

Effect of continuous nutrient enrichment on microalgae colonizing hard substrates

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Abstract

In order to understand the effect of changing nutrient conditions on benthic microalgae on hard substrates, *in-situ* experiments with artificial substrates were conducted in Kiel Fjord, Western Baltic Sea. As an extension of previous investigations, we used artificial substrates without silicate and thus were able to supply nutrient media with different Si:N ratios to porous substrates, from where they trickled out continuously. The biofilm developing on these substrates showed a significant increase in biovolume due to N + P enrichment, while Si alone had only minor effects. The stoichiometric composition of the biomass indicated nitrogen limitation during most of the year. The C:N ratios were lowered by the N + P addition. The algae were dominated by diatoms in most cases, but rhodophytes and chlorophytes also became important. The nutrient treatment affected the taxonomic composition mostly at the species level. The significance of the results with regard to coastal eutrophication is discussed.

Introduction

Whereas benthic microalgae inhabiting sediments may receive nutrients from the water column and from the sediment porewater, the benthic microflora on hard substrates depends upon water column nutrients and the internal recycling of nutrients (Riber & Wetzel, 1987; Hillebrand & Sommer, 1997). The influence of nutrient supply on marine microalgae on hard substrates has rarely been investigated as the effect of nutrient competition on epipelagic diatoms was thought to be minor compared to abiotic factors (Admiraal, 1984). It became clear, however, that nutrient limitation is possible in a variety of benthic habitats (Sundbäck & Snoeijs, 1991; Nilsson, 1995; Borchardt, 1996). It can be assumed that nutrients should be more important for the structure of benthic microflora on hard substrates lacking the sediment porewater as an additional nutrient pool.

It was shown in laboratory experiments with periphyton that diatoms are replaced by cyanobacteria and chlorophytes at low supply ratios of Si:N and

Si:P (Sommer, 1996). In a recent field study, the effect of nutrients on biomass and species composition of epilithic microalgae was tested experimentally in Kiel Fjord, Western Baltic Sea (Hillebrand & Sommer, 1997). This study revealed a positive response of total algal biovolume to nitrogen enrichment, indicating previously N-limited conditions. The experimental supply of nitrogen and/or phosphorus changed the species composition on artificial substrates and influenced biomass stoichiometry and community diversity. However, changes mostly occurred at the species level. Even at highest N and P supply rates, the diatoms stayed dominant in all experiments except one, which may be due to the fact that the kieselgur substrates themselves released silicate (Hillebrand & Sommer, 1997).

Here we report results of *in-situ* experiments conducted with Si-free substrates and two different modes of silicate enrichment (with or without additional enrichment of N and P). We were interested in the question whether compositional changes on the level of higher taxa would occur if the Si:N ratio was altered.

Table 1. Names, dates and treatments of *in-situ* experiments. Nutrient concentrations in ambient seawater in the Kiel Fjord at the end of the experiments are given in $\mu\text{mol l}^{-1}$. Nutrient treatments are given combined for all experiments

Name of experiment	Duration	Ambient N ($\text{NO}_2^- + \text{NO}_3^- + \text{NH}_4^+$)	Ambient P (PO_4^{3-})	Ambient Si ($\text{Si}(\text{OH})_4$)	Nutrient treatment (for all 3 experiments)
Autumn 1997	2 Sep–14 Oct 1997	8.4	1.11	18.5	- C: control ($n=2$) without addition of nutrients
Winter 1997	14 Oct–9 Dec 1997	16.2	1.19	31.5	- N + P + Si: ($n=6$) 450 N (as NaNO_3) + 30 P (as KH_2PO_4) + Si (0, 10, 50, 100, 250, 500)
Spring 1998	17 Mar–28 Apr 1998	24.6	0.27	13.2	- Si: ($n=5$) (10, 50, 100, 250, 500). Si as $\text{Na}_2\text{O}_3\text{Si} \cdot 5\text{H}_2\text{O}$

Methods

The experiments were conducted in Kiel Fjord, Western Baltic Sea, from September 1997 to May 1998 (Table 1). The experimental setup was similar to that used by Hillebrand & Sommer (1997). Artificial substrates were suspended from the pier of the institute at a water depth of ca. 1.5 m. The area is virtually non-tidal, but wind-driven fluctuations of the water-level occur. Nevertheless, the substrates were at least 0.5 m below the water surface at low water levels. The substrates consisted of lime tree wood blocks used for aeration of aquaria (15×15×30 mm, with a plastic connection for tubes on the top). These substrates were connected via silicone tubes to a darkened 10 l bottle placed upon the pier. These bottles were filled with 0.2 μm -filtered seawater (cellulose-acetate filters) from Kiel Fjord, to which nutrients were added (Table 1). The flow of these liquid media to the artificial substrates was controlled via a precise mechanic regulation device designed for intravenous infusion (Angiokard AK 5505), adjusting the flow rate to 1 (± 0.1) l d^{-1} . The supply bottle was refilled twice per week and the flow rate readjusted, if deviations $>0.1 \text{ l d}^{-1}$ were recorded. The nutrient supply rates were arranged in an increasing gradient of silicate concentrations, each with or without additional increase of N and P (Table 1). Additions of N and P were supplied in a 15:1 ratio, silicate concentrations in the

media ranged from 0 to 500 $\mu\text{mol l}^{-1}$. Although these concentrations in the medium appear to be high, the maximum supply rates were low due to the low flow rate (N: 26 $\mu\text{mol cm}^{-2} \text{ d}^{-1}$, Si: 29 $\mu\text{mol cm}^{-2} \text{ d}^{-1}$, P: 1.7 $\mu\text{mol cm}^{-2} \text{ d}^{-1}$).

At the end of the experiments, the artificial substrates were collected and the biomass was removed with a razor blade until no pigment colour could be detected on the substrate. The biomass was transferred to 100 ml of 0.2 μm -filtered seawater. The suspension was cautiously stirred, while subsamples were taken for microscopical analysis (fixation with Lugol's iodine), taxonomic identification and chemical analysis of biomass stoichiometry (processing of subsamples and determination of C, N and P as described in Hillebrand & Sommer, 1997). Very few wood particles were found in the counting samples, indicating that C:N:P measurements were not biased by wood scraped off the artificial substrate.

Cell abundance was determined with an inverted microscope (Leitz DMIRB) and standard Utermöhl counting chambers (Hydrobios), counting up to 1500 cells per sample at a magnification of 400×. Biovolume was calculated by measuring linear dimensions of 20 cells of each species and fitting nearest geometric models to the cell shape (Hillebrand et al., 1999).

Statistical analysis comprised two-factor ANOVA on gross variables (biovolume and cellular stoi-

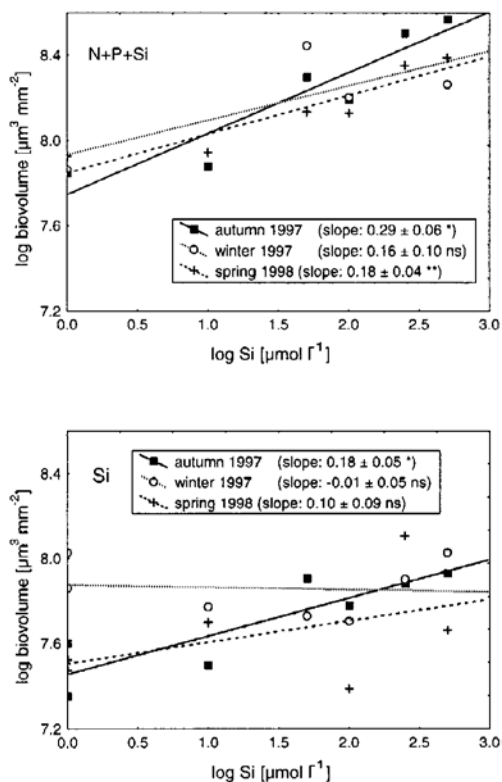


Figure 1. Total biovolume as a function of Si-concentrations, plotted for three experiments. Top: Enrichment of Si with additional supply of N+P. Bottom: Enrichment of Si alone. Regression slopes are given with standard error and significance level (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns: not significant).

chiometry) and the factors nutrient treatment and season of experiment. Homoscedasticity was assured by Bartlett's χ^2 -test. Combining different Si concentrations in one category is a conservative approach increasing the variation within categories. We applied linear regression models to evaluate the response of total biovolume to Si enrichment. Since single species could not be expected to respond in a linear way to nutrient enrichment, we adopted Spearman rank correlation to test the response of dominant species to Si treatments, whereas the Mann-Whitney U -test was used to test for differences between treatments with or without N + P enrichment. For both non-parametric tests, the biovolume of each species was normalized to the control in order to compare experiments with different biomass accrual.

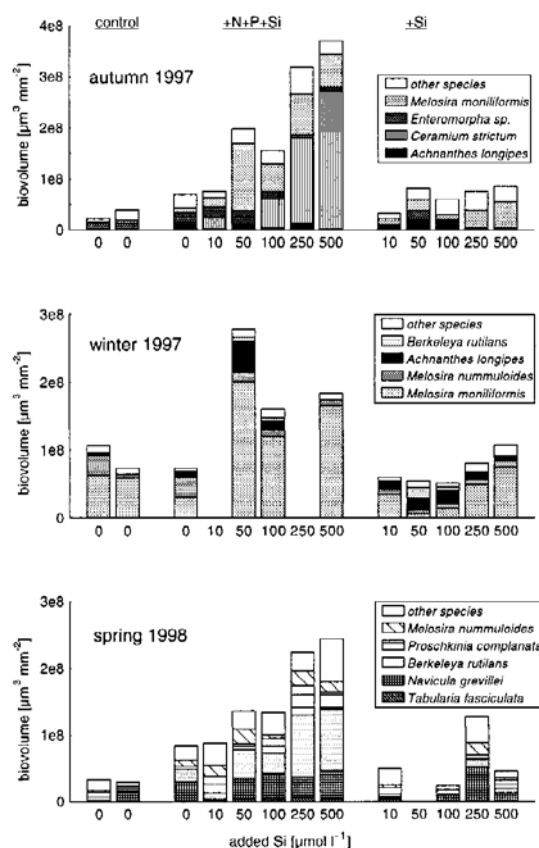


Figure 2. Species composition in three experiments conducted in the Kiel Fjord. Treatments are C (controls), N+P+Si (enrichment of N, P and Si), and Si (enrichment of Si alone). Si concentrations are given in $\mu\text{mol l}^{-1}$.

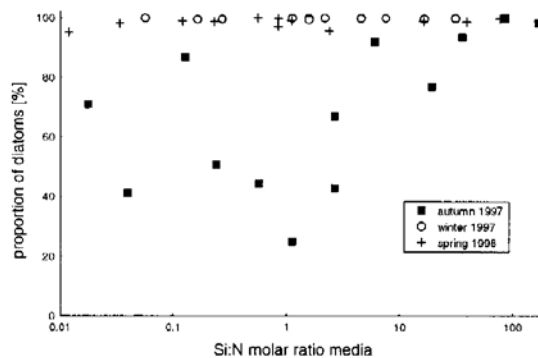


Figure 3. Proportion of diatoms in the biofilm as a function of the Si:N molar ratio in the medium.

Table 2. Results of a two-factor ANOVA on log-transformed total biovolume with treatment (control, N+P+Si or Si) and season of experiment (autumn, winter, spring) as factors. Log-transformation resulted in normal distribution ($\chi^2 = 1.95$, $p = 0.930$) and homogeneity of variances (Bartlett's $\chi^2 = 6.09$, $p = 0.637$)

Effect	df	MS effect	F-ratio	p-level
Treatment	2	0.907	18.218	<0.001
Season	2	0.109	2.192	0.131
Treatment \times season	4	0.051	1.015	0.417
Error	27	0.050		

Results

Total biovolume of microalgae was affected by nutrient treatment, but not by the seasonal setting of the experiment (Figure 1, Table 2). The biomass increased significantly in the N + P-enriched treatments compared to controls and Si-enrichments (Tukey's HSD, $p < 0.01$). The ANOVA detected no significant difference between controls and Si-treatments (Figures 1 and 2). However, Si-enrichment resulted in a significant linear increase in total biovolume in autumn 1997 and in spring 1998; but in the latter experiment, only in combination with N + P enrichment (Figure 1). In winter 1997, the Si enrichment had no significant effects.

In general, diatoms were the most dominant group (Figure 3). They stayed dominant throughout all treatments in winter 1997, when ambient Si-concentrations were highest (Table 1). In spring 1998, the proportion of diatoms declined at the lowest Si:N supply ratios, but they still contributed more than 90% to total biovolume in every case. Only in autumn 1997 the proportion of diatoms decreased significantly with decreasing Si:N ratios ($r = 0.588$, $p = 0.035$, $n = 13$), although the proportion was still very variable.

The species composition was changed in response to the nutrient treatment (Figure 2, Table 3). The addition of N + P favoured *Berkeleya rutilans*, *Ceramium strictum*, *Melosira moniliformis*, *M. nummuloides*, *Navicula grevillei* and *Tabularia fasciculata*. The biomass increase of *Proschkinia complanata* in spring 1998 and of the chlorophyte *Enteromorpha* sp. due to N + P enrichment was slightly insignificant (Table 3). *Enteromorpha* dominated at low Si concentrations and was negatively correlated to Si enrichment. The *Enteromorpha* individuals were small (up to 5 cm) compared to the size reported in other studies conducted

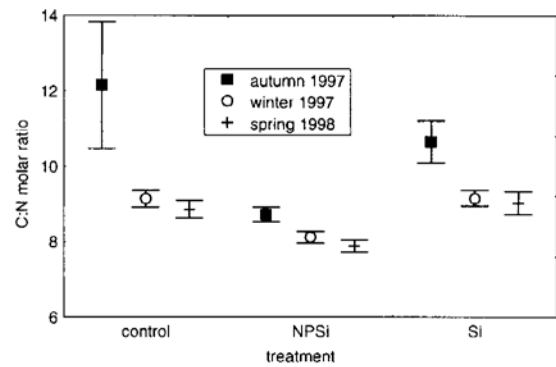


Figure 4. C:N ratios of algal biomass in control, N+P+Si and Si treatments of three experiments. Mean and standard errors are plotted.

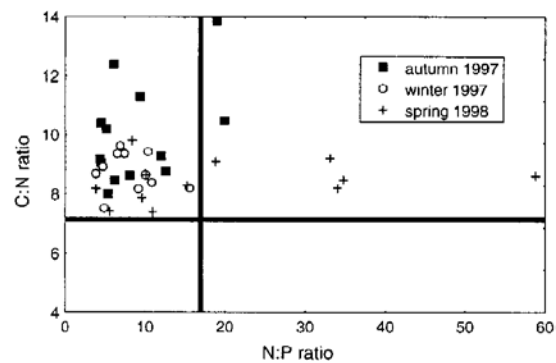


Figure 5. C:N and N:P ratios of algal biomass, with optimal ratios *sensu* Hillebrand & Sommer (1999) plotted as lines.

in the Baltic Sea (Lotze, 1998). The diatom *Melosira moniliformis* increased in high Si and medium Si + N + P treatments. At the highest Si + N + P conditions, the rhodophyte *Ceramium strictum* became dominant (Figure 2). The diatoms *Haslea crucigera* (in spring 1998) and *Pleurosigma elongatum* (in autumn 1997) showed significant positive correlations to Si-enrichment (Table 3). *Licmophora abbreviata* was most abundant in the control and decreased with enrichment. In winter 1997, the species composition showed no trends with nutrient enrichment.

In all experiments, the biomass stoichiometry changed with the nutrient treatment. C:N ratios decreased in the N + P-enriched treatments (Figure 4, Table 4), whereas the controls and Si treatments showed similar C:N ratios. The cellular C:N ratio was also influenced by the season of the experiments, since nutrient conditions changed during the year (Table 1). N:P ratios were generally lower than 17 in autumn and winter 1997 and varied broadly in spring 1998 (Figure 5).

Table 3. Response of dominant species to nutrient enrichment: Spearman-rank-correlation coefficients between species biovolume and Si-enrichment and Mann–Whitney *U*-test between samples enriched with N+P and those without. The table lists species name, rank correlation coefficient *r* for effect of Si enrichment, *z*-statistic for effect of N+P enrichment, with significance level (ns: not significant, * *p* < 0.05, ** *p* < 0.01). Moreover, the seasonal occurrence of the respective species is annotated together with the number of valid observations (*n*)

Species	Si <i>r</i> (Spearman)	N + P <i>z</i> (Mann–Whitney)	Annotation	<i>n</i>
<i>Achnanthes longipes</i>	0.14 ns	0.21 ns	only autumn	13
<i>Berkeleya rutilans</i>	0.27 ns	−2.42 *	all	36
<i>Ceramium strictum</i>	0.29 ns	−2.50 *	only autumn	13
<i>Enteromorpha spec.</i>	−0.56 *	−1.71 ns	only autumn	13
<i>Haslea crucigera</i>	0.75 **	−0.80 ns	only spring	12
<i>Melosira moniliformis</i>	0.49 **	−2.64 **	all	36
<i>Melosira nummuloides</i>	0.21 ns	−2.39 *	all	36
<i>Navicula grevillei</i>	0.24 ns	−2.88 **	only spring	12
<i>Pleurosigma elongatum</i>	0.71 **	−0.71 ns	only autumn	13
<i>Proschkinia complanata</i>	0.50 ns	−1.92 ns	only spring	12
<i>Tabularia fasciculata</i>	0.41 *	−2.39 *	all	36

Table 4. Results of a two-factor ANOVA on C:N ratios with treatment (control, N + P + Si or Si) and season of experiment (autumn, winter, spring) as factors. Homogeneity of variances was achieved by squareroot transformation of the dependent variable (Bartlett's $\chi^2 = 12.58$, *p* = 0.127), whereas normal distribution was not achieved ($\chi^2 = 13.80$, *p* = 0.008)

Effect	df	MS effect	<i>F</i> -ratio	<i>p</i> -level
Treatment	2	0.060	20.667	<0.001
Season	2	0.057	19.637	<0.001
Treatment × season	4	0.006	2.146	0.101
Error	28	0.003		

Discussion

Evaluation of the experimental setup

The method of direct and continuous supply of liquid media to benthic microalgae was already discussed earlier (Hillebrand & Sommer, 1997). The major change to previous experiments concerned the type of the substrate. Searching for a substrate with a surface not too smooth to hinder the attachment of microalgae (Snoeijs, 1991), we decided to use wood substrates, which additionally allowed the connection of tubes. The microbenthic community on the artificial substrates was generally similar to the community found on kieselgur substrates (Hillebrand &

Sommer, 1997). Dominant species were largely the same, with special emphasis on the diatoms *Berkeleya rutilans* and *Proschkinia complanata* in spring and *Melosira moniliformis* in autumn, as well as the rhodophyte *Ceramium strictum* in autumn. However, *Enteromorpha* sp. dominated at low Si-enrichment, but occurred only rarely on kieselgur substrates (Hillebrand & Sommer, 1997). Wood substrates were thus equally suitable for the attachment of microalgae as the kieselgur substrates used in previous experiments. Differences in colonization on rock and wood substrates were reported for stream periphyton, but it was also indicated that these initial differences may vanish with colonization time (Sabater et al., 1998).

As reported earlier, suspending the substrates effectively prevented the appearance of slow moving grazers like gastropods or large isopods (Hillebrand & Sommer, 1997). Few micrograzers like amphipods, nematodes and ciliates were recorded, but they never contributed more than 1% of total organism abundance. Bacteria were not analyzed in our experiments, but it can be assumed that bacteria may profit from the enhanced algal production (Hepinstall & Fuller, 1994). However, the microbial response to these kinds of enrichment experiments has rarely been assessed (but see Nilsson et al., 1991) and it remains a future task to unravel the interaction between periphyton and

benthic bacteria under different degrees of eutrophication.

Effect of nutrient treatment

Biomass stoichiometry is a useful tool for detecting nutrient limitation and has been widely applied to phytoplankton (Hecky et al., 1993). Recently, this concept has been transferred to benthic microalgae (Kahlert, 1998; Hillebrand & Sommer, 1999). Optimal C:N:P ratios in periphyton were proposed as indicators of saturated internal nutrient pools and thus optimal growth conditions. Hillebrand & Sommer (1999) derived optimal ratios of C:N:P of 119:17:1 from laboratory experiments, while Kahlert (1998) surveyed *in situ* freshwater data and found an optimal ratio of 158:18:1. Indication of N-limitation was proposed by Hillebrand & Sommer (1999) with a C:N >9 and a N:P <13.

The high C:N ratios and low N:P ratios in the control and the Si-treatments indicated nitrogen limitation in the autumn experiment. No N-limitation was detected during winter (C:N decreased) and spring, where high N:P ratios with high C:P ratios indicated P-limitation in some treatments. In early spring, a shift from N- to P-limitation in Kiel Fjord was observed before (Hillebrand & Sommer, 1997; Deegen, 1997) and was indicated also by ambient nutrient concentrations (Table 1). A shift from spring P-limitation to summer N-limitation was also reported from other sites, especially those with a freshwater inflow (D'Elia et al., 1986; Malone et al., 1996). Furthermore, the decrease in C:N:P ratios following the experimental addition of N indicated the effectiveness of the nutrient treatment and created a more nutrient replete situation for the periphyton.

Nutrient limitation is additionally indicated by the biomass increase following the N + P enrichment. Additional Si increased the total biovolume as well, but this increase was significant only in autumn 1997 and in spring 1998, for the latter time only in combination with N + P enrichment. Generally, Si enrichment was more effective (higher slopes, Figure 1) when N + P were enriched simultaneously. This indicates that Si limitation was less important than N limitation. Si-enrichment resulted in an biovolume increase only in autumn 1997 (Figure 1), when water column concentrations were $>15 \mu\text{mol l}^{-1}$ Si (Table 1). This concentration was obviously not sufficient to meet the demands of the developing biofilm, so there must be high diffusive barriers preventing the effective up-

take of water column Si by the biofilm. Freshwater periphyton was shown to have limited access to water column nutrients (Bothwell, 1985, 1989; Riber & Wetzel, 1987). The degree of limitation was shown to depend on low diffusion and low flow within the microbenthic assemblages, which is dependent on the biomass or the height of the mat (Wetzel, 1996). By supplying nutrients from the bottom, we presumably overcame this nutrient barrier, but also changed the vertical gradient of nutrient supply. Nevertheless, mainly erect growing species profited from the nutrient supply, indicating the importance of other factors related to vertical position in the periphyton (e.g. light).

Nutrient competition

From the nutrient-limited scenario shown in this and a previous study (Hillebrand & Sommer, 1997), it can be assumed that nutrient competition may play an important role for the structure of periphyton. However, the factors influencing the taxonomic composition of benthic microflora are not as well understood as those influencing the pelagic microalgae. For sediment-inhabiting microflora, Admiraal (1984) emphasized the role of physical and chemical conditions rather than nutrient competition, whereas the importance of nutrient supply was stressed later on (Sundbäck & Snoeijs, 1991; Flothmann & Werner, 1992; Nilsson, 1995). Whereas several studies on nutrient competition in freshwater periphyton have been published (see reviews in Borchardt, 1996; McCormick, 1996), only few studies have compared the combined effects of Si- and N + P- enrichment on benthic microalgae. In fact, few studies have regarded Si in any way, which is surprising since diatoms are often very dominant in periphyton (Borchardt, 1996). In experiments with combined control of Si and N + P, the community composition was mainly altered by enrichment of N + P (Carrick et al., 1988; Nilsson, 1995). The enrichment of Si alone did not increase the biomass (Carrick & Lowe, 1988) and enrichment of N + P + Si did not change the dominance of diatoms (Nilsson, 1995). A dominance shift to other taxa, e.g. cyanobacteria or chlorophytes, took place only at very low Si:N supply ratios (Nilsson, 1995; Sommer, 1996). These results are in agreement with our experiments, which showed a greater influence of N + P enrichment as well. The effect of Si on higher taxonomic levels was comparably low since only in one of our experiments a distinct dominance shift could be observed (Figure 3).

Also, the increase of biovolume with Si addition was more pronounced in combination with N + P enrichment (Figure 1). The variability of diatom dominance (Figure 3) in autumn 1997 was mostly due to the unexpected dominance of *Ceramium* (see below).

On the species level, several trends could be affirmed, which became already visible in previous experiments. *Berkeleya rutilans*, *Ceramium strictum*, *Melosira moniliformis*, *Navicula grevillei* and *Tabularia fasciculata* responded positively to N + P enrichment in our experiments (Table 3) and in similar studies (Sommer, 1996; Hillebrand & Sommer, 1997). Although species specific information about nutrient demands of benthic microalgae is widely lacking, the consistency of the taxonomic response to nutrient treatments between studies makes clear that physiological traits determine the competitive outcome. For *Berkeleya rutilans*, a high N-demand can be proposed from analysis of the gelatinous tubes of this species (Daniel et al., 1987). The chlorophyte *Enteromorpha* sp. decreased with increasing Si-concentrations, replaced by species with high Si-demand. These were mainly diatoms, especially *Haslea crucigera*, *M. moniliformis* and *Pleurosigma elongatum*.

Quite unexpectedly, however, the red algae *Ceramium strictum* became increasingly dominant with N + P enrichment and increasing Si addition. *Ceramium strictum* was also dominant in late summer 1996 at high N concentrations on Si-containing substrates (Hillebrand & Sommer, 1997). The reliance on high N-concentrations can be explained by high N-demands of *Ceramium* species (Pedersen & Borum, 1997). For the positive response to Si-enrichment, two possible explanations can be discussed: a direct explanation assumes Si-utilization by this rhodophyte, which to our knowledge has not been reported up to now. There are in fact hints that Si is utilized by filamentous algae, but these reports do not comprise rhodophytes (Parker, 1969). An indirect explanation is based on the succession often found during the colonization of free substrates by periphyton (e.g. Hudon & Bourget, 1981; Hoagland et al., 1982). This would mean that microorganisms (in this case diatoms profiting from the additional Si) alter the surface of the substrate and thus facilitate the adherence of *Ceramium*, by this increasing the abundance of attached individuals, as well as prolonging the growth phase of the attached individuals until the experiment was harvested.

Eutrophication and silicate

Eutrophication represents an imbalanced nutrient supply, since only N and P are anthropogenically enriched, while Si and several micronutrients are not. This imbalance is intensified by an alteration of biogeochemical cycles due to enhanced diatom production, transferring more dissolved Si (DSi) to particulate Si (PSi) (Conley et al., 1993). Since the turnover time of PSi is quite long (Conley et al., 1993), the pool of DSi is gradually depleted. Together with increased loadings of N and P, this leads to a decrease of the DSi:DIN ratio, as has been found in the Baltic Sea for the time period 1970–1990 (Rahm et al., 1996). For phytoplankton, the consequences of this development for species composition and algal blooms became increasingly obvious (Riegman et al., 1992; Bodeanu, 1993; Sommer, 1994). For benthic microalgae, however, predictions are less clearcut. A decrease of DSi in the water column may affect benthic diatoms lacking the sediment-associated pool of PSi. A decrease of diatoms at N + P enriched conditions has been shown experimentally on a local scale (Carrick et al., 1988; Nilsson, 1995; Sommer, 1996). However, the decrease of Si:N supply ratios may be less influential for diatoms inhabiting sediments which represents an undepletable pool of PSi. This pool may prevent Si-limitation (Sundbäck & Snoeijs, 1991), if the temperature-dependent dissolution rates meet the demand of the biofilm (Sigmon & Cahoon, 1997) or if bacterial mediated PSi-dissolution is fast enough (Bidle & Azam, 1999).

Conclusions

Total biovolume of microalgae colonizing hard substrates was more strongly influenced by N + P enrichment compared to Si enrichment. Only in one experiment the dominance of diatoms decreased with decreasing Si:N supply ratios. N-limitation was indicated by the enrichment experiments and by biomass stoichiometry of the benthic microalgae. However, the supply of nutrients had strong effects on the species composition of the periphyton.

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