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Effect of ciliates on nitrification and nitrifying bacteria in Baltic Sea sediments

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ABSTRACT: Nitrification in aquatic sediments is catalyzed by bacteria. While many autecological studies on these bacteria have been published, few have regarded them as part of the benthic microbial food web. Ciliates are important as grazers on bacteria, but also for remineralization of organic matter. We tested the hypothesis that ciliates can affect nitrification. Experiments with Baltic Sea sediments in laboratory flumes, with or without the addition of cultured ciliates, were conducted. We found indication of a higher nitrification potential (ammonium oxidation) in one experiment and increased abundances of nitrifying bacteria in treatments with ciliates. This is likely due to higher nitrogen availability caused by excretion by ciliates and enhanced transport processes in the sediment.

KEY WORDS: Nitrifying bacteria · Ciliates · Nitrification · Bacteria · Sediment · FISH · Nitrification slurry assay

INTRODUCTION

The nitrogen cycle is one of the most important biogeochemical pathways, with nitrogen being an essential nutrient for all organisms. Various types of bacteria are responsible for many transformations within the nitrogen cycle. A large number of studies on these bacteria have been conducted in the past, but few investigators have looked at them as part of a food web, in which these bacteria have to compete with other organisms for nutrients/substrates and in which they might be prey for other components of the benthic food web (Verhagen & Laanbroek 1992), particularly for ciliates (Lavrentyev et al. 1997, Strauss & Dodds 1997). Our study focussed on nitrifying bacteria in sediments of the Baltic Sea and their possible trophic interactions with ciliates. Nitrification, the sequential oxidation of ammonium (NH_4^+) to nitrite (NO_2^-) and then nitrate (NO_3^-), is predominantly accomplished by chemolithotrophic bacteria under oxic conditions. This process is a dominant biogeochemical pathway in the upper layer of aquatic sediments, which are inhabited by large numbers and a high diversity of ciliates. Nitrifying bacteria consist of 2 functionally different groups of proteobacteria: ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) (Spiek & Bock 1998), as well as Archaea (Könnecke et al. 2005). The latter were only recently discovered and were not taken into consideration in this study, although they might be quantitatively important. Ciliates are important bacterial grazers in aquatic systems (Sherr & Sherr 2002), including sediments (Epstein 1997, Cleven 2004), with a wide array of feeding strategies. Grazing pressure of protists can greatly affect bacterial activity and community structure (Šimek et al. 1997, Rønn et al. 2002, Matz & Jürgens 2003). Hence, ciliate grazing is likely to affect the biogeochemical pathways in which bacteria play an essential role.

We designed a simple laboratory experiment using flumes filled with natural sediments, analyzing the effects of ciliates on the nitrification potential, the abundance and taxonomic composition of nitrifying bacteria in sediments.

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MATERIALS AND METHODS

Experimental setup. Experiments were conducted in 2 laboratory flumes (length: 32.44 cm; width: 17.4 cm; height: 18.7 cm; depth of sediment layer: ~7 cm). The upper 20 cm of natural sediments were collected from the Baltic Sea (Bay of Kiel, intertidal zone, approximately 54° 21′ N, 10° 09′ E), close to the shoreline, passed through a 2 mm sieve and incubated in the flumes for 40 d prior to the experiments in order to allow vertical gradients to re-establish. Water circulation was ~10 l h^{-1} with artificial seawater (salinity: 26‰; temperature: 20°C); each flume had an independent water circuit. Illumination was 12:12 h light:dark with artificial light (25 µE m^{-2} s^{-1}).

A ciliate cocktail consisting of several ciliate species initially isolated from the same sediments (for list of species see Table 3) and grown in the laboratory was added at a final density of 2.45×10^3 cells ml^{-1} of sediment to one of the flumes (+ ciliate treatment, +Cil). The ciliates were isolated 6 wk prior to the experiments and were cultured in artificial seawater (salinity: 24‰) in the dark at 20°C, with autoclaved wheat grains as the carbon source. No nitrifying bacteria were found among the food bacteria that developed in the cultures. An identical volume of 1.2 µm filtered ciliate cocktail was added to the other flume as a control (– ciliate treatment, –Cil). Some ciliates in low natural abundances were still present in –Cil (see 'Results'). Water circulation was paused for 1.5 h after addition of the ciliates to allow them to settle and migrate into the sediments, followed by an incubation time of 3 d.

Sampling procedure. All samples were taken with a plastic sediment corer (diameter: 16 mm). Bacteria, flagellate and ciliate samples were transferred into preweighed 50 ml plastic centrifugation tubes and fixed with glutardialdehyde (GA; 2% final concentration). Samples for nitrifying bacteria were also transferred into 50 ml centrifugation tubes and were fixed with paraformaldehyde (4% final concentration). For each group 3 independent replicates per flume were collected. Samples for the determination of the nitrification potential were collected with the same type of corer, but transferred directly into the Erlenmeyer flasks used for the slurry assay (see below), with 6 independent replicates per flume. Samples for abiotic parameters such as sediment porosity, ash-free dry weight and grain size distribution were collected with a spoon and stored in 50 ml centrifugation tubes until further processing (6 independent replicates per flume).

Enumeration of bacteria, flagellates and ciliates. Total bacterial and flagellate abundances were enumerated using epifluorescence microscopy. Fixed samples were sonicated (Labsonic M, pulse: 0.9; amplitude: 60%; 60 s) in order to separate attached bacteria from sediment particles and to break up bacterial aggregates. For extraction of bacteria and flagellates, samples were then resuspended in sterile artificial seawater with 2% GA and gently shaken by hand for 60 s. Thereafter, 3.5 ml of supernatant was immediately pipetted off into a 50 ml centrifuge tube. To achieve a quantitative extraction, this washing procedure was repeated at least 5 times per sample. Then, 200 or 250 µl of the supernatant was diluted with 1800 or 1750 µl of particle-free artificial seawater, stained with DAPI (Porter & Feig 1980) and filtered onto a black 0.2 µm polycarbonate filter (Nuclepore) or a 0.8 µm filter (Nuclepore) for bacterial and flagellate counts, respectively.

The NOB *Nitrospira* and *Nitrobacter* and the AOB *Betaproteobacteria* (β-AOB) were detected with fluorescence *in situ* hybridization (FISH). Probes Ntspa712 and cNtspa712 (Daims et al. 2001) and NIT3 (Wagner et al. 1996) were used for NOB and Nso1225 (Mobarry et al. 1996) and for β-AOB, respectively (for details on these oligonucleotide probes see probeBase; Loy et al. 2003). All probes were labelled with the fluorescent dye Cy3. Hybridization procedures followed a protocol described previously by Pernthaler et al. (2001). Washing and filtration were identical to the procedures applied for the DAPI method, except that white 0.2 µm filters (Nuclepore) were used.

Ciliate extraction followed the methods of Epstein (1995) and Cleven (2004). Samples were washed at least 7 times with 5 ml of sterile artificial seawater amended with 2% GA to remove larger sand grains. After separation from the remaining sediment particles by density gradient centrifugation, ciliates were filtered onto 1.2 µm cellulose nitrate filters (Sartorius), then counted and taxonomically identified using a silver-staining method that stains nuclei, cilia and ciliary basal bodies (Skibbe 1994). All counts were conducted using a Nikon Eclipse E800 microscope at 1000× magnification for bacteria and flagellates and 400× to 1000× for ciliates.

Nitrification potential (slurry assay). Sediment samples were slurried (~6.5 g sediment + 100 ml of 0.2 µm filtered artificial seawater) and placed into 200 ml Erlenmeyer flasks. Each flask was amended with NH_4^+ (50 µM) and $NaClO_3$ (20 mM) for NH_4^+ oxidation rates or with NO_2^- (25 µM) and allylthiourea (ATU; 10 mg l^{-1}) for NO_2^- oxidation rates. $NaClO_3$ and ATU are inhibitors for the respective complementary step of nitrification. Thus, in the treatment with $NaClO_3$, the NO_2^- concentration increased, while it decreased in the treatment with ATU. The Erlenmeyer flasks were aerated and stirred continuously. Sub-samples were taken from each flask after 0, 1.5, 5 and 22 h (t_0, t_1, t_2 and t_3,

respectively). Prior to sampling, stirring and aeration were stopped for 5 min. Samples were centrifuged for 5 min (Heraeus Labofuge 200, 2860 × *g*) to remove remaining sediment particles. NO_2^- concentrations were measured photometrically using a Bran + Luebbe AutoAnalyzer (AA3, Method No. G-029-92 for nitrite-N, Dollhopf et al. 2005, recommended by P. Stief pers. comm.). Nitrification potentials were calculated from the change in NO_2^- concentration over time.

Abiotic parameters. Temperature, pH, oxygen saturation and salinity in the water circuit were measured with a multiprobe (WTW Multimeter 350i, ConOX and a SenTix 41 probes; Table 1), NH_4^+, NO_2^-, NO_3^- and PO_4^- concentrations were determined photometrically (Bran + Luebbe AutoAnalyzer AA3, Method No. G-029-92 for nitrite-N and G-016-91 for nitrate-N). Dry weight, ash-free dry weight, porosity (calculated according to Hölting 1996) and grain size distribution were determined (Retsch AS 200 basic wet sieving machine; Table 2). All results given per gram refer to dry weight. SigmaXL (Version 2000) and SPSS (Version 11.0) software were used for statistical analyses.

In addition to the data shown, we tried to obtain *in situ* concentrations of NH_4^+, NO_2^- and NO_3^- with LIX microelectrodes (DeBeer et al. 1997); however, due to the salinity of the system this was not possible.

Table 1. Abiotic parameters in the different treatments in the experimental flumes. Data are means ± SD (+Cil: + ciliate treatment; –Cil: – ciliate treatment)

	+Cil	–Cil
pH	8.6	8.5
Water O_2 saturation (%)	87	92
Temperature (°C)	20.3	20.9
Salinity (‰)	26.2	25.8
NH_4^+ (µM)	47 ± 14	31 ± 7
NO_2^- (µM)	9 ± 0.6	6 ± 0.8
NO_3^- (µM)	985 ± 128	828 ± 107
PO_4^- (µM)	186 ± 0.6	188 ± 23
Sediment porosity	32.8 ± 1.1	31.4 ± 0.6
Sediment ignition loss (%)	0.9 ± 0.9	1.7 ± 2.1

Table 2. Sediment grain size distribution in the experimental flumes. Data are mean ± SD

Grain size (mm)	Percent portion
>0.5	32.2 ± 3.7
0.49–0.125	63.1 ± 3.4
0.125–0.063	1.1 ± 0.6
0.063–0.025	0.3 ± 0.3
<0.025	3.3 ± 0.7

RESULTS

Bacteria and flagellates

In our sediment incubations, total bacterial abundances after the incubation period ranged from 5.4 × 10^8 to 6.0 × 10^9 cells g^{-1} and numbers were similar in treatments with and without ciliates (*t*-test, p = 0.955; Fig. 1). Abundances of the 3 investigated types of nitrifying bacteria (*Nitrosomonas*, *Nitrospira* and *Nitrobacter*) were always lower in the treatments without ciliates (Fig. 1). Together they contributed 1.45 and 0.35% of total bacteria in the treatments with and without ciliates, respectively. Although not statistically significant, the difference was most distinct for *Nitrospira* (*t*-test, p = 0.0585), followed by *Nitrobacter* (*t*-test, p = 0.0702) and β-AOB (*t*-test, p = 0.2613). Total flagellate abundance at the end of the incubation period was 2.5×10^5 (±2.0×10^4) cells g^{-1} in the +Cil treatment and 1.5×10^5 (±3.8×10^4) cells g^{-1} in the –Cil treatment; no significant difference between treatments was found. Total biovolumes of flagellates were similar in both treatments, with 6.5×10^6 (±3.2×10^6) µm^3 g^{-1} in the +Cil treatment and 6.4×10^6 (±2.9×10^6) µm^3 g^{-1} in the –Cil treatment. The proportion of autotrophic flagellates did not differ significantly between the treatments either, being 54 and 60% in terms of total flagellate abundance, but 84 and 91% of the total flagellate biovolume.

Ciliates

Ciliate abundance in +Cil was 1478 (±578) cells g^{-1}, i.e. 52% of the ciliates initially added to the sediment were recovered. The abundance in –Cil was 189 (±120) cells g^{-1}, which is significantly lower (*t*-test, p =

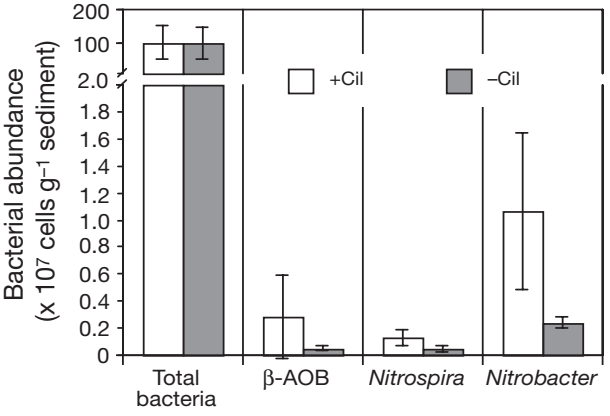

Fig. 1. Bacterial abundances in the experimental sediments after the incubation period (error bars = ±1 SD). β-AOB: ammonium-oxidizing *Betaproteobacteria*. +Cil: + ciliate treatment; –Cil: – ciliate treatment

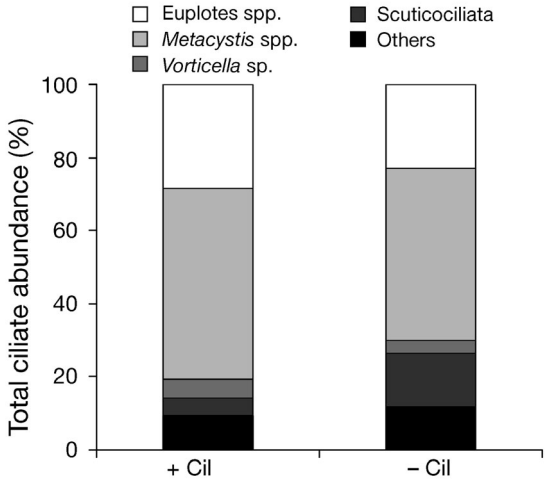

Fig. 2. Taxonomic composition of ciliates in the experimental flumes after the 3 d incubation period

0.006), reflecting the natural sediment abundance. The taxonomic composition of the ciliate community was very similar in both treatments (χ^2-test, p = 0.999; Fig. 2) and was dominated by *Metacystis* spp. and *Euplotes* spp. Together these 2 taxa contributed 80.5% (+Cil) and 70.0% (–Cil) to the total ciliate abundance. In addition, *Aspidicsa* sp., *Coleps* spp., *Vorticella* sp., *Paramecium* spp., *Litonotus* sp., *Loxophyllum helus* and various scuticociliates were found in lower abundances (Table 3).

Nitrification potential

Nitrification potentials ranging from 0.08 to 5.10 µg N g^{-1} h^{-1} were measured in the different treatments (Fig. 3). In –Cil, the rates were generally lower than in +Cil (except for nitrite oxidation t_1 to t_2). The difference was significant for ammonium oxidation t_1 to t_2 (t-test, p = 0.0294), with rates for ammonium oxidation of 4.47 (±1.60), 0.61 (±0.17) and 0.35 (±0.08) µg N g^{-1} h^{-1} for +Cil and 3.37 (±1.48), 0.29 (±0.27) and 0.31 (±0.14) µg N g^{-1} h^{-1} for –Cil for t_0 to t_1, t_1 to t_2 and t_2 to t_3, respectively. Nitrite oxidation rates were 5.10 (±2.88), 0.08 (±0.08) and 0.25 (±0.09) µg N g^{-1} h^{-1} for +Cil and 2.54 (±0.99), 0.10 (±0.07) and 0.18 (±0.05) µg N g^{-1} h^{-1} for –Cil. The high rates in the interval t_0 to t_1 caused a depletion of the substrates, and, consequently, the rates measured in the intervals t_1 to t_2 and t_2 to t_3 were much lower and not included in our further analysis.

Table 3. Taxonomic composition of the ciliate community (% of total abundance). Total abundances were 1478 (±578) cells g^{-1} in the + ciliate treatment (+Cil) and 189 (±120) cells g^{-1} in the – ciliate treatment (–Cil). Data are mean ± SD

Taxon	+Cil	–Cil
Euplotes spp.	28.5 ± 11.8	22.8 ± 8.6
Aspidisca sp.	1.0 ± 1.6	0.0 ± 0.0
Metacystis spp.	52.0 ± 13.8	47.2 ± 21.0
Coleps spp.	0.5 ± 0.8	2.2 ± 3.8
Vorticella sp.	5.4 ± 1.0	3.3 ± 5.8
Paramecium spp.	1.9 ± 2.2	3.3 ± 5.8
Scuticociliata	4.7 ± 1.0	14.7 ± 20.0
Undeterminate Hymentomatida	0.0 ± 0.0	2.1 ± 3.6
Litonotus sp.	0.5 ± 0.8	0.0 ± 0.0
Loxophyllum helus	1.6 ± 2.7	2.2 ± 3.8
Undeterminate Pleurostomatida	0.0 ± 0.0	2.1 ± 3.6
Undeterminate ciliates	4.0 ± 3.1	0.0 ± 0.0

DISCUSSION

Our experiments demonstrated that the increase in ciliate abundance could enhance the abundance of nitrifying bacteria and the nitrification potential in the sediments taken from the Baltic Sea. This was not always statistically significant, but reflected a distinct trend. A similar effect was found by Strauss & Dodds

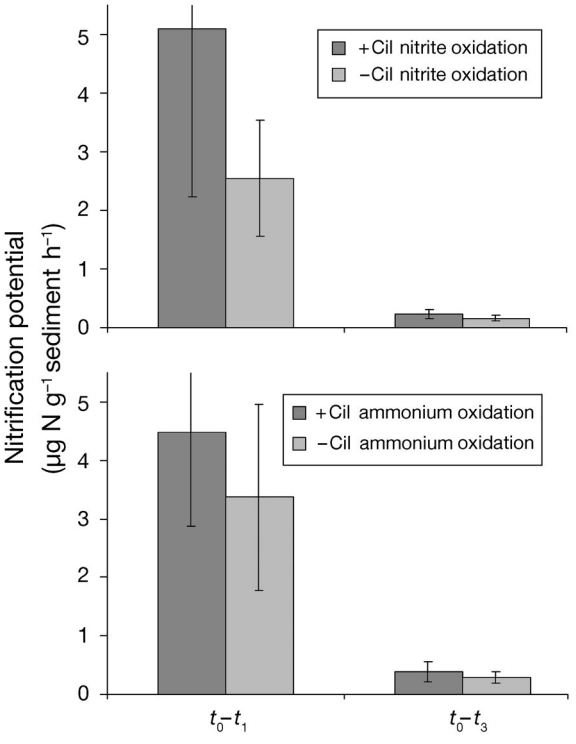

Fig. 3. Nitrification potentials of the sediments in the experimental flumes after the incubation period, as determined with slurry assays. Rates from t_0 to t_1 are shown. Due to the high rates, the substrates in the slurry assay were consumed very quickly and the rates from t_2 to t_3 were not used for further analysis (t_0, t_1, t_2, and t_3: 0, 1.5, 5 and 22 h, respectively; error bars: ±1 SD)

(1997) for submerged soil sediments. Although Lavrentyev et al. (1997) demonstrated that ciliates do ingest nitrifying bacteria, a recent study showed that selective grazing by a mixed ciliate community (ciliates preferring or discriminating against nitrifying bacteria; Neubacher et al. 2007) can be excluded. We conclude that the presence of ciliates, especially at high abundances, enhances transport processes in sediments (Glud & Fenchel 1999) and thus improves substrate availability to nitrifying bacteria. Furthermore, ciliate digestion of food leads to an ammonium excretion of 0.25 to 2 µg N h^{-1} mg^{-1} dry weight (Ferrier-Pagès & Rassoulzadegan 1994), which further contributes to an increased substrate availability for nitrifiers in the presence of ciliates. Lavrentyev et al. (1997) showed that in planktonic systems ciliate grazing pressure can lead to the formation of large colonies of bacteria, making it more difficult for nitrifying bacteria to obtain substrates. This is unlikely to happen in an interstitial system, where most bacteria are attached to sediment particles (Kasimir 1990). Flagellate and total bacterial abundances after the incubation period did not differ between +Cil or –Cil, indicating that grazing pressure from ciliates was rather low or that grazing losses of bacteria and flagellates were compensated by increased growth, possibly accompanied by a change in community composition. As indicated by the large portion of biovolume, the flagellate community was dominated by large phototrophic flagellates; heterotrophic flagellates were rather small in size. Other studies have shown that—in contrast to planktonic systems—in sediments, ciliates might be more important as bacterial grazers than heterotrophic flagellates, and top-down control of benthic bacteria by heterotrophic flagellates seems unlikely (Dietrich & Arndt 2000 and literature cited therein). Thus, flagellate grazing on bacteria was not considered as a factor interacting with the effect of ciliates on nitrification in our experimental system, but further studies might look at the role of flagellates more closely. In addition to bacteria and flagellates, diatoms were also found in our systems. Presumably they were too large as prey for the ciliate species in our sediments. As the ciliate abundance increased 8-fold after the incubation period in +Cil, we assume that ciliate grazing pressure on bacteria and flagellates was rather high. This is consistent with the ciliate community composition being strongly dominated by small bacterivorous forms. Thus, the grazing pressure of ciliates increased the availability of nitrogen compounds, thereby allowing compensation of grazing losses due to higher growth rates of bacteria and flagellates. Our results further indicate that nitrifying bacteria profit disproportionately from higher substrate availability due to ciliate grazing, because ammonium as a substrate for nitrification is the main excretion product of ciliates (Ferrier-Pagès & Rassoulzadegan 1994). Another explanation for the increase in nitrification potentials and the abundance of nitrifying bacteria in the treatments with high ciliate abundances might be an enhanced transport of solutes such as NH_4^+, NO_2^-, NO_3^- and O_2 through the interstitial spaces due to the movement and filtration activity of the ciliates (Glud & Fenchel 1999). Fenchel (1986) showed that ciliates can generate a significant flow, which may contribute to a better distribution of substrates in the interstitial spaces of the sediments, where transport is otherwise mostly limited to diffusion (Huettel & Webster 2001).

Despite the significant difference of ciliate abundance between the treatments, a true control without any ciliates would have been desirable, but there is no practical method to completely remove ciliates selectively from sediments without damaging other components of the microbial community. Generating an artificial sediment system with sterile sand and bacteria, flagellates, ciliates, algae and metazoans from cultures does not seem to be a sufficient solution either, as such a system can hardly represent the complex interactions found in natural sediments. However, besides the rather high variability in the abundance of nitrifying bacteria in our samples, the presence of low numbers of ciliates in the control likely caused the lack of statistical significance in some of the effects of the experimental manipulation. In addition to the nitrification potentials, knowledge of *in situ* concentrations of nitrogen compounds and *in situ* nitrification rates would have been very useful. Such direct measurements would have avoided possible effects on the components of the microbial food web due to the use of inhibitors. However, because of the difficulties with the use of microsensors these data are not available. Furthermore, it cannot be completely ruled out that the use of other ciliate species might have attenuated or increased the effect on nitrifying bacteria. We are, however, confident that this is unlikely, since the ciliates used in our experiment represented the natural community very well. Here, 52% of the ciliates that were added initially were recovered after the incubation period. The remaining 48% probably did not find their way into the sediment and, consequently, were destroyed in the pump. As the measurements of NH_4^+, NO_2^-, NO_3^- and PO_4^- in the water column showed, this did not have a significant effect on the nutrient level and can be ruled out as a source of the increased nutrient supply in +Cil.

Total bacterial abundances were well within the range of those reported for other marine and freshwater sediments (Llobet-Brossa et al. 1998, Kuwae & Hosokawa 1999, Altmann et al. 2004). In some studies,

bacteria of the genus *Nitrospira* have been identified as the dominating NOB in marine and freshwater systems (Hovanec et al. 1998, Altmann et al. 2003). In our study, bacteria of the genus *Nitrobacter* were the quantitatively most important NOB. Abundances of *Nitrobacter* exceeded *Nitrospira* abundances >5-fold in –Cil and 8-fold in +Cil. In both treatments, *Nitrobacter* accounted for 71% of the detected nitrifying bacteria. Other NOB, such as *Nitrospina* or *Nitrococcus*, might have also been present, but are unlikely to be numerically important (Watson et al. 1981). During the course of our experiments, a new ammonia-oxidizing Crenarchaeota was described (Könneke et al. 2005). Future experiments should consider the presence and abundance of this new type of ammonia-oxidizing microorganism, which might show a different reaction to ciliate grazing.

CONCLUSIONS

Ciliates affect the abundance of nitrifying bacteria in sediments and, hence, the nitrification potential. The likely reason for the increased abundances of nitrifiers and nitrification potentials in the presence of ciliates is probably nitrogen recycling, which leads to enhanced nitrogen availability for nitrifying bacteria. This indicates that nitrifying bacteria are subject to bottom-up control by substrate availability rather than top-down control through ciliate grazing. Ciliate grazing did not negatively affect the nitrifying bacteria quantitatively, although Lavrentyev et al. (1997) and Neubacher et al. (2007) showed that they are ingested by ciliates. Another possible explanation for the increased abundances of nitrifying bacteria and nitrification potentials in +Cil might be an increased transport of substrates and oxygen in the sediments due to the movement and filtration activities of the ciliates.

Although the results of the present study lead to the conclusion that ciliates should be considered as a controlling factor for nitrifying bacteria and nitrification, further studies on this relationship are necessary. These should focus on a comparison with other marine and freshwater systems; *in situ* experiments should be conducted, and seasonal effects should be taken into account.

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