Influence of bacterial activities on nitrogen uptake rates determined by the application of antibiotics

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Abstract

The influence of bacterial activities on inorganic nutrients has always affected total phytoplankton uptake rates owing to the absence of a reliable method that can exclude these effects. The use of natural samples to determine the contribution of bacterial activities has been based on the size fractionation method which, unfortunately, is encumbered with uncertainties, especially because of the size overlap between bacteria and phytoplankton communities. In this paper, the results are reported of an estimation of bacterial activities by the use of inhibitors (antibiotics). It was shown that the contribution of bacterial activities to the uptake of nitrogenous nutrients was highest for ammonium (79%), followed by nitrate (72%) and urea (62%). In a second set of experiments the concentration of ammonium was raised by 5 μM. This was done to avoid nutrient limitation resulting

from the absence of recycled nutrients following the addition of antibiotics and the maximum contribution of bacterial activity to the uptake rate of ammonium increased to 87%. It can be concluded that the use of inhibitors is a good method, a reliable alternative to the fractionation method. However, it is important to note that inhibitors can affect both phytoplankton growth and the nutrient recycling process. Our results indicate that the application of antibiotics had measurable effects not only on the target bacteria but also on the uptake behaviour of phytoplankton. Our observations were therefore limited to the period when there was no effect on the phytoplankton, as was demonstrated by a carbon protein incorporation experiment.

1. Introduction

In an aquatic environment bacterial activities make a significant contribution to the control of nutrient availability through uptake, mineralisation and nitrification. Mineralisation and nitrification are important processes and recycled nutrients are an important factor in the production of euphotic phytoplankton. Bacteria can therefore control phytoplankton productivity depending on the rate of these activities. Varying rates have been reported in different regions. Maguer et al. (1998) investigated English Channel waters. Their findings indicated that during spring in deep waters (0–70 m), the regeneration rates of ammonium varied between 7–26 nM h^{-1}, while during summer the variation was between 2–14 nM h^{-1}. In shallow waters (0–40 m) the rates of ammonium regeneration were higher than in deep waters. The regeneration of ammonium was between 8–50 nM h^{-1} in spring and 14–21 nM h^{-1} in summer. Nitrification rates measured by Brion (personal communication) in the North Sea at two different stations near the Belgian coast indicated a rate between 34 nM h^{-1} and 66 nM h^{-1} in early May.

Generally, the reported phytoplankton nutrient uptake rates have included the influence of bacteria; this is because of the difficulties in excluding their influence from that of phytoplankton. For example, Kirchman et al. (1994) measured bacterial activities by the thymidine and leucine incorporation method and found that the Whatman^{TM} GF/F filters used in the determination of uptake by phytoplankton retain all bacterial activities. However, according to earlier observations by Kirchman et al. (1989), the Whatman^{TM} GF/F retains 50% of the heterotrophic bacteria assemblage.

The reported contributions of bacterial activities to the uptake of different inorganic nutrients are significant but similarly variable. Wheeler & Kirchman (1986) used the ^{15}N method and did not observe nitrate uptake in a bacteria size fraction sample, while the uptake of ammonium was as high as $> 70\%$ of the total ammonium utilisation. However, Horrigan et al.

(1988) found evidence of nitrate uptake by heterotrophic bacteria. These findings were later corroborated by Kirchman et al. (1991), who reported the uptake of nitrate, ammonium and urea by the size fraction of < 0.8 μm, a fraction that was confirmed to contain the majority of the bacteria population. In another study by Kirchman et al. (1992), it was found that bacteria preferred ammonium rather than nitrate. This supported an earlier observation by Wheeler & Kirchman (1986), who estimated the ammonium uptake by the bacterial fraction to be $> 50\%$ of inorganic nitrogen. In addition, in Antarctic water it has been found that the bacterial contribution to ammonium uptake is very significant, especially during the phytoplankton bloom period (Tupas et al. 1994). However, bacterial uptake of inorganic nutrients is not restricted to nitrogenous nutrients. The uptake of phosphate by bacteria (Currie & Kalff 1984) and their preference for dissolved free amino acids rather than nitrate and ammonium have also been reported as being significant (Kirchman et al. 1992).

Measurements of nutrient uptake rates by bacteria in order to distinguish them from those of phytoplankton N-uptake have been based on the fractionation method. This method has been attempted with varying success: its variability and efficiency have commonly caused problems. For example, in the experiment by Harrison & Wood (1988), the size fraction < 1.0 μm was used and indicated substantial $^{15}NO_3^-$ uptake by bacteria; the simultaneous fixation of $^{14}CO_2$ by this fraction was also observed, which was deduced to indicate contamination by picophytoplankton. Kirchman et al. (1991) used the size fraction < 0.8 μm to determine nitrate and urea uptake by bacteria, but later Kirchman et al. (1992) reported that in a similar size fraction, there was 4% of chlorophyll and other phaeopigments. Fuhrman et al. (1988) investigated the uptake of ammonium by using a ^{13}N tracer isotope with 0.2–0.6 μm size fractions; their results supported the findings of Wheeler & Kirchman (1986), i.e. that heterotrophic bacteria and phytoplankton overlap in size and are therefore difficult to separate. Ducklow & Carlson (1992) reported the presence of intact autotrophs in < 1 μm filtrates as one of the factors that complicated their observations. In general, the size fractionation method continues to pose problems.

In order to overcome the difficulties of separating heterotrophic bacteria from phytoplankton, Wheeler & Kirchman (1986) used the antibiotics chloramphenicol and cycloheximide, respective inhibitors of prokaryotes and eukaryotes. For prokaryotes, chloramphenicol reduced amino acid uptake by 58% and inhibited ammonium uptake by between 25 and 50%. By contrast, the cycloheximide inhibition effect in eukaryotes was uncertain. This antibiotic was reported to inhibit amino acid uptake by about 20% and significantly inhibit ammonium uptake by 36–98%.

In natural samples there are mixtures of nutrients and also processes which make it difficult to obtain correct phytoplankton uptake rates for each of the dissolved nutrients by the conventional ^{15}N technique. As mentioned, there are a limited number of reliable practical methods, which can discriminate or estimate correct values due to bacterial activities. The influence of bacterial activities needs to be excluded; then only will it be possible to obtain more reliable estimates of phytoplankton uptake rates for different nitrogenous nutrients.

Although the use of antibiotics by Wheeler & Kirchman (1986) was not very successful, the use of inhibitors in preventing bacterial activities can provide a better estimation of their activities. In this paper, we report results where the activities of bacteria were limited by the use of a different set of antibiotics – streptomycin sulphate and polymyxine B sulphate, which were tested by the ^{14}C incorporation method. While they did not inhibit phytoplankton growth, they did bring bacterial activity to a total halt, as was reported by Lei Chou (pers. comm.) and also tested in the present study. The contribution of bacterial activities was assessed by comparison of two groups of samples: in one, their activities were stopped by addition of antibiotics, whereas in the other there was no addition of antibiotics.

2. Material and methods

Bacteria biomass

Samples were collected from Belgian coastal waters. Water samples for bacteria counts were preserved with formaldehyde (2%) and stored at 4°C. Before counting 1 to 5 ml of the water samples were stained for 15 min with 4,6 diamidino-2-phenylindole (DAPI) to make a final concentration of 0.1 μg l^{-1} (Porter & Feig 1980). The stained bacteria were collected by filtration on 0.2 μm pore-size black polycarbonate filters (Nuclepore), then mounted on microscopic slides and stored at –20°C until examination. The abundance of free-living bacteria was determined with an epifluorescence microscope (Leitz, Laborlux D). The bacteria were counted from a minimum of 20 different microscopic fields at 1000 × magnification. Between 300 and 600 bacteria were counted for each sample, and the carbon biomass estimated using the biovolume-dependent carbon conversion factor of Simon & Azam (1989).

Nutrient concentrations

Samples for determining NO_3^- concentrations were immediately filtered through Whatman^{TM} glass-fibre filters (GF/F), preserved in plastic bottles and frozen until analysis. Nitrate concentrations were determined by

an automated diazotation method (detection limit 0.1 μM; D'Elia 1983, Elskens & Elskens 1989). Ammonium and urea samples were kept in glass bottles and fixed by the immediate addition of reagents. The ammonium concentrations were determined by the indophenol-blue method (detection limit 0.03 μM) according to Koroleff (1969), and urea concentrations were determined by an adapted diacetylmonoxime method (detection limit 0.1 μM; Goeyens et al. 1998). All sample concentrations were measured in duplicate. The reagents used in the analyses and standards preparation were all analytical grade. Solutions were prepared in deionised water from a Millipore^{TM} Milli-Q ion exchange unit.

Incubation experiment

Sample incubations were done in two sets, each of 4 polycarbonate bottles. After three bottles from the first set had been filled with sea water, each was enriched with a different tracer, i.e. $^{15}NO_3^-$, $^{15}NH_4^+$ and $CO(^{15}NH_2)_2$ and then immediately placed in the incubator. The fourth bottle of the set was treated in a similar manner, but the concentration of NH_4^+ was raised by 5 μM. In the second set, the samples were first treated with a cocktail of two different antibiotics – streptomycin sulphate (2 g/20 litres) and polymyxin B sulphate (2 g/20 litres) about half an hour before starting the incubation, in order to allow the antibiotics to act against the bacteria. The experimental set-up and addition of tracers were similar to those of the first set. The two sets were incubated in a floating incubator under natural sea conditions.

A monitoring experiment was performed parallel to the above two sets of experiments in order to examine the efficiency of the two antibiotics used in the inhibition of bacterial activities. Seawater samples were pre-filtered through Whatman^{TM} glass microfibre filters (GF/F) to remove phytoplankton from the sample. In the sample, the above-stated quantities of antibiotics were added and the sample incubations were done following the same procedure as above. This method was used in accordance with documented information on the filtration efficiency of GF/F filters. It was assumed that the findings by Kirchman et al. (1989), that GF/F can retain 50% of bacteria, was useful for a satisfactory establishment of the effects of bacteria on the nutrients in the sample. The ammonium concentration was used as a sensitive parameter readily susceptible to marine bacterial activities. The ammonium concentrations were therefore measured before and after the incubation of each experiment; the differences in concentrations were recorded.

After incubation for about 24 hours, each sample was filtered through a Whatman^{TM} glass microfibre filter (GF/F) preheated to 450°C to

eliminate residual carbon and nitrogen and the particulate matter was dried at 50°C for a minimum of 24 hours. The particulate matter (PM) was converted to dinitrogen by a modified Dumas method (Fiedler & Proksch 1975), and its ^{15}N abundance was measured by emission spectrometry using Jasco NIA–1 or N–151 ^{15}N Analysers. Calibration was done with certified standards (Goeyens et al. 1985). PON/POC analyses were done on a Carlo Erba NA 1500 CN Analyser.

3. Results

Bacteria biomass

In 1998, the highest biomass increase was recorded from late April (3 μgC l^{-1}) to early May (61 μgC l^{-1}); it then decreased to 29 μgC l^{-1}

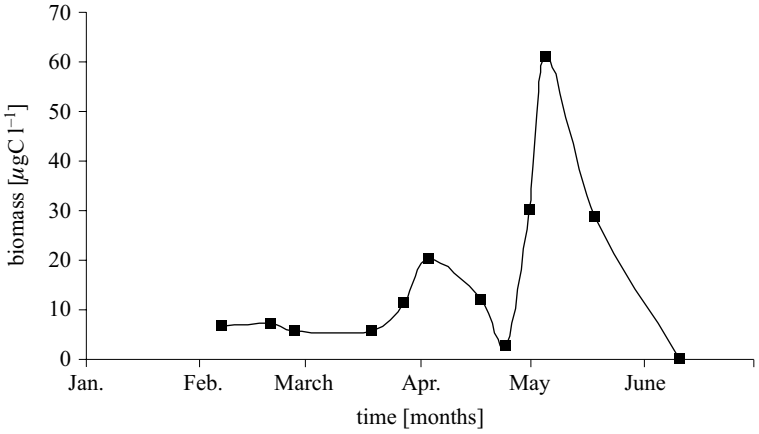

Fig. 1a. Evolution of bacteria biomass during the 1998 sampling season

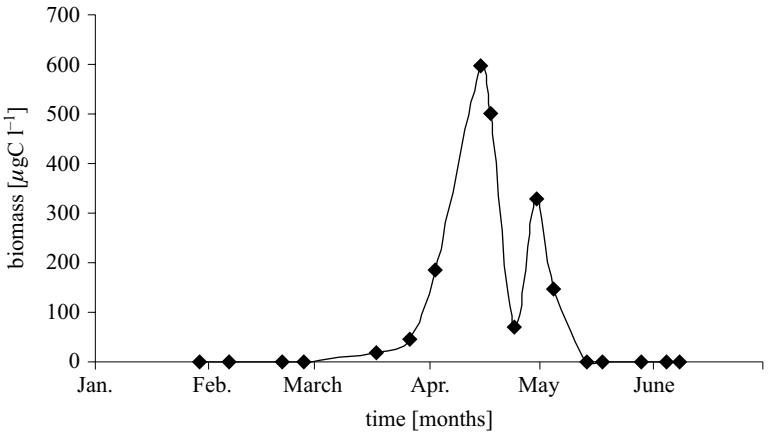

Fig. 1b. Seasonal changes in *Phaeocystis* biomass during the 1998 sampling season

in mid-May. In early June there were no recorded planktonic bacteria. In 1998 *Phaeocystis* made up the major phytoplankton community. Figs. 1a and 1b show that the changes in *Phaeocystis* biomass with season did not coincide with those of the bacteria biomass. However, when the bacteria biomass reached its maximum, there was a small peak (on May 5) in the *Phaeocystis* biomass.

Ammonium regeneration

From the incubation experiment of the pre-filtered samples treated with antibiotics (Table 1), it was noted that there was no difference between ammonium concentrations recorded before and after the incubation. This implied that mineralisation/uptake processes associated with bacterial activities were insignificant.

Table 1. Comparison of ambient ammonium concentration and concentration at the end of incubation for samples filtered before incubation to monitor the inhibitory effect of antibiotics

Date	Ambient concentration [μM]	End of incubation concentration [μM]
27 March 1998	0.39	0.37
24 April 1998	0.44	0.47
5 May 1998	1.02	1.00
10 June 1998	0.86	0.88

Uptake rates

There were differences in nutrient uptake rates for samples treated with antibiotics and those without the treatment (Figs. 2 and 3). The absolute nitrate uptake rates in samples treated with antibiotics (Fig. 2a) showed lower rates in almost all measurements than those without antibiotics. In natural samples, the highest absolute nitrate uptake rates were recorded in late April (0.127 μM h^{-1}), while for samples treated with antibiotics the highest uptake rates were in late March (0.082 μM h^{-1}). From the maximum, the uptake rates decreased to a minimum in May and June. The percentages of nitrate uptake reduction (Table 2) after the addition of antibiotics were between 8% and 72%. In March, the difference was at a minimum (8%) and increased to a maximum by the end of April (72%). In May and June the uptake was at a minimum but the differences were between 38% and 66% respectively.

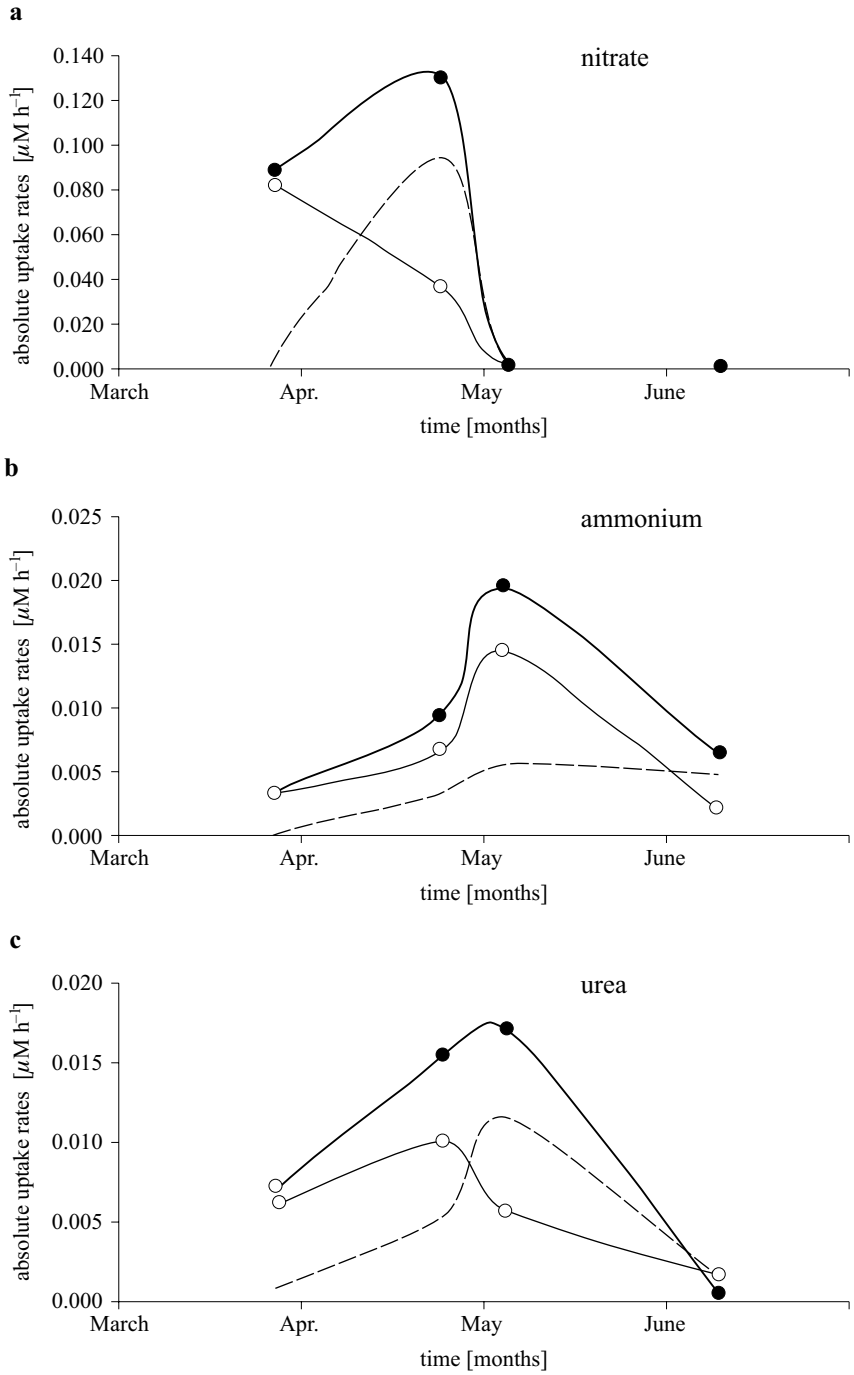

Fig. 2. Nutrient uptake rates (a–c) in natural water samples (•) and samples treated with antibiotics (○). The dotted lines represent the difference in uptake rates of the two types of samples

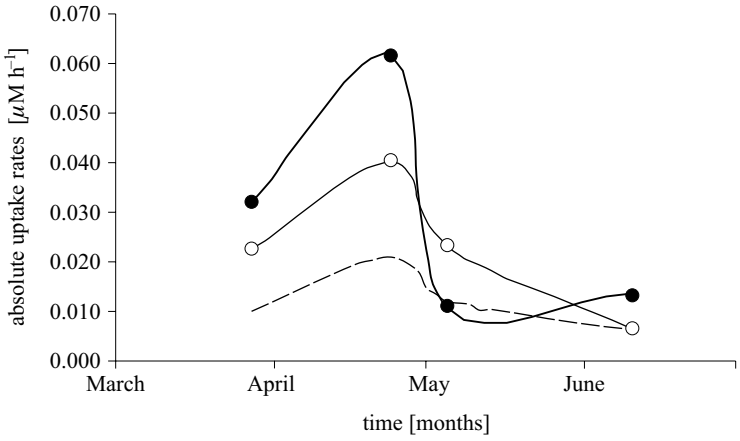

Fig. 3. Measured uptake rates of ammonium from samples with enhanced ammonium concentrations and samples treated with antibiotics after enhancement of the ammonium concentration. The symbols are identical to those in Fig. 2

Table 2. Percentage reduction of the nutrient uptake rates after the addition of antibiotics and their ambient concentrations on each date of incubation. Two sets of incubations were done: (1) with enhanced ammonium concentration ($+NH_4^+$) and (2) without ammonium concentration enhancement ($-NH_4^+$)

Date	Nitrate		Ammonium			Urea	
	conc. [μM]	reduction [%]	conc. [μM]	($-NH_4^+$) [%]	($+NH_4^+$) [%]	conc. [μM]	reduction [%]
27 March 1998	17.41	7.81	0.39	3.44	72.00	0.24	13.02
24 April 1998	0.69	72.41	0.44	29.95	87.39	0.21	35.18
5 May 1998	0.00	38.47	1.02	26.43	82.65	0.74	67.14
10 June 1998	0.19	65.97	0.86	76.65	79.46	0.37	***

*** no reduction observed.

For ammonium incubations (Fig. 2b) samples with antibiotics again showed low uptake rates compared to natural samples. Both samples reached a maximum uptake rate on 5 May, when the absolute uptake rate was 0.0192 μM h^{-1} for the sample without antibiotics and 0.0141 μM h^{-1} for the sample with antibiotics. Towards mid-June the uptake rate fell to a minimum. In general, the addition of antibiotics reduced ammonium uptake rates by between 3% and 77% (Table 2). In March, there was no significant difference between uptake rates measured in natural samples (0.0032 μM h^{-1}) and antibiotic-treated samples (0.0030 h^{-1}). Towards the end of April the difference in uptake rate increased to 0.0027 μM h^{-1}, which

was about 29% of the uptake rate in the natural sample. In early May, the difference in uptake rate was at a maximum (0.0051 μM h^{-1}), which was about 26% of the total uptake in the natural samples. Between May and June, the difference remained almost constant, but the percentage decrease was the highest ($\sim$77%).

Absolute urea uptake rates for natural samples increased from 0.007 μM h^{-1} in late March to a maximum, 0.017 μM h^{-1}, on 5 May (Fig. 2c). From the maximum, the uptake rates decreased to a minimum of about 0.0002 μM h^{-1} in June. Samples treated with antibiotics showed reduced uptake rates in an almost similar trend to the natural sample uptake rates. The absolute uptake rates increased from 0.006 μM h^{-1} in March to the maximum of 0.010 μM h^{-1} on 24 April (earlier than in natural samples). The minimum absolute urea uptake rate was 0.003 μM h^{-1} in June, higher than the uptake rates in natural samples. The difference between the natural and antibiotic-treated samples ranged from 13% to 67% (Table 2): 13% in March, increasing to 67% in May. In June, there was no reduction in the sample treated with antibiotics, but the uptake rate did increase by a factor of more than 10.

Effects of enhancing ammonium concentration

In Fig. 3, the ammonium uptake rates were measured in two incubation bottles with enhanced concentrations of ammonium (+5 μM). In the first set, uptake rates were measured without the addition of antibiotics, in the second set antibiotics were added. The absolute ammonium uptake rates increased from March and peaked in April, when the value for samples without antibiotics was 0.06 μM h^{-1} and for those with antibiotics 0.04 μM h^{-1}. Both samples displayed a decrease in early May. Minimum absolute uptake rates were 0.007 and 0.01 μM h^{-1} for samples with and without antibiotics, respectively. The difference between samples without antibiotics and those with antibiotics ranged from 72% to 87% (Table 2). It increased from March to a maximum in late April (87%). In May and June the difference was less (82 and 79%, respectively).

4. Discussion

The trend of bacteria biomass and nutrient concentrations (Figs. 1a and 4) indicated a possible relationship between bacteria biomass and urea, and between nitrate and ammonium concentrations. In 1998, the bacteria biomass peaks were related to minimum urea concentrations. Moreover, in both years, ammonium concentrations increased when bacteria biomasses were at a maximum. We therefore deduced that the increase in ammonium

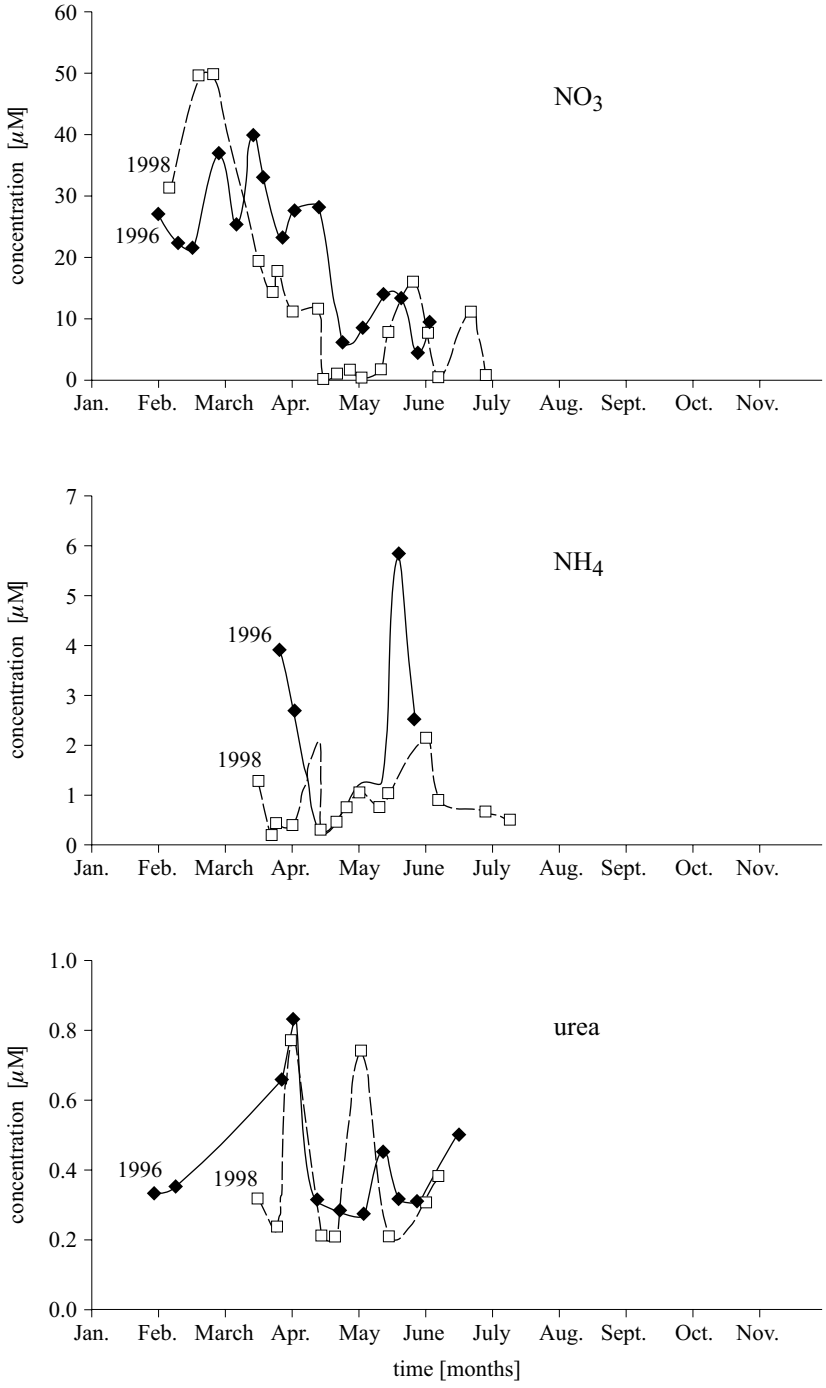

Fig. 4. The general trend in nitrogenous nutrient concentrations during the two years corresponding to the bacteria biomass

concentration with bacteria biomass was the result of bacterial activities, especially mineralisation. This could also be an indication that ammonium uptake by bacteria was too low to influence the concentrations, and the trend was not dependent on bacterial activities. However, there was strong evidence that bacterial uptake did significantly influence the concentration of urea during the early part of 1998.

The use of antibiotics showed up significant differences between samples treated with antibiotics and those without antibiotics. The absence of measurable differences between pre-filtered samples subsequently treated with antibiotics and those without antibiotics indicated there were no biological activities affecting changes in ammonium concentration. This therefore indicated that antibiotics were satisfactorily effective against bacterial activities. However, these results must be interpreted in the light of the problems arising out of the use of antibiotics. In this investigation it was logical first to consider the specificity of antibiotics in blocking bacterial activity without disrupting phytoplankton production. We therefore compared observations from experiments designed simultaneously with this work, in order to investigate the possible effects of antibiotics on phytoplankton uptake behaviour. Fig. 5 is a scatter plot of the results.

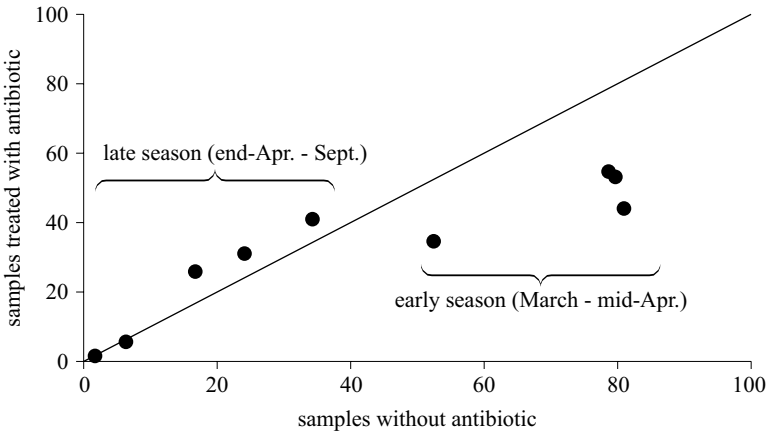

Fig. 5. Phytoplankton protein synthesis [μgC l^{-1} d^{-1}] measured from samples treated with antibiotics and those untreated (without) antibiotics measured by ^{14}C incorporation. The diagonal line is a bisect of expected linearity if no effect of antibiotics on protein synthesis was observed

In this experiment, incorporation into protein indicated that during the early part of the season, from March to mid-April, protein synthesis was reduced as a result of the addition of antibiotics. This was a period of high chlorophyll *a* concentration and phytoplankton protein synthesis. However,

from late April to September there was no difference between the protein synthesis rates of samples treated with and those without antibiotics.

Difficulties thus arose in understanding the possible physiological effects of antibiotics on the phytoplankton. Compared to the above results, it was to some extent likely that antibiotics, most probably polymyxine B-sulphate, were exerting a physiological stress on the phytoplankton. Polymixines are sometimes toxic towards eukaryotic cells because of the fatty acid group their molecules contain. The fast-growing phytoplankton might have counteracted the stress by (most probably) increasing their respiration rate and therefore affecting carbon incorporation into protein. The uptake rate of nitrogen may not be directly affected, but if carbon incorporation into protein is reduced, less nitrogen will be needed for protein synthesis, this therefore resulting in the removal (exudation) of excess nitrogen from the phytoplankton body. Such an effect might not have been so pronounced in the later part of the season owing to low phytoplankton activities.

Bacteria are responsible for not only the uptake but also the regeneration of nutrients, and both processes are inhibited by antibiotics. The inhibition of regeneration processes can have two side effects: the isotopic dilution of the nutrient pool under consideration may be prevented and the availability of this nutrient for phytoplankton uptake may be reduced. As explained in the introduction, regeneration is particularly important for ammonium. The regeneration of nitrate (nitrification) is generally believed to be less important, although few measurements have shown that it can be significant (refer to the introduction).

On the basis of the above explanations, when interpreting uptake rate results from the use of antibiotics in order to separate phytoplanktonic from bacterial processes, we have to take certain considerations into account: (1) for urea uptake, the percentage reduction of N-uptake by the use of antibiotics can be seen as a contribution of bacteria, but only in the late season, because in the early season phytoplankton protein synthesis is affected by the added antibiotics; (2) for ammonium and nitrate uptake, we need to consider the effect of blocking regeneration during incubation by the use of antibiotics. The experimental set-up was needed to explain the influence of bacteria during the early part of the season, when biological activities are at an optimum level, but the first observation above limits the justification of the method, since it indicated that antibiotics cannot be used efficiently during the season. The second consideration could easily be ignored when the ambient ammonium and nitrate concentrations did not limit the uptake during the incubation period. Indeed, theoretically, if the ambient ammonium

and nitrate pool is large, the quantity of regenerated nutrients during incubation will not influence the uptake behaviour of phytoplankton. On the other hand, at low ambient concentrations, regenerated nutrients can be important in supporting phytoplankton growth. In this investigation, during the incubation season ammonium concentrations were between 0.4–2 μM, those of nitrate between 0.2–7 μM.

From the nature of our results, we limited our interpretations of uptake rate differences to the period from late-April to June (Fig. 2), a period when antibiotics had no effect on phytoplankton activities. This included the period of maximum bacteria biomass. This period was characterised by peak ammonium and urea uptake rates, and it was also a period during which nitrate uptake rates were already decreasing from the maximum for the season.

On the basis of percentage reduction in uptake rates (Table 2), ammonium was the leading nutrient to have a maximum reduction (79%), followed by nitrate (72%) and urea (67%). However, for nitrate and ammonium uptake rates, this reduction was not only a result of inhibition of bacterial uptake. It could also result from the inhibition of ammonium and nitrate regeneration. As explained above with regard to ammonium, the inhibition of bacterial activities also decreases regenerated ammonium owing to interference with the ammonification process. Such interference can result in a low ammonium concentration and, therefore, a low uptake by phytoplankton cells. By assuming that this happened then, the percentages of reduction observed after the addition of antibiotics were both a result of the inhibition of bacteria ammonium uptake and of a reduced phytoplankton ammonium uptake. The same can be said about the nitrate uptake rates, although ambient nitrate concentrations were commonly higher than ammonium. For urea, the main process able to affect its bacteria-associated concentration is uptake. We can therefore assume that the observed difference is a result of uptake by bacteria and conclude that bacteria were responsible for between 35 and 67% of urea uptake during the late season.

In the experiments with ammonium enhancement, we reduced the importance of isotope dilution and the possible limited availability of regenerated ammonium. In this case, the percentage reductions observed after the addition of antibiotics were mainly the result of inhibited bacterial uptake. The contribution of bacteria to ammonium uptake was about 82–87% in the late season, which were comparable to the values reported by Wheeler & Kirchman (1986), whereby bacteria size fractionation uptake was estimated to be more than 70% of the total ammonium uptake.

5. Conclusion

The application of antibiotics can be a good method for distinguishing between phytoplankton and bacterial nitrogen uptake. However, careful selection of the type of antibiotics and the required concentration for effective and selective inhibition should be taken into account and further investigated. Antibiotics can separately indicate the extent of bacteria and phytoplankton activities. From this study it can generally be noted that antibiotics affected the activities of both bacteria and phytoplankton during the early period of their growing season. It should also be noted that blocking ammonium and nitrate regeneration processes can modify the isotope dilution and the availability of regenerated ammonium and nitrate. This can influence the phytoplankton nitrate and ammonium uptake rates. In the incubation experiment urea regeneration was mainly linked to zooplankton excretion, a process not affected by antibiotics. Thus, results must be interpreted with great care, especially those concerning nitrate and ammonium uptake. Before any nitrogen uptake results can be trusted, it should have been demonstrated that antibiotics did not affect phytoplankton uptake behaviour during the period of observation.

Bacterial activities remain an important part of nitrogen uptake rates and contribute to the inaccurate estimation of phytoplankton uptake rates, although in most cases, phytoplankton uptake remains the determinant of uptake trend of nutrients over the season. Our results showed that urea uptake by bacteria could be as high as 67% of the total urea uptake during a period of high bacteria biomass and in the decreasing phase of phytoplankton biomass. For nitrate and ammonium uptake, the exact percentage of bacteria contribution cannot be established with certainty because of the inhibition of regeneration processes. Nevertheless, in situations where ambient nitrate and ammonium concentrations are high, the role of regenerated nutrients is not significant and cannot influence the uptake behaviour. In these cases, for example, we found that the contribution of bacteria to ammonium uptake could be as high as 87% when ammonium concentrations were enhanced.

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