′ N; 5° 24′ E), a stream with low metal concentrations, but subjected to nutrient-rich runoff from agricultural activities. The extremely polluted strain (EP) originated from biofilms grown in the Eindergatloop stream (51° 15′ N; 5° 25′ E) polluted with Zn and Cd for more than a century by the seepage of a former Zn foundry and exposed also to partially purified sewage (Admiraal et al. 1999). Micro-algal biofilms were grown on sandblasted glass discs of 1.5 cm² surface. The sampling and collection procedures were carried out according to Ivorra et al. (2000). All field (and laboratory) material was cleaned before use with a 1% HNO₃ solution and rinsed with double distilled water.
Table 1. Physical and chemical characteristics from the water of the reference (Keersop; R) and extremely polluted (Eindergatloop; EP) streams during the 6-week colonisation period of the micro-algal biofilms used for the isolation of the strains. Mean (n=3) (±SD).

**Isolation of G. parvulum**

In the laboratory, R and EP field biofilms were scraped from the glass discs with a razor blade and suspended in 2 ml double distilled water. A portion of the scraped algal material was spread on agar plates for inspection with a stereomicroscope and subsequent isolation. For the two locations, a single cell of the diatom *G. parvulum* was isolated with a hand-pulled glass micropipet and transferred to a sterile culture tube containing 3 ml of sterile WC medium (Guillard and Lorenzen 1972) and a cover glass to support cell attachment. Culture tubes were incubated at 20 °C, under illumination of ca. 60 µmol photon·m⁻²·s⁻¹ fluorescent light and at 16: 8 h LD (light: dark) cycle. Tubes were continuously gentle rotated until an algal pellet was apparent (about 2-3 wks). The mono-specific character and condition of cultures were verified by microscopy. Species determination was performed according to Krammer and Lange-Bertalot (1986/91). At this point, the strain originating from the polluted stream (EP) Eindergatloop, was divided into two sterile culture tubes one containing 3 ml plain
WC medium (EP) and one containing 3 ml WC medium enriched with 1000 \( \mu g \text{ Zn} \cdot \text{L}^{-1} \) and 50 \( \mu g \text{ Cd} \cdot \text{L}^{-1} \) (EP+). This metal enriched medium was used to mimic the growth conditions at the polluted field station. The strain from the reference stream (R) was cultured in WC medium without metal addition.

A suspension (2 ml) of strain material was pipetted from the tubes into sterile acid cleaned glass Erlenmeyer flasks containing 100 ml sterile WC medium (with or without metal enrichment depending on the strain) and 2-3 sand-blasted glass discs to obtain mono-specific biofilms. The strains were grown under the conditions specified above for 2-3 wks. Discs with strains were transferred then to Erlenmeyer flasks containing 250 ml sterile WC medium and new clean discs for colonisation. Erlenmeyer flasks were sonicated in a cold bath for 60 s in order to detach the cells from the colonised discs and to favour an uniform inoculum for the new replicate discs. Culture conditions were maintained constant until uniform mono-specific biofilms were just visible (1 wk). The discs were collected in Erlenmeyer flasks containing 1000 ml WC medium and ca. 30 discs for the performance of the toxicity experiments and after sonication allowed to grow for 1 wk.

**Short-term toxicity tests**

One day before the start of the experiments, WC medium was substituted by WC medium without Fe-EDTA (WC-). The short-term Zn and Cu toxicity tests (5 h) were also performed in WC- medium to avoid chelation of the test metals (Ivorra et al. 1995). Strains from the reference (R) and the extremely polluted stream cultured in the metal enriched medium (EP+) were exposed to a range of Zn (0-6.10^4 \( \mu g \text{ Zn} \cdot \text{L}^{-1} \)) and to a range of Cu (0-1.3.10^3 \( \mu g \text{ Cu} \cdot \text{L}^{-1} \)) concentrations that were added to the WC- medium. The reference (R) and the extremely polluted strain (EP) cultured in plain WC- medium were also exposed to 0-2.10^4 \( \mu g \text{ Zn} \cdot \text{L}^{-1} \) in a third experiment. Four disc replicates of each strain were exposed to each metal concentration. Each disc was placed on the bottom of a separate transparent glass vial, with the colonised side face up, in 22 ml test solution. During the tests, the discs were incubated on a rotary plate (70 rpm), at 20 °C and at a constant light intensity of 70 \( \mu \text{mol photon} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \).
Two years after these experiments were performed, the R and EP strains were again exposed for 6 h to Zn under the same experimental conditions and setup as described above, to check the persistence of the Zn toxicity thresholds in the strains. In this experiment, eight replicates per treatment were used.

**Dissolved metal concentrations and pH in tests solutions**

At the beginning and at the end of the tests, 2 ml water samples were taken for the determination of the actual dissolved Zn and Cu concentrations in the different strain treatments. The samples were centrifuged (3000 rpm; 3 min) and the supernatants were acidified to pH 1-2 with reagent grade HNO$_3$. Zn and Cu concentrations were determined by atomic absorption spectrophotometry (AAS; with background correction). Quality control of metal analysis was performed using reference material (NIST [SRM 1643]: simulated freshwater, National Institute of Standards and Technology, Gaithersburg, MD, USA); the measured values deviated less than 10% from certified values. Actual dissolved metal concentrations were expressed as μg dissolved metal·L$^{-1}$. The pH was measured in fresh medium and at the end of the experiments.

**DW, metals and pigments in strains**

At the end of the tests, several discs were frozen for determination of metal contents and algal pigments concentrations in the strains. Metal concentrations in 3 discs per test concentration were analysed. The discs were suspended in double distilled water and cells were detached from discs by sonication in a cold water bath. The cell suspensions of *G. parvulum* were freeze-dried and the dry weight (DW) was determined. The DW of all tested strains on discs was below 1 mg·cm$^{-2}$. Mean DW values ranged from 0.2 to 0.6 mg·cm$^{-2}$.

Freeze-dried cells were digested in 50 μl HNO$_3$ (70%, ultrapure reagent grade [Ultrex]) using a microwave oven equipped with temperature and pressure control program. Metal analyses were performed, as in the water samples, with a flame and graphite furnace AAS. Quality control of metal analysis was carried out by analysing digestion
blanks and reference material (NIST [SRM 2704]: Buffalo River Sediment). The measured values deviated less than 10% from the certified values and digestion blanks were near the detection limits. Metal concentrations results were normalised as $\mu$g metal·cm$^{-2}$.

One disc replicate of each test concentration was used to analyse plant pigments in the *G. parvulum* strains. Pigments were extracted from freeze-dried discs with methanol 95% buffered with ammonium acetate 5% and were sonicated for 10 min. Pigments were analysed by HPLC according to Barranguet *et al.* (1997). Concentrations were expressed as $\mu$g pigment·cm$^{-2}$.

**Toxicity endpoints**

The photon yield or photochemical efficiency of PSII per absorbed photon ($\phi_p$) and the minimal chlorophyll fluorescence in dark adapted cells ($F_0$) were measured in the experiments using a PAM 101-103 Walz fluorometer. $F_0$ is related to the amount of algal biomass (chlorophyll) of samples (Falkowski and Kiefer 1985). $\phi_p$ gives a good estimation of the efficiency of the linear electron flow in the photosynthetic apparatus in light adapted cells (Genty *et al.* 1989; Hofstraat *et al.* 1994). $\phi_p$ is defined as:

$$\frac{F_m' - F}{F_m'}$$

where $F$ is the actual chlorophyll fluorescence under actinic light and $F_m'$ is the maximal fluorescence in light adapted cells. $F_m'$ is measured after exposure to a high-intensity (10000 $\mu$mol photon·m$^{-2}$·s$^{-1}$), but short (600 ms), saturating pulse capable of closing all reaction centers and disabling any photochemical reaction. The saturating light pulses were provided by a KL-1500 lamp and controlled with the PAM 103 unit. Six pulses were applied per replicate every 20 s.

The settings of the PAM were optimised and fixed before proceeding with the measurements and kept constant throughout the tests. The vials were placed above the sensor of the PAM.
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At the beginning and the end of each experiment, $F_0$ was measured after adaptation of biofilms to darkness for 20 min. The change in $F_0$ during the exposure was calculated as:

$$\Delta F_0 = F_{0\text{final}} - F_{0\text{initial}}$$

The measurements of $\phi_p$ were started after 2 h of incubation of the strains in the test solutions and under actinic light (ca. 70 $\mu$mol photon·m$^{-2}$·s$^{-1}$). The $\phi_p$ was calculated as the average of the last 3 successive pulses per disc. The measurement was done on two replicate discs per treatment.

**Calculation of effect concentrations**

The metal concentrations causing a reduction of 50% (EC$_{50}$) of the $\phi_p$ after exposure of the G. parvulum strains were calculated by fitting the following equation:

$$Y = \frac{c}{1 + e^{(X-a)}}$$

(log-logistic response model by Haanstra et al. 1985)

through sigmoidal dose-effect curves created with Kaleidagraph™. Where $Y$= inhibition %, $X$= log$_{10}$ actual metal concentration ($\mu$g·L$^{-1}$), $a$= log$_{10}$ EC$_{50}$, $b$=slope of the log-logistic curve and $c$= $\phi_p$ of controls (100%). The $\phi_p$ of treatments was expressed as percentage of the mean of the corresponding controls. EC$_{50}$'s and 95% confidence limits (CL) were expressed as $\mu$g metal·L$^{-1}$. Differences in effect concentrations were regarded as significant when the 95% confidence limits did not overlap.

**Variability within natural G. parvulum populations**

To estimate the variability in sensitivity to Zn within the in situ populations of G. parvulum in the R and EP streams, an additional experiment was conducted in the laboratory with 2 weeks old intact microbenthic communities from these streams. Communities were colonised in the field on glass discs as described above and in Ivorra et al. (2000). In the laboratory, the colonised discs from the EP and R stream were pre-incubated separately for 1 week in (6 L) WC$^-$ medium, in glass aquaria under continuous water movement (0.1 m·s$^{-1}$), before the 96 h exposure to 0 and 6000 $\mu$g Zn·L$^{-1}$ started. The temperature, light intensity and regime during the pre-incubation and exposure was identical as for the cultured strains. Renewal of the medium and test
solutions was done after 48 h. The pH and Zn concentrations in the aquaria were routinely monitored.

After 96 h of exposure, several discs from each treatment were sampled for immediate observation of *in vivo* chloroplast morphology of the *G. parvulum* cells with an epifluorescence microscope (1250x). Healthy (fluorescent) chloroplasts of *G. parvulum* are typically H-shaped. Non-viable or expiring cells show strongly condensed H-chloroplasts, irregularly shaped or fully spread through the cell which is also vacuolised. Fluorescence of chlorophyll may or may not exist in degraded cells.

Three discs per treatment were preserved in formaldehyde 4% and used to determine the percentage of viable *G. parvulum* cells in each treatment. At least 100 *G. parvulum* cells per disc were enumerated.

**Statistics**

Significant differences between strains and treatments of the mono-specific biofilms of *G. parvulum* were analysed using one-way ANOVA and the post-hoc Scheffé tests (Sokal and Rohlf 1995). When the assumptions for ANOVA were not met, non parametric Kruskal-Wallis test (Sokal and Rohlf 1995) was applied. Significant differences between the percentage of viable cells of *G. parvulum* in Zn-exposed and control populations from the R and EP stream were analysed with student-t tests (Sokal and Rohlf 1995).

**RESULTS**

**Dissolved metal concentrations and pH**

The actual Zn and Cu concentrations to which the different strains were exposed at the start of the experiments were very similar and matched rather well with the nominal dose (Table 2). After 5 h exposure, the concentrations of Zn and Cu had decreased down to respectively 55% and 70% of the initial Zn and Cu concentrations.
The pH of the medium used in the experiment was 7.1 ±0.1 (±SD). The pH of the Zn and Cu tests solutions showed a similar evolution in both R and EP strains, after the 5h incubation period. The pH ranged from 7.1± 0.1 to 7.2 ± 0.1 at the highest Zn concentrations to 7.6± 0.1 and 7.7± 0.1 in controls. The pH in the Cu test ranged from 7.7± 0 in the highest concentration to 7.8± 0.1 in controls.

<table>
<thead>
<tr>
<th>Nominal Zn conc.</th>
<th>Experiment 1</th>
<th></th>
<th>Experiment 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>EP+</td>
<td>R</td>
<td>EP+</td>
</tr>
<tr>
<td>0</td>
<td>28 (15)</td>
<td>20 (18)</td>
<td>8 (8)</td>
<td>4 (3)</td>
</tr>
<tr>
<td>3000</td>
<td>3285 (73)</td>
<td>3286 (9)</td>
<td>3640 (741)</td>
<td>3523 (0)</td>
</tr>
<tr>
<td>6000</td>
<td>6059 (110)</td>
<td>6074 (103)</td>
<td>6446 (250)</td>
<td>7125 (708)</td>
</tr>
<tr>
<td>20000</td>
<td></td>
<td>21736 (424)</td>
<td></td>
<td>22185 (117)</td>
</tr>
<tr>
<td>60000</td>
<td>693433 (3003)</td>
<td>73001 (1114)</td>
<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
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<th>Nominal Cu conc.</th>
<th>Experiment 3</th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>R</td>
<td>EP+</td>
</tr>
<tr>
<td>0</td>
<td>3 (0)</td>
<td>4 (1)</td>
</tr>
<tr>
<td>572</td>
<td>588 (2)</td>
<td>607 (6)</td>
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<tr>
<td>763</td>
<td>818 (14)</td>
<td>755 (38)</td>
</tr>
<tr>
<td>1271</td>
<td>1344 (12)</td>
<td>1341 (16)</td>
</tr>
</tbody>
</table>

Table 2. Nominal and actual Zn and Cu concentrations (µg·L⁻¹) to which the strains R, EP and EP+ of G. parvulum were exposed in the laboratory. Mean (n=4-6) (±SD).

**Metal concentrations in G. parvulum strains**

Zn, Cu and Cd concentrations in the mono-specific G. parvulum biofilms reflected the dissolved concentrations of these metals in the test solutions and the metal concentrations in the culture medium. Hence, the EP+ strain had higher Cd concentrations than the R strain (Fig. 1) due to the culturing of this strain in the synthetic medium suplemented with 50 µg Cd·L⁻¹. Non parametric Kruskal-Wallis tests indicated significant (p<0.05) differences in metal concentrations between treatments after 5 hours exposure. In general, the Zn concentrations in the EP+ strains were higher than in the R and EP strains (Fig. 1). However, the Zn and Cu concentrations measured in the R, EP and EP+ mono-specific biofilms that were exposed to the highest Zn and Cu concentrations at each experiment did not differ significantly from each other after the 5 hours exposure period. The increased Zn
concentrations in the EP+ strain exposed to $6 \cdot 10^4 \, \mu g \, Zn L^{-1}$ seemed to revert into a significant (p<0.05) reduction of Cd.

**Figure 1.** Metal concentrations determined in the R, EP and EP+ G. parvulum mono-specific biofilms after 5 h exposure in the laboratory. Mean (n=3) (±SD).

**Figure 2.** Chl. a/Chl. c ratios of the R, EP and EP+ G. parvulum mono-specific biofilms after 5 h exposure in the laboratory.

**Pigments concentrations in G. parvulum strains**

Microscopical inspection of the G. parvulum cells of the R and EP+ strains revealed the total loss of the characteristic H-shaped chloroplast of healthy (control) cells, at the
highest metal concentrations. However, after 5 hours of metal exposure no allomerisation of Chl. \textit{a} was detected. The absence of phaeophytin \textit{a} and phaeophorbid \textit{a} in the exposed strains indicated that the short-term metal exposure period did not induce degradation of Chl. \textit{a} to coloured products.

Higher Chl. \textit{a}/Chl. \textit{c} ratios were found in the polluted strains and in the highest metal treatments (Fig. 2). High Chl. \textit{a}/Chl. \textit{c} ratios may indicate a higher vulnerability of the Chl. \textit{c} than Chl. \textit{a} to metal exposure. Conversely, Chl. \textit{a}/fucoxanthin ratios were rather constant (2.9 ±0.2) and did not indicate differences between strains or metal treatments. Low concentrations of the carotenoids fucoxanthin and diadinoxanthin were detected in all controls and metal treatments. Yet, trace concentrations of diatoxanthin (about 0.01-0.1 μg∙cm\(^{-2}\)) were only detected in the metal exposed strains.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>(R) F(_0) (F.U.)*</th>
<th>EP+ F(_0) (F.U.)</th>
<th>EP F(_0)</th>
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</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>0.189 (0.02)</td>
<td>0.184 (0.02)</td>
<td></td>
</tr>
<tr>
<td>Experiment 2</td>
<td>0.164 (0.01)</td>
<td></td>
<td>0.136 (0.01)</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>0.192 (0.02)</td>
<td>0.145 (0.02)</td>
<td></td>
</tr>
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</table>

* F.U.= fluorescence units.

**Table 3.** Initial F\(_0\) values of \textit{G. parvulum} biofilms from experiments 1, 2 and 3. Mean (n=8) (±SD).

**Toxicity of metals to \textit{G. parvulum} strains**

The initial F\(_0\) values, of the studied \textit{R}, EP and EP+ strains, were rather similar among strains and treatments (Table 3).

After 5 hours incubation, the F\(_0\) of all control treatments increased, while that of the metal-exposed treatments decreased (Fig. 3). ΔF\(_0\) was significantly (p<0.05) lower in the highest Zn and Cu treatments than in the controls (Fig. 3); yet, at each test concentration there were no significant differences in ΔF\(_0\) between strains (Fig. 3).
In agreement with the results for $F_0$, the highest $\phi_p$ values were measured in all control treatments (Fig. 4). The $\phi_p$ of controls was very similar, between 0.500-0.600 FU (Fig. 4), and remained constant from the beginning to the end of the incubation period. The yield of all strains exposed to the highest Zn concentrations was severely reduced, already after 2 hours of exposure, to values below 0.100, indicating a total inhibition of the photosynthetic activity (Fig. 4). On the other hand, all treatments of the EP+ strain exposed to Cu had lower $\phi_p$ than the Cu-exposed treatments of R strain, from the beginning of the experiment onwards (Fig. 4).

The final and lowest EC$_{50}$'s for Zn were determined after 5 hours of exposure (Fig. 4). As expected, the EC$_{50}$ values of the EP+ strain exposed to Zn were significantly higher than the EC$_{50}$ values of the R strain indicating a lesser sensitivity of the EP+ strain to enhanced Zn concentrations (Fig. 4). The EP strain (cultured without metals) had also a higher 5h EC$_{50}$ and was thus less sensitive to Zn than the R strain (Fig. 4). The $\phi_p$ and EC$_{50}$'s for Cu of the R and EP+ strains after 5 h exposure indicated a higher sensitivity to Cu of the EP(+) strain (Fig. 4) in contrast to its low Zn sensitivity.

The differences in Zn-toxicity observed between the R and EP strain were still recorded two years after the performance of the first Zn experiment. The EC$_{50}$'s (based also on
reduction of the $\phi_p$) of the R ($EC_{50} = 1064 \mu g \text{ Zn}\cdot\text{L}^{-1}$; CL= 418-2708) and EP ($EC_{50} = 5655 \mu g \text{ Zn}\cdot\text{L}^{-1}$; CL= 4370-7317) strains still differed by a factor five and matched the values previously obtained.

![Graph showing photon yield and corresponding EC50 values for R and EP strains at different times.](image)

**Figure 4.** Time evolution of the photon yield ($\phi_p$), and corresponding EC50 values, of the R, EP and EP+ G. parvulum mono-specific biofilms during Zn and/or Cu exposure. Mean (n=6) (±SD). (FU= fluorescence units; CL= confidence limits of EC50 values).

**Variability within G. parvulum from natural communities**

About 90% of both R and EP communities was constituted by diatoms. G. parvulum made up 20% of the R and 6% of EP diatom cells in these communities. After 4 days, the percentage of viable G. parvulum cells in controls was $40\% \pm 11$ in the R and $51\%$
±11 in the EP community. The non-viable cells were mostly empty frustules. In the Zn-exposed treatments the percentages of viable cells were lower after 4 days. Only 7% ±2 of the G. parvulum cells in the R communities presented intact chloroplasts after Zn treatment, while in the exposed EP communities 21% ±9 of the cells had intact chloroplasts. The percentage (41%) of viable G. parvulum cells in the exposed EP respect to the EP control population was much higher than this percentage (19%) in the exposed R population respect to the R control.

The measured Zn concentrations matched well the nominal Zn doses. The mean pH in the aquaria was 7.1 and pH differences between control and Zn exposed treatments were small (0.05 pH units).

**DISCUSSION**

Our results demonstrated that the strain of the benthic diatom G. parvulum isolated from a stream chronically subjected to high Zn (and Cd) concentrations was more tolerant to Zn in the laboratory than the strain from a stream with markedly lower (29 fold) dissolved Zn concentrations. The photosynthetic activity of the polluted strain was affected at much higher Zn concentrations than the reference strain. A genetical based difference in tolerance between the polluted and the reference strain was suggested by the higher Zn-tolerance found in the polluted strain pre-incubated in the metal-free culture medium in the laboratory and by the persistance of this tolerance for over two years. Though differences in tolerance between strains from polluted and unpolluted environments have been demonstrated (Jensen et al. 1974; Takamura et al. 1989; Behra et al. 1999), care should be taken in the interpretation of the ecological significance of results obtained from single strains of a particular taxa (Wood and Leatham 1992). Genetical strain differentiation can only be established by DNA and RNA analyses and enzymatic electrophoretic techniques (Murphy 1978; Brand et al. 1981), not being used in the present study nor in Jensen et al. (1974), Takamura et al. (1989) and Behra et al. (1999). Also in nature, strains of a same species might show different morphological traits (Wood and Leatham 1992) and populations of a certain
taxa might be composed by a mixture of strains with possibly different sensitivities to several physical or chemical environmental factors at the same time (Gavis et al. 1981; Brand et al. 1981). Thus, tolerance and co-tolerance to a single factor can not be discriminated. An additional field experiment showing that individual cells in communities from the polluted river are surviving Zn stress in greater numbers than at the Zn clean reference site, is consistent with genetic adaptation to Zn. Still absence of unspecific adaptations can not be ruled out completely at this stage. Between-strain differentiation to chemical exposure could be partitioned into genetic and environmental variability and their interaction (Hoffmann and Parsons 1991; Behra et al. 1999).

Though *G. parvulum* has been described as most tolerant to highly polysaprobic conditions by Lange-Bertalot (1979), abundant in urban waste water river effluent (Vis et al. 1998) and organically polluted streams (Kelly and Whitton 1995; Van de Vijver and Beyens 1998), Medley and Clements (1998) found *G. parvulum* rather sensitive to metals compared to other earlier successional taxa. In their experiments, *G. parvulum* densities dropped below 50% of control communities after 10 days exposure to 54 µg Zn·L⁻¹, 6.7 µg Cu·L⁻¹ and 0.8 µg Cd·L⁻¹. However, Leland and Carter (1984) observed that after an initial decline (first 7 weeks) in density of *Gomphonema* spp. (*G. parvulum*, *G. subclavatum* and *G. truncatum*) no differences between treatment and controls were encountered in microbenthic communities exposed to 10 µg Cu·L⁻¹ for 1 year. These results suggest a certain acclimation capacity of this species to long-term metal exposure. According to Biggs et al. (1998) *G. parvulum* is a taxon associated with eutrophic and physically disturbed environments, with low to moderate growth rates, and is a strong competitor that may tend to dominate in disturbed conditions. These features are similar to those observed in metallophyte plants, frequently involved in plant adaptation to metal-contaminated soils (Schat and Verkleij 1998). Therefore, we may conclude from our results and those of others, that *G. parvulum* is a species able to adapt (genetically) to a wide range of physical and chemical stress.

The absence of tolerance for Cu of the polluted strains showed the selectiveness of induction of Zn-tolerance in the studied polluted strains through chronic metal
exposure in the field. Recent studies of the co-segregation patterns of genetical based metal-tolerances in vascular plant species, have found that Cu and Zn tolerance are controlled by different genes (Schat and Vooijs 1997). However, although genetical adaptation to Cu or Zn might be highly specific, the underlying physiological mechanisms of adaptation supporting tolerance may be similar. Tolerance to Cu and to Zn might rely on differential metal uptake rates and sequestration of these metals by phytochelatins (Gekeler et al. 1988) or in vacuole structures of cells (Nassiri et al. 1997). Indications for such processes were also found in the present study. The Zn and Cd concentrations determined in the EP biofilms were similar or even higher than in the reference strains, but they did not seem to impair the photosynthetic performance of the polluted strains as severely as in the reference biofilms. The results indicated that adaptive differences in the binding and inactivation capacity of Zn (and Cd) on or into the cells between the strains of the same species exist. Physiological adaptation to Zn, Cu and Cd in cells have been related to differences in intracellular pools of glutathione, a major metal-binding ligand in metal homeostasis mechanisms (Stauber and Florence 1990; Rijstenbil et al. 1994a,b; Miller et al. 1990; El-Naggar and El-Sheekh 1998; Morelli and Pratesi 1997).

Both Zn and Cu exposure decreased the initial \( F_0 \) of the \( G. \ parvulum \) cells after 5 hours incubation time. However, no differences between strains were observed in the effect of Zn on \( F_0 \). Because a decrease of the initial \( F_0 \) of the polluted strain due to the metal exposure was also observed. \( F_0 \) is the fluorescence intensity emitted when all primary electron acceptors in the electron-tranfer chain are fully oxidized (thus all PSII reaction centers are open) and the photosynthetic membrane is in the non-energized state (Van Kooten and Snel 1990; Brack and Frank 1998). In vivo, at physiological temperatures, almost all chlorophyll fluorescence in algae is associated with photosystem II (Falkowski and Kiefer 1985). Hence, the observed decrease in \( F_0 \) values in the metal-exposed treatments indicated a reduction of the number of photosynthetic centers as a result of the Zn and Cu exposure that could lead to a reduction of the photosynthetic affinity and ability to produce \( O_2 \) (Rijstenbil et al. 1994a).
The combination of higher Chl. a/ Chl. c ratios and the presence of higher diatoxanthin concentrations in metal-inhibited biofilms seemed to confirm that Zn and Cu induced structural changes in the chloroplasts of cells. The increase of diatoxanthin in cells has been correlated with an increase of heat release in the antenna complex that results in the reduction of fluorescence (Olaizola et al. 1994). Corroboration of this observation is needed and will be the object of further research.

In contrast to \( F_\nu \), clear differences between the strains were observed in the effect of Zn on \( \phi_p \). \( \phi_p \) supplies information on how the light-energy absorbed by antenna pigments is processed at the photosystem II level. Although inhibition of photosynthesis by Zn in algae has been previously reported (Fisher et al. 1981; Stauber and Florence 1990, Takamura et al. 1989; De Filippis and Ziegler 1993) a direct action of Zn at photosystem level is still under discussion (Rijstenbil et al. 1994a,b). Inhibition of algal photosynthesis by Zn may be the result of the altered activity of enzymes involved in the fixation of CO\(_2\) (De Filippis and Ziegler 1993).

Conversely, many Cu-toxicity studies with algae and higher plants have demonstrated that Cu directly affects photosystem II (Shioi et al. 1978; Samson et al. 1988; Clijsters and Van Assche 1985; Pätsikkä et al. 1998). Samson et al. (1988) found that Cu exposure also decreased quantum yield of photosystem II in the marine planktonic diatom Dunaliella tertiolecta. A reduction of active photosystem II centers in illuminated leaves of Phaseolus vulgaris was observed by Pätsikkä et al. (1998). Disruption of the photosynthetic electron transport by Cu may be the consequence of Cu competition for metal sites at photosystem II (Rijstenbil et al. 1994a; Pätsikkä et al. 1998). Moreover, the Cu induction of highly toxic oxyradicals may cause protein damage (Weckx and Clijsters 1996) and peroxidation of lipids and chlorophyll (Sandmann and Böger 1980; Rijstenbil et al. 1994a) that may contribute to the observed inhibition of \( \phi_p \).

Our results showed that both Zn and Cu affected the electron transport flow of photosystem II. Considering the effects of \( F_\nu \) and \( \phi_p \) jointly, it can be concluded that the
adaptation of the polluted strain is based on maintaining the efficiency of the PS II rather than the number of reaction centers.

The ongoing development of a PAM fluorometer that allows the measurement of $F_0$ and $\phi_p$ at an individual cellular level (Micro-PAM) will certainly add information to the assessment of metal tolerance in subpopulations within one microphytobenthic species.

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