Studies on the impact of food web effects on nitrification in aquatic sediments

Dissertation zur Erlangung des Grades eines
Doktors der Naturwissenschaften

- Dr. rer. nat. -

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Ein Fluss passt sich dem Weg an, der möglich ist, vergisst aber nie sein Ziel, das Meer. Zart an der Quelle, schwillt er, durch die Flüsse gespeist, auf die er unterwegs trifft, stetig an.

Paolo Coelho
Handbuch des Kriegers des Lichts

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PART I

1. SYNOPSIS

1.1a Abstract

Nitrification is an important biogeochemical pathway in the upper, oxic layer of aquatic sediments and is predominantly accomplished by two groups of chemolithotrophic nitrifying bacteria. While these bacteria have been subject to numerous autecological studies before, they have rarely been regarded as part of food webs, in which they have to compete with other organisms for nutrients and substrates and in which they are prey to other organisms. The impact of ciliates as important bacterial grazers in sediments on nitrification and nitrifying bacteria was investigated, both in marine and freshwater systems. *In vitro* experiments were conducted to detect and evaluate a possible grazing selectivity of ciliates for or against nitrifying bacteria. Furthermore, the effect of a bioturbating omnivorous polychaete (*Nereis diversicolor*) in marine sediments was studied. Natural sediments in laboratory flumes were used to detect quantitative effects of ciliates or *Nereis*.

The results indicate that increased ciliate abundances led to higher nitrification potentials, higher abundances of nitrifying bacteria and higher nitrate concentrations. The effects were strongest in marine sediments. Selective feeding could be ruled out as a cause. Increased bacterial abundances in the presence of the *Nereis* and changes in the community composition of nitrifying bacteria were found.

The long-known and widely used settling method for the enrichment of fixed ciliate cells from plankton samples was critically reviewed in an additional study. The settling times commonly used are based on experience or estimation, but not on experimentally derived data. Settling times for seven ciliate cultures were determined empirically and theoretical sinking velocities were calculated. A method for the determination of the ciliate density (in terms of g ml^{-1}) had to be developed for the theoretical approach. The results revealed that the sinking velocity is much higher than assumed, which allows to save up to 95 % of settling time and improves the quality of the results.

1.1b Zusammenfassung

Die Nitrifikation ist ein bedeutender biogeochemischer Prozess in den oberen, oxischen Bereichen von aquatischen Sedimenten, der vor allem von zwei Gruppen chemolithotropher Bakterien katalysiert wird. Diese Bakterien wurden bereits in vielen autökologische Studien untersucht. Jedoch wurden sie bisher selten als Teil von Nahrungsnetzen betrachtet, in denen sie mit anderen Organismen um Nährstoffe und Substrate konkurrieren und in denen sie Beute für andere Organismen darstellen. Der Einfluss von Ciliaten als wichtige Räuber von Bakterien in Sedimenten auf die Nitrifikation und die nitrifizierenden Bakterien wurde sowohl in marinen- als auch in Süßwasser-Sedimenten untersucht. *In vitro*-Experimente zur Bestimmung der Fraß-Selektivität der Ciliaten für oder gegen nitrifizierende Bakterien als mögliche Ursache wurden durchgeführt. Darüber hinaus wurde der Effekt eines bioturbierenden omnivoren Polychaeten (*Nereis diversicolor*) in marinen Sedimenten ermittelt. Versuche mit natürlichen Sedimenten in Labor-Fließrinnen wurden eingesetzt, um einen möglichen quantitativen Einfluss von Ciliaten oder *Nereis* festzustellen.

Erhöhte Abundanzen von Ciliaten führten zu höheren Nitrifikationspotentialen, höheren Nitratkonzentrationen und höheren Abundanzen von nitrifizierenden Bakerien. Dieser Effekt war in marinen Sedimenten stärker ausgeprägt. Selektiver Fraß der Ciliaten für oder gegen nitrifizierende Bakterien konnte ausgeschlossen werden. Die Anwesenheit von *Nereis* führte zu erhöhten Bakerienabundanzen und einer veränderten Gemeinschaft der nitrifizierenden Bakterien.

In einer weiteren Studie wurde die häufig eingesetzte Sedimentations-Methode zur Anreicherung von fixierten Plankton-Proben kritisch betrachtet. Die bislang verwendeten Sedimentationszeiten beruhen auf Erfahrung oder Schätzung. Für sieben Ciliatenkulturen wurde die Sinkgeschwindigkeit empirisch bestimmt und zusätzlich theoretisch berechnet. Hierfür musste zunächst eine Methode zur Bestimmung der Dichte (g ml^{-1}) entwickelt werden. Die Ergebnisse zeigen, dass die Sinkgeschwindigkeit deutlich höher ist als erwartet. Dadurch können bis zu 95 % der Sedimentationszeiten eingespart werden und die ergebnisse werden zuverlässiger.

1.2 General introduction

Among the biogeochemical element cycles, the nitrogen cycle (Fig. 1.1) is one of the most important ones. Nitrogen is an essential nutrient for all organisms, and nitrogen compounds are relevant for a number of environmental problems such as eutrophication (Rabalais 2002) or the green house effect (Lent et al. 1999). Within the nitrogen cycle, numerous transformations are catalyzed by various prokaryotes, some of which were identified only very recently (Strous et al. 1999, Könnecke et al. 2005). Many autecological studies on these organisms have been conducted, but despite their importance, these prokaryotes have only rarely been looked at as a part of a food web, in which they have to compete for substrates and nutrients and in which they might be prey to other organisms (Verhagen & Laanbroeck 1992), ciliates (Lavrentyev et al. 1997, Strauss & Dodds 1997). In order to start filling this

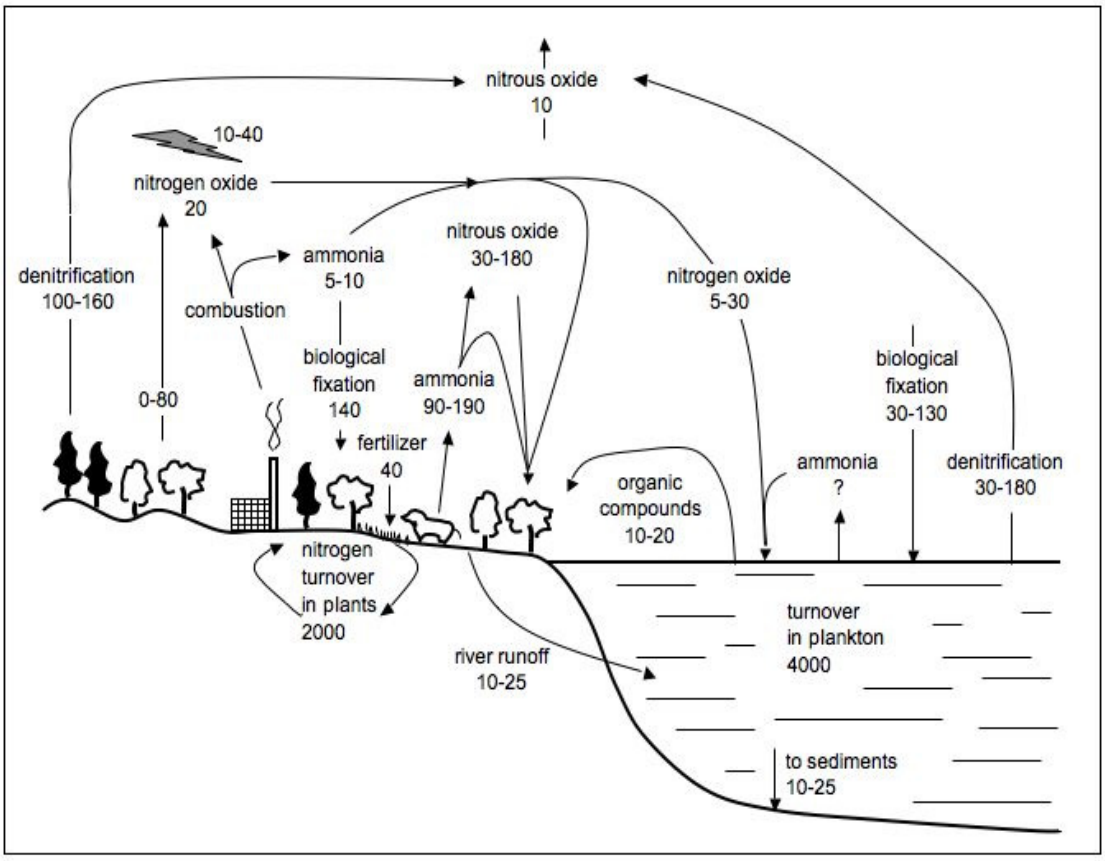

Fig. 1.1: The global nitrogen cycle. Units are Tg (10^{12}g) N yr^{-1}. From Söderlund & Rosswall (1982) based on Söderlund & Svensson (1976)

gap, this PhD thesis focussed on ecological aspects of nitrification and nitrifying bacteria. Nitrification is the sequential oxidation of ammonium (NH_4^+) to nitrate (NO_3^-) via nitrite (NO_2^-), and it is predominantly accomplished by chemolithotrophic bacteria. The first step is the ammonium oxidation, which proceeds by a series of oxidation stages through hydroxylamine and pyruvic oxime to nitrous acid:

$$NH_4^+ + 1½\ O_2 \quad \leftrightarrow \quad 2H^+ + NO_2^- + H_2O$$

($\Delta G_0' = -66.0$ kcal)

$$NH_4^+ \rightarrow NH_2OH \rightarrow H_2N_2O_2 \rightarrow HNO_2$$

This transformation is catalyzed by bacteria, which are largely confined to the genus *Nitrosomonas* (Nitrobacteriaceae, order Pseudomonadales), although *Nitrosococcus, Nitrosospira, Nitrosolobus, Nitrosovibrio* and several other taxa, including methane-oxidizing bacteria and crenarchaeota, are also known to be capable of this process (Wetzel 2001 and literature cited herein, Könnecke et al. 2005). The second step is the nitrite oxidation:

$$NO_2^- + ½\ O_2 \quad \leftrightarrow \quad NO_3^-$$

($\Delta G_0' = -18.0$ kcal)

The bacteria catalyzing this step are *Nitrobacter, Nitrospira, Nitrospina* and *Nitrococcus*. Nitrification is an aerobic process and requires a pH close to neutral. It can be inhibited by certain organic compounds such as tannins and by high intensities of light (Horrigan & Springer 1990, Wetzel 2001 and literature cited herein). Nitrification is an important process, as it transfers the primary excretion product ammonium into nitrate, which can then be utilized as nutrient or removed from the system by denitrification to N_2. Ammonium can also be oxidized in an anaerobic process (anaerobic ammonuim oxidation; Anammox) (Jetten et al. 1999), but this pathway is catalyzed by different organisms (Strous et al. 1999).

Nitrification does not only take place in the water column, but also in sediments, where it is spatially closely coupled to other biogeochemical processes due to the steep gradients at the sediment/water interface and where bacterial abundances are higher than in the water column. Only recently, sandy sediments have been identified

as places of strong microbial activity, both in freshwater and in marine systems (Hendricks 1996, DeBeer et al. 2005). Besides bacteria, the interstitial system of a sandy sediment is inhabited by a broad variety of other microscopical organisms, such as flagellates, ciliates, gastrotrichs, algae, nematodes and other benthic meiofauna, forming a complex benthic food web (e.g. Epstein 1997a, Schmid-Araya & Schmid 2000). While in pelagic systems heterotrophic flagellates are considered to be the main bacterial grazers (Berninger et al. 1991), in the benthic food web, ciliates are the more important bacterivores (Kemp 1988, Epstein 1997b, Dietrich & Arndt 2000, Cleven 2004). Ciliates belong to the protists and are a very heterogeneous group regarding their metabolism, feeding mechanism, locomotion, and habitat preferences (Hausmann & Bradbury 1996). They occur not only in aquatic environments, but also in soils and as parasites in other organisms. Interstitial and epibenthic ciliates inhabiting sediments (Fig. 1.2) feed mainly on bacteria, flagellates, other ciliates and algae and are well adapted to this special environment (Berninger & Epstein 1995, Wickham et al. 2000).

The main aim of this PhD study was to find out, whether ciliates have an impact on nitrification in aquatic sediments. In theory, ciliates can have an effect on the nitrogen cycle in several ways. Ciliates take up nitrogen compounds with their food and excrete ammonium, so their own metabolism contributes to the processing of nitrogen in the sediments (Daumas 1990, Ferrier-Pagés & Rassoulzadegan 1994, Hassink et al. 1994). Furthermore, ciliates can use nitrate as an electron acceptor (Finlay et al. 1983, Finlay 1985), but that does only occur under anoxic conditions and is not relevant for nitrification.

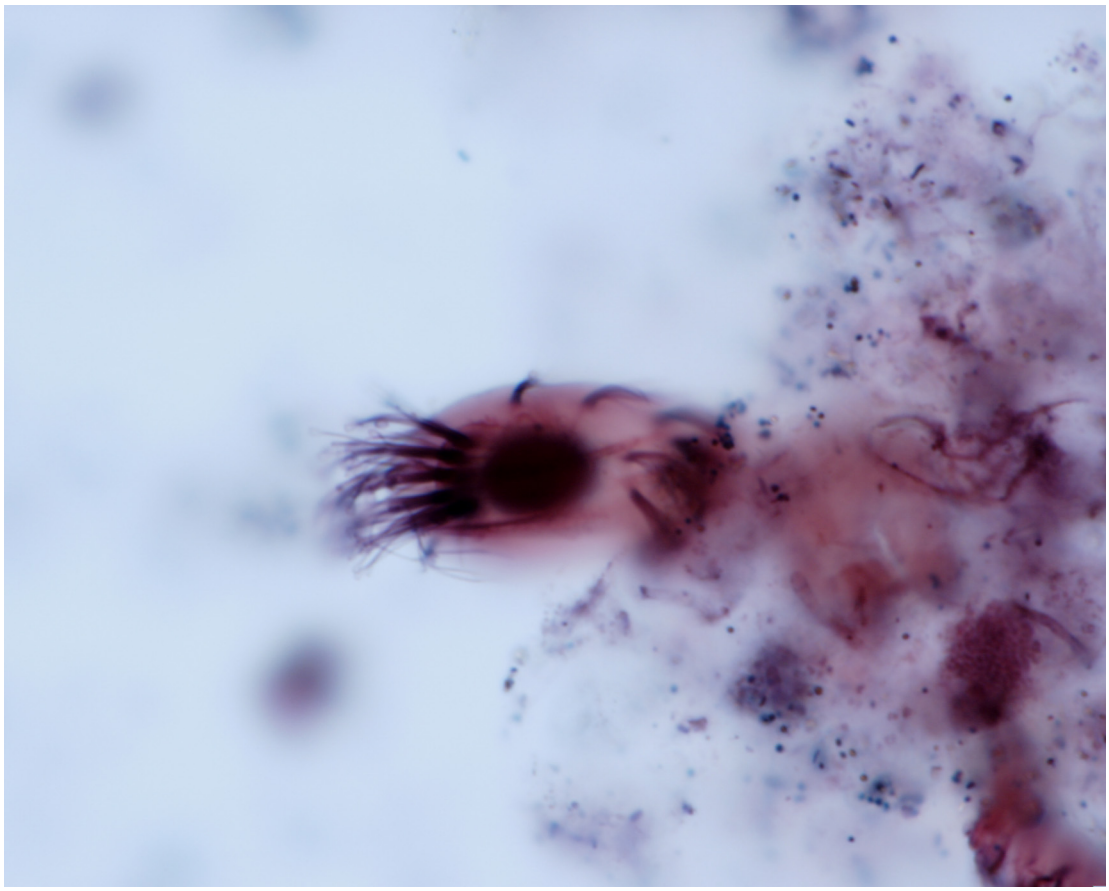

Fig. 1.2: Specimen of *Euplotes* sp. from sediments of the Baltic Sea, as an example for a typical interstitial ciliate (length of cell body approx. 60 µm); details made visible by the quantitative protargol staining (QPS) method.

In addition to these direct effects ciliates might also have an indirect impact on nitrification by their grazing on bacteria. Protistan grazing is known to be able to change the activity, productivity and composition of the bacterial community (Šimek et al. 1997, Hahn & Höfle 2001, Rønn et al 2002, Matz & Jürgens 2003). As nitrification is predominantly accomplished by bacteria, these changes in activity and community structure might also affect the nitrifying bacteria, and thus, nitrification, as the nitrifying bacteria have to compete for nutrients/substrates with the other bacteria or other organisms (Verhagen & Laanbroeck 1992, Verhagen et al. 1993, Riisgard-Petersen et al. 2004). Furthermore, ciliate grazing can be selective, and resulting effects on the bacterial community were reported by Fenchel (1980) and Šimek et al. (1994). The selectivity can be based on the size (e. g. Kivi & Setaelae 1995) and/or the chemical properties (Verity 1991) of the prey. Because nitrifying bacteria account only for a very small proportion of total bacteria (e. g. Altmann et

al. 2003) and have very low growth rates (Spieck & Bock 1998), a selective grazing with ciliates prefering nitrifying bacteria over other types of bacteria might have dramatic effects on their population and composition. Mallory et al. (1983) showed that selective protist grazing might even eliminate slow-growing bacteria. Several studies have shown that bacteria have morphological and physiological defence mechanisms such as formation of microcolonies, production of exopolymers or change of cell size or -shape (e. g. Lebaron et al. 1999, Hahn & Höfle 2001, Hahn et al. 2004). No such mechanism is known for nitrifying bacteria. Chemical defence mechanisms have not been reported either, but are known from other aquatic organisms (e. g. Wolfe et al. 1997). If a defence mechanism was present, it might lead to selective grazing where ciliates prefer „common" bacteria over nitrifying bacteria.

In addition to their own metabolism and their indirect effects due to grazing, ciliates might also affect nitrification simply through their movement and feeding activites, which is realized by ciliary movement („beating"), creating water currents. A single cell of a typical interstitial ciliate (such as *Uronema* or *Euplotes*) can filter a water volume of 6 to 50 µl per day (Fenchel 1986). Consequently, Glud & Fenchel (1999) demonstrated that high ciliate densites such as found in estuarine sediments can enhance the transport coefficient of biogeochemically important solutes by a factor of 1.1 to 10 above the diffusive coefficient. The effect might even be stronger than the impact of bioturbation of meiofauna organisms (Glud & Fenchel 1999; compare section 1.6). Thus, ciliates might improve the supply of oxygen and substrates for nitrifying bacteria and enhance nitrification.

So far, studies that examined the interaction of the nitrogen cycle and protists are very scarce, and were mostly conducted in soil systems (Stout 1980, Verhagen et al. 1993, Hassink et al. 1994, Strauss & Dodds 1997). This PhD thesis consists of four studies (chapters 2 to 5), which address individual aspects of the interaction between ciliates, benthic food webs and nitrification in aquatic ecosystems. In addition, a fifth study dealing with a standard method for concentration of ciliate cells has been conducted. In the following sections, a brief overview of these studies is presented.

1.3 Grazing selectivity of ciliates

In the first study (chapter 2) the question was addressed, whether ciliates can affect nitrification via selective grazing on nitrifying bacteria. Lavrentyev et al. (1997) demonstrated that ciliates feed on nitrifying bacteria, but their study was conducted in a pelagic system and used fluorescently labelled nitrifying bacteria (FLNB). This method is not suitable for the detection of selectivity, as FLBs are significantly discriminated by protists (Boenigk et al. 2001). Our study was designed as a series of *in-vitro* experiments and used fluorescence-*in-situ*-hybridization (FISH) as main method. The aims of the study were (i) to detect nitrifying bacteria within the food vacuoles of ciliates, (ii) to learn about possible selection for or against nitrifying bacteria, and (iii) to investigate a possible size- and morphology-dependant food selectivity by the ciliates.

For the experiments we used cultures of four ciliate species (*Paramecium aurelia*, *Euplotes octocarinatus*, *Tetrahymena pyriformis* and *Cyclidium glaucoma*), which resemble the natural spectrum of size and feeding types of bacterivorous interstitial ciliates (Šimek et al. 1994, Eisenmann et al. 1998, Wilks & Sleigh 1998, Hausmann et al. 2003). Cultures of *Nitrosomonas europaea* as ammonium-oxidizing bacteria and *Nitrospira moscoviensis* as nitrite-oxidizing bacteria were offered as food. A mixture of isolates from free-living bacteria was used as additional „food bacteria mix“. These did not contain any nitrifying bacteria. The experiments were designed as *in-vitro* experiments, using 50 ml centrifuge tubes and constant light and temperature conditions. The four ciliate cultures were fed with the bacterial food mix and the nitrifying bacteria in a ratio of approx. 1:10. Bacteria were counted using the DAPI method (Porter & Feig 1980), Fluorescence-*in-situ*-hybridization (FISH) was used to detect the nitrifying bacteria and differentiate them from the food bacteria mix. FISH has already been used to detect ingested bacteria within the food vacuoles of protists (Diederichs et al. 2003) (Fig. 1.3). For details on experimental setup, probes and hybridization conditions, counting procedures, processing of data etc. see chapter 2.3.

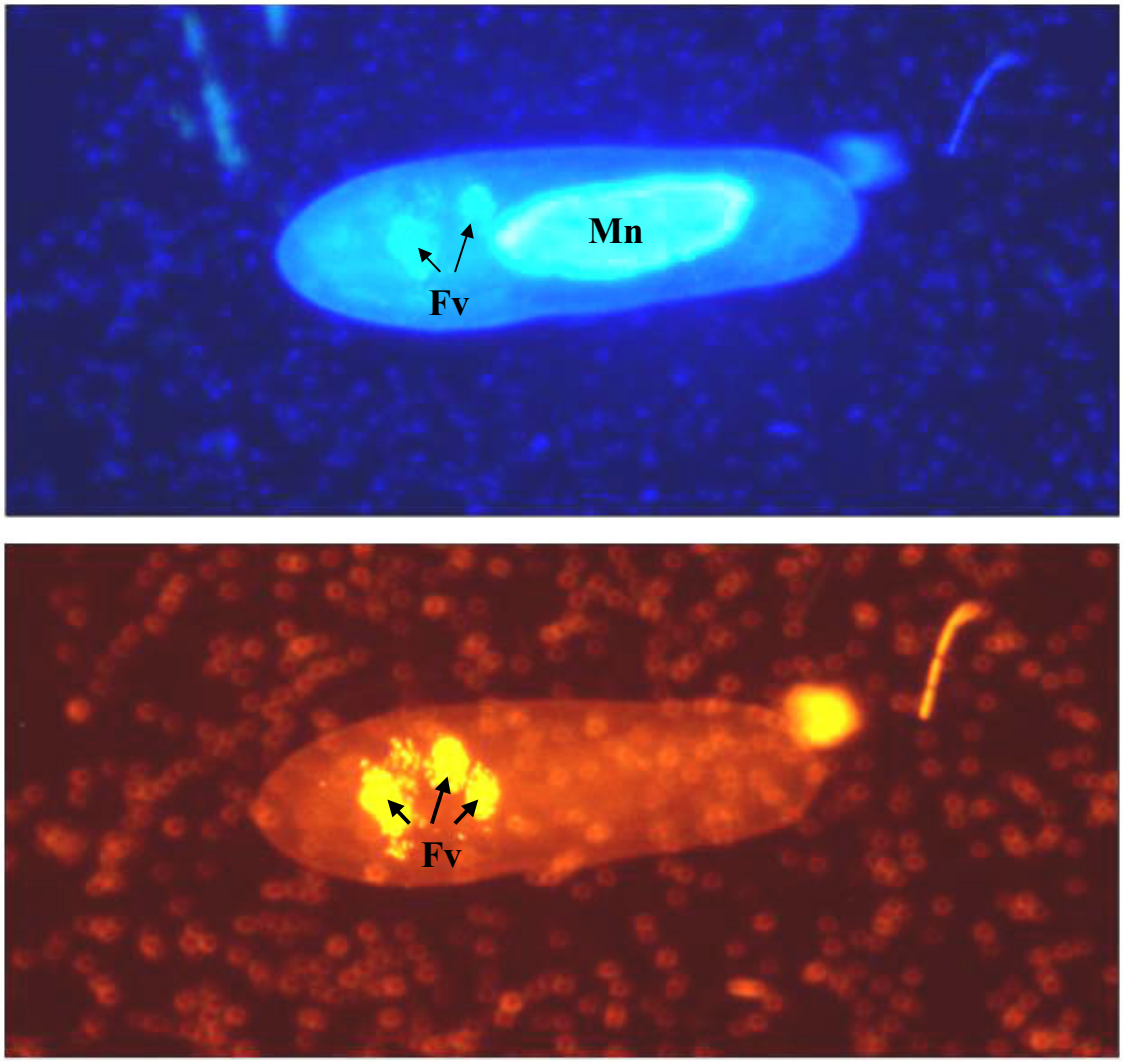

Fig. 1.3: The ciliate *Paramecium aurelia*; A: DAPI-staining, B: FISH-staining with probe Ntspa712 and cNtspa712, ingested bacteria of the genus *Nitrospira* are clearly visible in the food vacuoles. Magnification 1000x; Mn = macronucleus, Fv = food vacuoles.

The results have shown that all four ciliate species efficiently fed on the bacterial food offered, which is supported by their positive growth rates, and that they all ingested both *Nitrosomonas* and *Nitrospira*. Ingestion rates ranged from 1 to 285 bacteria $cell^{-1}$ h^{-1}. To detect a selectivity for or against the nitrifying bacteria, the relative clearance rates for food bacteria mix and for the two types of nitrifying bacteria were calculated (Tab. 1.1) and compared according to Chesson (1983). This is equivalent to the relative preference of the ciliate for each food type without considering the ciliate abundance. *P. aurelia*, *E. octocarinatus* and *T. pyriformis* showed no preference for either the nitrifying bacteria or the food bacteria mix. Only

C. glaucoma showed a slight trend towards a preference for the food bacteria mix, but this was not statistically significant (Fig. 1.4).

Table 1.1: Abundance [cell ml^{-1}] and biovolume [μm^3 $cell^{-1}$] of ciliates, ingestion rates (I) [bacteria $cell^{-1}$ h^{-1}] (mean of 3 replicates ± 1 SD) and clearance rates (C) [nl $cell^{-1}$ h^{-1}] of *P. aurelia, E. octocarinatus, T. pyriformis* and *C. glaucoma* on different bacterial food after grazing periods of 90 min and 240 min.

ciliate grazing period	ciliate abund. [cell ml^{-1}]	ciliate biovol. [μm^3 $cell^{-1}$]	food bacteria mix I	food bacteria mix C	*N. moscoviensis* I	*N. moscoviensis* C	*N. europaea* I	*N. europaea* C
Paramecium aurelia								
t_1 – 90 min	3.14E+02	1.40E+05	285 ± 235	345 ± 162	56 ± 23	93 ± 14	120 ± 11	235 ± 548
t_2 – 240 min	3.71E+02	1.46E+05	125 ± 178	212 ± 232	1 ± 2	99 ± 49	9 ± 7	117 ± 390
Euplotes octocarinatus								
t_1 – 90 min	2.62E+02	3.64E+05	148 ± 112	138 ± 228	47 ± 7	71 ± 69	124 ± 54	233 ± 283
t_2 – 240 min	2.67E+02	4.54E+05	280 ± 67	213± 132	91 ± 23	72 ± 72	144 ± 225	362 ± 434
Tetrahymena pyriformis								
t_1 – 90 min	2.98E+03	3.86E+04	87 ± 73	68 ± 21	13 ± 4	15 ± 18	12 ± 74	12 ± 15
t_2 – 240 min	3.07E+03	3.77E+04	17 ± 18	34 ± 9	9 ± 1	17 ± 21	6± 14	16 ± 25
Cyclidium glaucoma								
t_1 – 90 min	3.62E+03	1.43E+03	34 ± 25	17 ± 36	2 ± 8	20 ± 53	4 ± 3	18 ± 10
t_2 – 240 min	3.19E+03	1.46E+03	143 ± 63	16 ± 23	13 ± 19	11 ± 58	8 ± 5	15 ± 23

The first aim of the study, the detection of ingested nitrifying bacteria in the food vacuoles of ciliates, was achieved (Fig. 1.3). While the FISH method qualitatively proved the ingestion of nitrifying bacteria for all four ciliate species, a quantitative analysis, i. e. counting of ingested bacteria, was not possible. The bacteria were too densely packed or already in a state of digestion, thus preventing the counting of individual bacteria inside the food vacuoles. A rough estimation of the number of ingested cells using the ratio between the volume of the bacteria and the volume of food vacuole could have been possible (Eisenmann et al. 1998), but was not the scope of this study. Ingestion- and clearance rates were calculated from the abundance of nitrifying bacteria (see chapter 2.3 for details). Further refinement of the FISH method and the use of a confocal laser scanning microscope might allow the analysis of individual ingested bacteria inside of the food vacuoles in the future.

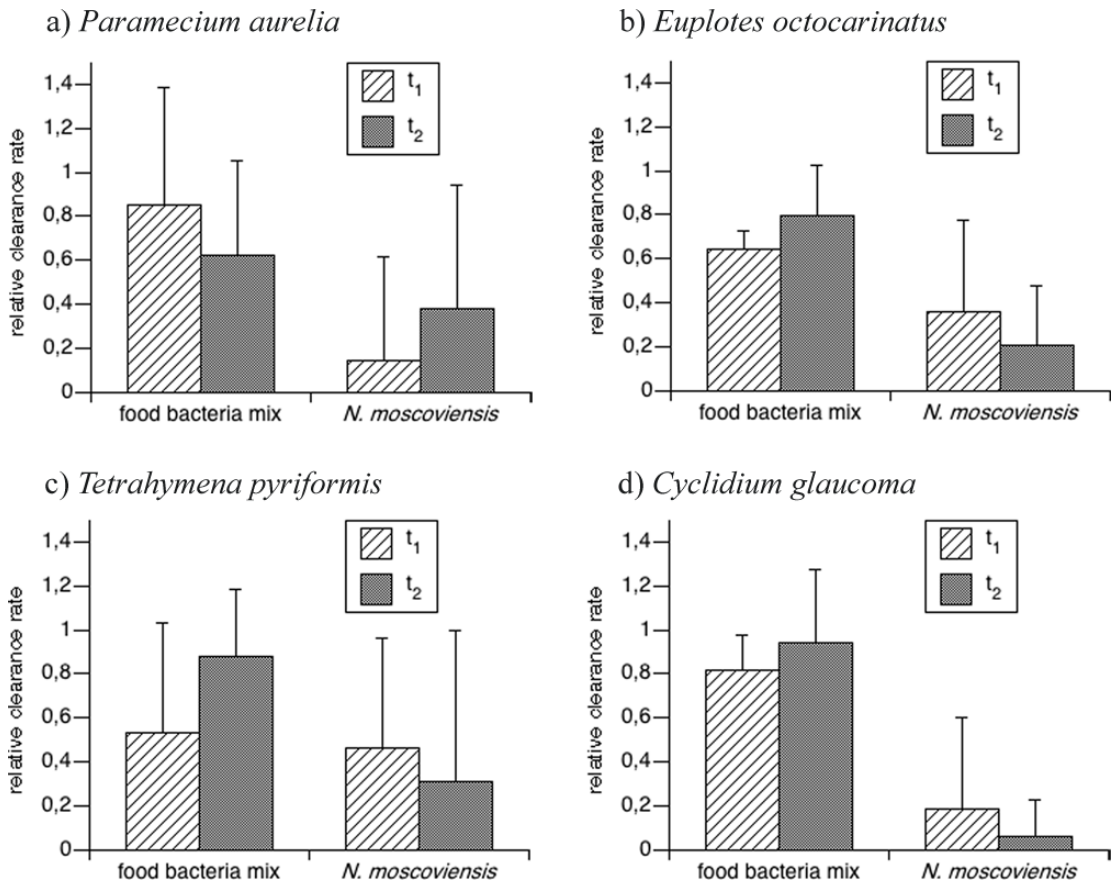

Fig. 1.4: Relative clearance rates of (**a**) *P. aurelia* (**b**) *E. octocarinatus* (**c**) *T. pyriformis* (**d**) *C. glaucoma* on food bacteria mix and *N. moscoviensis*; values are means of 3 replicates ± 1 SD.

The second aim of this study was to establish whether selective grazing pressure on nitrifying bacteria might affect their population dynamics and consequently nitrification rates in natural systems. In all of our experiments, with all ciliate grazers, ingestion rates for both ammonium-oxidizing bacteria and nitrite-oxidizing bacteria were even lower than those for the food bacteria mix. This does not just refer to absolute ingestion rates, but is also valid on a relative scale, taking the ratio of nitrifying bacteria to food mix into account. Statistical analysis showed no significant difference between relative clearance rates for nitrifying bacteria and food bacteria mix. Consequently, grazing had no positive or negative effect on the abundances of nitrifying bacteria. Given that in natural environments, such as stream sediments, where the contribution of nitrifying bacteria to the entire bacterial community is even lower (approx. 0.16%; Altmann et al. 2004a), factors other than

grazing appear to be responsible for their low population sizes, low growth rates and long doubling times (at least 12 hours, even under optimal nitrite and temperature conditions; Ehrich et al. 1995).

The third aim of our study was to find out about food selection of ciliates due to size and morphology of the bacteria. *N. moscoviensis* and *N. europaea* appear in cocci of the size range 0.4 to 0.5 µm diameter, equivalent to a cell volume of approx. 0.524 μm^3 $cell^{-1}$ (Ehrich et al. 1995). This puts them into a size class especially relevant as food for small ciliates, such as *C. glaucoma* and *T. pyriformis* (Fenchel 1986, Šimek et al. 1994, Posch et al. 2001). Both ciliate species are well known as bacterivores. *C. glaucoma*'s bacterial uptake remains almost linear at particle concentrations of up to 10^7 ml^{-1} (Fenchel 1986), and for *T. pyriformis* ingestion rates of up to 100 % of their body volume per hour are recorded (Eisenmann et al. 1998). In our study, *T. pyriformis* ingested bacteria equivalent to 75% of their own volume per hour, while rates for *C. glaucoma* reached values of 97% (on food mix bacteria and *N. moscoviensis* combined). However, despite these high ingestion rates no significant decrease of bacteria of defined size classes was recorded during the grazing periods (t-test, $p > 0.05$). Consequently, abundances and biovolume of the bacteria in our experiments were not noticeably affected. Larger ciliates typically have the capacity to ingest 10 – 30% of their own cell volume per hour (Fenchel 1980). This was confirmed in our experiments. *P. aurelia* took up approximately 397 μm^3 bacteria h^{-1} which corresponds to 44% of its cell volume. However, this grazing activity caused a non significant (t-test, $p > 0.05$) decrease among the bacterial mix and had no effect on total biovolume of the bacterial community. *Euplotes octocarinatus* was feeding constantly on bacteria throughout each treatment but the rates differed between the two incubation periods. The absolute ingestion rates of *E. octocarinatus* and *P. aurelia* on *N. moscoviensis* were similar. In addition to total ingestion rates, clearance rates, i. e. the volume of water a ciliate clears of food particles per unit time, were calculated. The rates from our experiments were equivalent (*E. octocarinatus*) or slightly lower (*P. aurelia* and *T. pyriformis*) than those recorded by Fenchel (1980).

The results of our laboratory experiments imply that nitrifying bacteria do not underlie disproportionately high or low grazing pressure by a diverse ciliate community (*P. aurelia, E. octocarinatus, T. pyriformis* and *C. glaucoma*), and that

the ciliates did not influence the species composition or the composition of morphotypes within the bacterial community. Ingested bacteria were detected within the ciliates' food vacuoles, but the rates measured were too low to infer a significant reduction of these bacteria through grazing. Further, no quantitative interpretation of food vacuole contents was possible. Nitrite- and ammonium transforming bacteria were detected within the food vacuoles of the ciliates using a combination of DAPI and FISH marking techniques. However, we still see a need for further improvement of the approaches and the methods used in our study. The FISH technology has to be improved for quantitative registration of consumed bacteria by protistan grazers from natural systems. In addition, experiments using ammonium-oxidizing and nitrite-oxidizing bacteria as potential food are still missing, allowing us to draw conclusions on possible effects on the whole nitrogen cycle in natural systems. It would further be desirable to apply our approach to natural systems.

1.4 Impact of ciliates on nitrification in fluvial sediments

While the first study aimed at a likely qualitative mechanism of the interaction between ciliates and nitrifying bacteria, in the second and third study we investigated if ciliates have a quantitative effect on nitrification in a near-natural system. Similar studies were conducted in fluvial sediments from the River Salzach (Austria) and in marine sediments from the Baltic Sea (Bay of Kiel, Germany). The latter is described in chapters 1.5 and 2, while this chapter focusses on the experiments using the fluvial sediment. One experiment (V5) is portrayed in detail in chapter 3, but a total of five similar experiments (V1-V5) were conducted. If not stated otherwise, the data presented in this chapter refer to V5.

Rivers contain only 0.0001% of the total water on earth (Wetzel 2001). In spite of these low quantities, running waters are of enormous geological significance, as they move large amounts of dissolved and particulate matter from the land to the sea, and this matter is subject to biogeochemical processes during the transport. Compared to lakes, they are longliving and highly dynamic systems (Grishanin 1974). Furthermore, rivers have a tremendous importance for human civilization, as they are

used as drinking water resources, as waterways for the transport of goods, for the disposal of waste water, for the production of energy, for fishery and as recreation areas.

In streams and rivers the surface-bound bacterial activity in the hyporheic interstitial (Orghidan 1959) greatly exceeds the activity of free-living bacteria. Buesing & Marxen (2005) identified riverine sediments as places of high bacterial production, similar to marine systems. From an ecosystem perspective it seems that the water column is the medium that transports carbon and nutrients to the foci of heterotrophic metabolism. These foci are located in the sediments and the epiphyton and serve as important sinks of organic matter (Fischer 2003). It was shown, for example, that in the Ogeechee River (USA) benthic bacteria accounted for >90% of the system metabolism (Edwards et al. 1990). The sediments of the hyporheic interstitial characterize the ecotone between the river, the riparian zone and the groundwater (Dahm et al. 1998, Pusch et al. 1998, Fischer et al. 2003). The effectiveness of sorption and transport processes into sediments, and thus the importance of the contribution of sediments to the total balance of matter in a river and the rivers self-purification ability also greatly depends on the flow characteristics such as the hydraulic exchange between water and the hyporheic zone (diffusive vs. advective transport) or the boundary layer (laminar vs. turbulent flow) (Hunt & Parry 1998, Battin 2000, Huettel & Webster 2001, Battin et al. 2003, Fischer et al. 2003, Huettel et al. 2003, Fischer et al. 2005).

The nitrogen cycle in river sediments in general (e. g. Kemp & Dodds 2002, Cavanaugh et al. 2006) and nitrification in fluvial sediments in particular (e. g. Strauss & Lamberti 2000, Strauss 2002, Strauss et al. 2002, Strauss et al. 2004) has been subject to research before, but these studies did not take the interaction between nitrification and ciliates into account, which was the focus of this study. Our aim was to find out whether a change in ciliate abundance would affect the activity and composition of nitrifying bacteria in fluvial sediments.

To avoid artefacts due to uncontrollable environmental factors, all experiments were conducted in laboratory flumes (Fig. 1.5) The flow velocity was adjusted to a very low value to prevent sediment transport within or out of the flume and to limit transport processes within the sediment to diffusion. This was done because investigating the effect of flow velocity and advective transport on the interaction of

ciliates and nitrifying bacteria was beyond the scope of this study, but it should be considered in future projects.

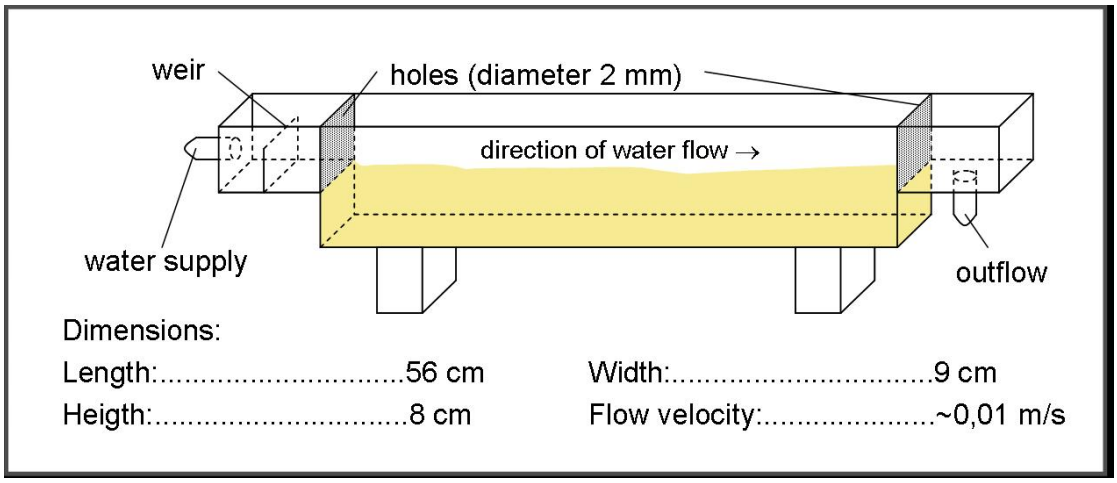

Fig. 1.5: Flume as used for the experiments with the sediments from the river Salzach.

Natural sediments were taken from the River Salzach near Salzburg (Austria) and were treated in three different ways for the experiments: for the first treatment, sediments were autoclaved to remove all natural organisms and release nutrients. The second treatment were natural sediments without any manipulation. For the third treatment, sediments were frozen (-20°C) and unfrozen three times over ten days and then also placed in the flumes. Freezing kills macrofauna organisms, but protists and bacteria survive this procedure. Furtheron, the treatments are refered to as „autoclaved“, „natural“ and „frozen“. Two flumes were established per treatment. To one flume of each treatment a ciliate cocktail comprising four ciliate species (*Euplotes octocarinatus*, *Tetrahymena pyriformis*, *Paramecium aurelia* and *Cyclidium glaucoma*; Tab. 3.2) was added, while the other flume recieved 1.2 µm-filtered ciliate free culture medium as control. This assemblage of ciliates represented the natural size spectrum and different feeding mechanisms (Šimek et al. 1994, Eisenmann et al. 1998, Wilks & Sleigh 1998, Hausmann et al. 2003) of bacterivorous interstitial ciliates. We analyzed total and nitrifying bacteria with the DAPI-method and FISH, ciliate abundance and taxonomic composition using the QPS-method, *in situ* concentrations of nitrate and ammonium with microsensors and nitrification potentials with a slurry assay. Some abiotic parameters were determined

additionally. For more details on experimental setup, incubation periods, sampling, methods, etc. see chapter 3.3.

The results of the oxygen microsensor measurements show that the oxic zone in the sediments was limited to the upper 4-5 mm (Fig. 3.2). The measurements of ammonium and nitrate microprofiles revealed that the addition of ciliates leads to higher *in situ* concentrations of nitrate. This effect was most significant in V3 (Fig. 1.6) were the highest number of ciliates was added (~2000 cells g^{-1}), but was also found in the frozen sediment treatment in V5 (Fig. 3.3), for example.

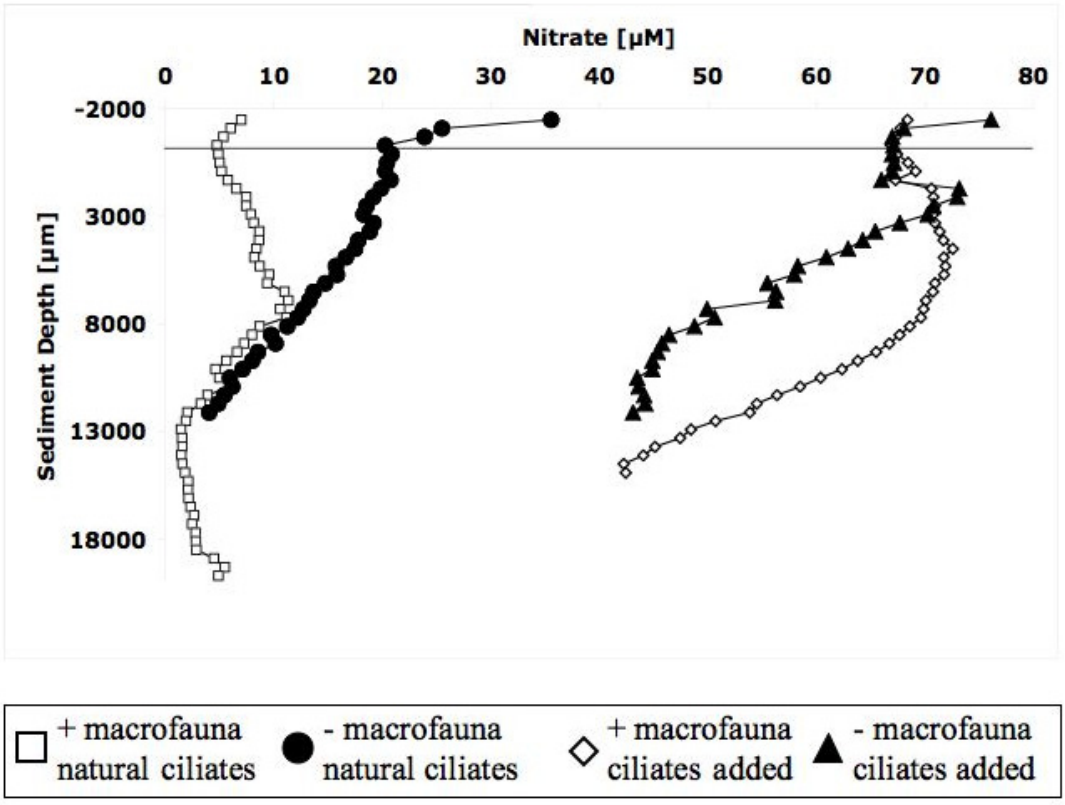

Fig. 1.6: Nitrate profiles from sediments with and without macrofauna organisms from V3. Macrofauna was removed by freezing. The treatment with ciliates added showed higher *in situ* nitrate concentrations. Standard deviations are not shown for clearance of the figure, differences between mean concentrations were statistically significant (t-test, $p < 0.05$).

Nitrification potentials were only measured in V4 and V5, but showed that the nitrification potential of the experimental sediments was too low to be detected (Tab. 3.5). Also, the contribution of nitrifying bacteria was very low, ranging from 0.012 to

0.030 % of total bacterial abundance, compared to other freshwater sediments (e. g. Altmann et al. 2003). The abundance of the ammonium-oxidizing *Nitrosomonas* was significantly higher in the flumes with ciliates added in all three treatments (Fig. 1.7). Total bacterial and flagellate abundances did not vary between the +Cil and –Cil flumes (Fig. 1.7 and 3.6). Ciliate abundances were very low in the sediments, and thus it was not possible to concentrate enough ciliate cells for analysis of abundance and taxonomic composition. However, the microscopic analysis of the QPS slides indicated that the abundances in the +Cil flumes was distinctly higher than in the –Cil flumes and based on the numbers from the Baltic Sea study (chapters 1.5 and 4) it can be assumed that ~50 % of the ciliates that were initially added remained in the sediments after the incubation period.

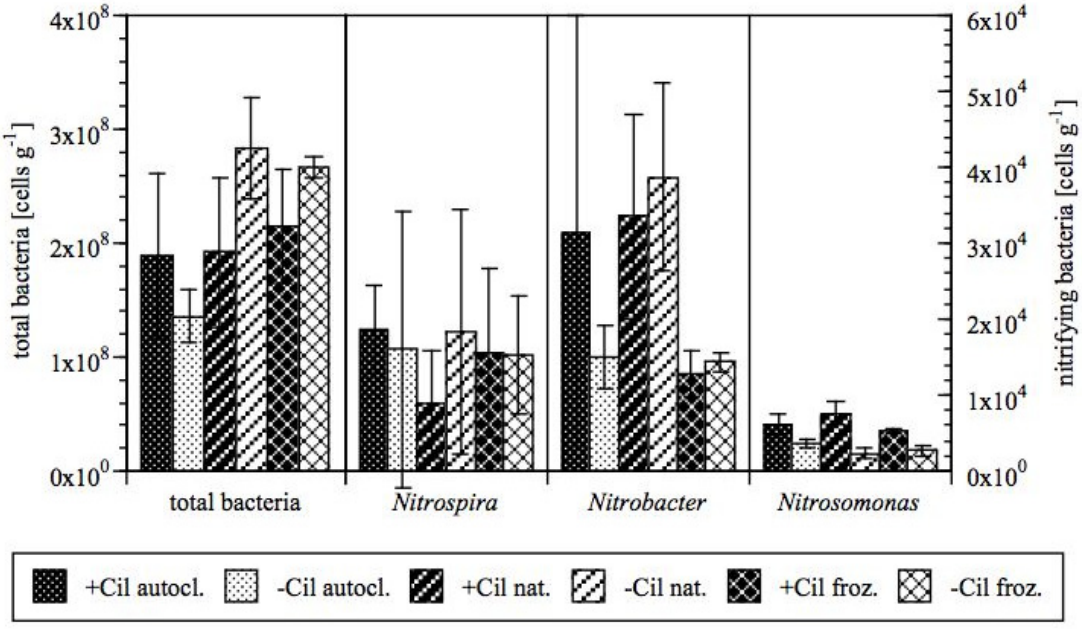

Fig. 1.7: Total bacterial abundances and abundances of the investigated nitrifying bacteria after the incubation period. Similar patterns indicate the autoclaved, natural and frozen sediments, the dark pattern refers to the +Cil treatment, the lighter pattern to the –Cil treatment. Error bars show ± 1 standard deviation.

Surprisingly, our treatments of the sediments – autoclaved, natural or frozen – did not affect the biotic or abiotic parameters measured in our study as strongly as we expected. Nitrate microprofiles for the autoclaved sediments were significantly

different from all other flumes. The very low concentrations together with abundances of bacteria and flagellates, that were similar to the natural flumes, indicate that all nutrients released by autoclaving were retransformed into biomass during the incubation period. Total bacterial abundances were all in the same range, no statistical difference could be detected, neither between the +Cil and –Cil flumes of one treatment nor between the treatments. Ammonium-oxidizing bacteria showed a difference between the +Cil and –Cil flumes for all treatments, but no difference between the treatments. No differences were found for the nitrite-oxidizing bacteria. There was no special inoculation after autoclaving the sediments, but the flumes were filled with natural sediment before and were not sterilized. Nevertheless the bacterial population grew back to an abundance similar to the untreated, natural sediment within the incubation period. For the nitrifying bacteria, there was no change in the community structure. Flagellate abundances were highest in the frozen sediment, but as well this difference was not significant. As indicated by the large proportion of biovolume, flagellate community was dominated by large phototrophs, heterotrophic flagellates were rather small. Other studies have shown that – in contrast to planktonic systems – in sediments ciliates are more important as bacterial grazers than heterotrophic flagellates and a top-down control of benthic bacteria by heterotrophic flagellates seems unlikely (Dietrich & Arndt 2000 and literature cited therein).

Despite the low abundances of ciliates, the low contribution of nitrifying bacteria and the very low nitrification potentials, we found two indications for enhanced nitrification in the presence of higher ciliate abundances. These were (1) the higher abundances of *Nitrosomonas* in all treatments and (2) the higher nitrate concentrations in the frozen sediment. This is consistent with the findings of Prast et al. (subm.) from marine sediments. As selective feeding can be ruled out as possible cause for this effect (Neubacher et al. 2007; chapters 1.3 and 2), the most likely mechanisms are the increase of substrate concentration due to ammonium excretion by ciliates (Ferrier-Pagès & Rassoulzadegan 1994) and the improved distribution of substrates and oxygen by ciliate activity (Fenchel 1986, Glud & Fenchel 1999), which will be explained in detail in the following chapter 1.5 (also see chapters 3 and 4).

Some factors that were not variable in the experiments are likely to have an impact on nitrification in fluvial sediments and its interaction with ciliates, and they need to be addressed in future studies. Among these are abiotic factors such as flow velocity, sediment grain size distribution and organic content. Biotic factors include bioturbation and other components of the benthic food web. Especially the role of flagellates requires further investigations.

1.5 Impact of ciliates on nitrification in marine sediments

This study (chapter 4) was similar to the studies conducted with the sediments from the River Salzach (chapter 1.4, 3), but a marine system instead of the fluvial sediments was investigated. Again, the aim of the study was to find out whether ciliates can affect nitrification and nitrifying bacteria in these sediments. While the abiotic characteristics such as grain size distribution and organic content of the sediment, that was taken from the Baltic Sea in the Bay of Kiel, were quite similar to the Salzach sediments, the differences between the fluvial and the marine sediment concerned not only salinity, but also natural abundance of bacteria and other components of the microbial food web. Furthermore, marine sediments are not subject to continuous strong flow and thus to constant transport and relocation, as sediments in rivers and streams are. For a comparison between the fluvial and the marine sediments and the results from both studies see chapter 1.8.

The experimental setup was similar to the previous study, but only two flumes were used, with the only treatment being the addition of ciliates from a culture to one flume and the addition of ciliate free 1.2 µm-filtered culture to the other flume as control. Bacteria, nitrifying bacteria, flagellates and ciliates were counted, nutrient concentrations in the water circuit were measured and nitrification potentials of the sediments were determined. For a detailed description of sampling, experimental setup and methods see chapter 4.3.

The ciliate abundance in the treatment with ciliates added (+Cil) was 1478 (±578) cells g^{-1}, which is significantly higher than in the treatment without the addition of ciliates (-Cil; 189 ± 120 cells g^{-1}). The increase in the abundance represents 52 % of

the ciliates that were added initially. The taxonomic composition in both treatments was similar (χ^2-test, p = 0.006; Fig. 4.3, Tab. 4.3).

The results showed that whilst the abundances of total bacteria and flagellates were similar in both treatments, the abundance of nitrifying bacteria was higher for all three investigated types of nitrifying bacteria *Nitrosomonas*, *Nitrospira* and *Nitrobacter*. 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) (Fig. 1.8). Together they contributed 1.45 and 0.35 % of total bacteria in the treatments with and without ciliates, respectively. Nitrification potentials also were higher in the +Cil treatments, although not statistically significant, too (Fig. 1.9).

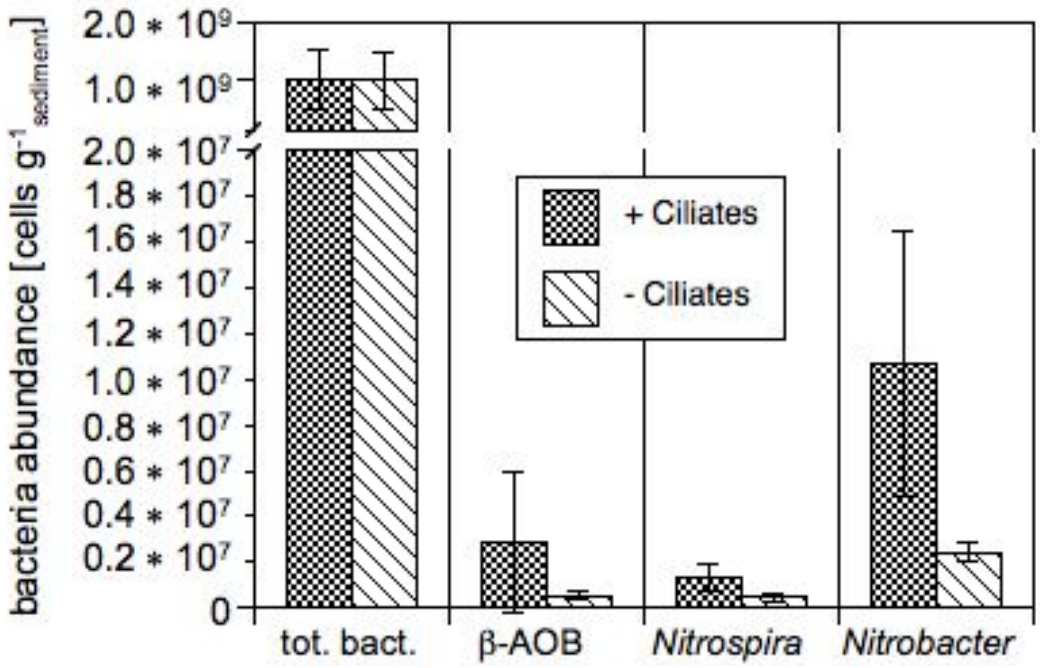

Figure 1.8: Bacterial abundances in the experimental sediments after the incubation period (error bars: ±1 SD).

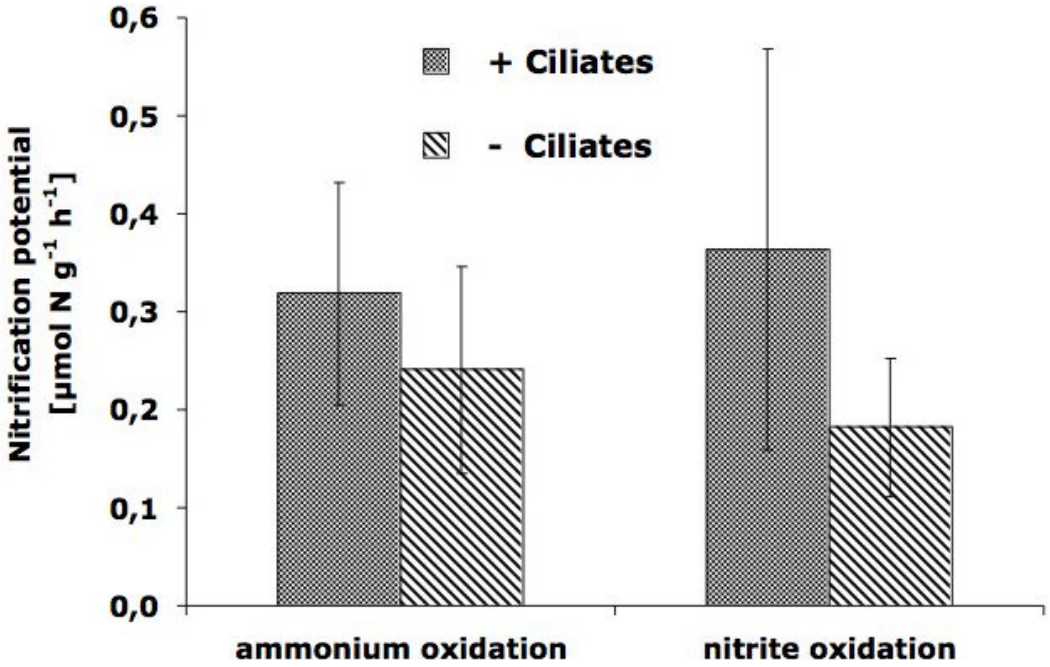

Figure 1.9: Nitrification potentials of the sediments in the experimental flumes after the incubation period, as determined with the slurry assays (error bars: ±1 SD).

The experiments demonstrated that an increase in ciliate abundance could enhance the abundance of nitrifying bacteria as well as the nitrification potentials 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 and Dodds (1997) for submerged soil sediments.

The results of the study on grazing selectivity (chapters 1.3, 2) showed that this can be ruled out as possible cause for the changes in abundance of nitrifying bacteria and nitrification potential. We conclude that the presence of ciliates, especially in 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 – 2 µg N h^{-1} mg^{-1} dryweight (Ferrier-Pagès & Rassoulzadegan 1994), which further contributes to an improved substrate availability for nitrifiers in the presence of ciliates. Flagellate and total bacterial abundances after the incubation period did not differ between the treatments with or without ciliates, indicating that grazing pressure from ciliates was

rather low or that grazing losses of bacteria and flagellates were compensated by increased growth, accompanied by a change in community composition. As indicated by the large portion of biovolume, flagellate community was dominated by large phototrophic flagellates, while 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 a 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.

Our results further indicate that nitrifying bacteria profit disproportionately from higher substrate availability due to ciliate grazing, because ammonium as substrate for nitrification is the main excretion product of ciliates (Ferrier-Pagès & Rassoulzadegan 1994). Another explanation for the increase in nitrification potentials and abundances 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 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 otherwise transport is mostly limited to diffusion (Huettel & Webster 2001).

Despite the significant difference in ciliate abundances 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 abundances of nitrifying bacteria in our samples, the presence of some, though low numbers of ciliates in the control likely caused the lack of statistical significance in the contrasts of some of the effects of the experimental manipulation. Furtheron, it cannot be completely ruled out that the use of other ciliates 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. 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 alone did not have a significant impact on the nutrient level and can be ruled out as a source of increased nutrient supply in the +Cil treatments. During the course of our experiments a new ammonia-oxidizing crenarchaeota was described by Könnecke et al. (2005). Future experiments should consider also the presence and abundance of this new type of ammonia-oxidizing microorganism, which might show a different reaction to ciliate grazing.

In short, the reason for the increased abundances of nitrifyers and nitrification potentials in the presence of ciliates probably is nitrogen recycling, that leads to an enhanced nitrogen availability for nitrifying bacteria. This indicates that nitrifying bacteria are rather subject to bottom-up control by substrate availability than top-down controlled by ciliate grazing. Ciliate grazing did not negatively affect the nitrifying bacteria, although Lavrentyev et al. (1997) and Neubacher et al. (subm.) showed that they are ingested by ciliates. Another possible explanation for the increased abundances of nitrifying bacteria and nitrification potentials in the +Cil treatments might be an increased transport of substrates and oxygen in the sediments due to movement and filtration activity 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.

1.6 Impact of bioturbation on nitrification

This study (chapter 5) focussed on the effect of a benthic macroinvertebrate, the marine polychaete *Nereis diversicolor* (Fig. 1.10), on nitrification in Baltic Sea sediments. Possible effects might be due to grazing on bacteria or bioturbation.

Fig. 1.10: *Nereis diversicolor* in its sediment burrow (length of worm: approx. 10 cm; picture: Adrian Bischoff).

Bioturbation is the relocation of sediment by the activity of animals, such as digging, ingestion, defaecation or building of burrows (e. g. Schaefer & Tischler 1983, Reible et al. 1996). This activity leads to a vertical and horizontal rearrangement of sediment particles and pore water, and associated with that to a mixis of sediment surface and deeper layers. Bioturbation has multiple effects on the sediment structure. The stability of the sediment decreases, the water content, porosity, and transport of solutes within the sediments increases (Davis 1993, Mulsow et al. 1998, Rowden et al. 1998). Especially the increased supply of oxygen (Frenzel 1990, Fenchel 1996) has a significant impact on the colonization of the sediments. For example, Daumas (1990) and Gilbert et al. (1995) report higher bacterial abundances and higher bacterial production and activity from bioturbated sediments. Thus,

bioturbation also affects biogeochemical processes (Mermillod-Blondin et al. 2003, Mermillod-Blondin et al. 2004). Some studies have already investigated the impact of bioturbation on the nitrogen cycle (e. g. Yamada & Kayama 1987, Svensson et al. 2001, Stief & DeBeer 2002, Altmann et al. 2004b, Marshall et al. 2004).

In addition to their function as bioturbators (Duport et al. 2006), marine polychaetes such as *N. diversicolor* are deposit feeders and described as effective bacterial grazers in sediments (Grossmann & Reichart 1991, Sherr & Sherr 2002, Lucas et al. 2003). Plante et al. (1989) concluded that grazing pressure of polychaetes had an effect on bacterial activity and alters the bacterial community structure in the sediment. Hence polychaete grazing might also affect metabolic pathways such as nitrification, which are catalyzed by bacteria.

The aim of the study was to detect whether *Nereis* has an impact on nitrification due to grazing and/or bioturbation. *Nereis* was chosen as a model polychaete and bioturbator because it is widely common in shallow sediments and euryoecius to salinity, temperature, oxygen availability and sediment grain size. We designed a laboratory study with four experimental flumes, two with a fine sediment and two with a coarse sediment. To one flume of each sediment type *N. diversicolor* was added (approx. 1000 ind. m^{-2}), the second flume of each sediment type was used as a control without worms (for details on experimental setup, incubation periods, sampling procedure, methods etc. see chapter 5.3). We used the DAPI-method (Porter & Feig 1980) to detect the number of total bacteria, and FISH to detect nitrifying bacteria. Nitrification potentials of the sediments were determined using a slurry assay (Dollhopf et al. 2005). Various abiotic parameters were recorded. In addition, we tried to obtain *in-situ* concentrations of NO_3^-, NO_2^- and NH_4^+ in the sediments using LIX-microsensors (De Beer et al. 1997), but due to salinity this was not possible.

The results showed some significant differences between the treatments. For the fine sediments, total bacterial abundance was almost doubled in the treatment with worms. For the coarse sediments, no significant difference was found, although that is most probably due to a sampling artefact and a resulting statistical outlier. Comparing the two types of sediment, total bacterial abundances were slightly higher in the coarse sediment (Fig. 5.1). For the nitrifying bacteria, results showed different trends within the two types of sediment. While the abundance of nitrifying bacteria

was higher in the treatment with *Nereis* in the fine sediment, it was higher in the treatment without worms in the coarse sediment. The contribution to total baceria abundance was also different between the two types of sediment: 4.65 and 3.80 % for the fine sediment, and 1.85 and 2.08 % for the coarse sediment, respectively. The composition of the three investigated taxa of nitrifying bacteria also differed between the treatments. The difference between with and without *Nereis* in the fine sediments were significant for *Nitrospira* and *Nitrobacter*. The total number and the relative contribution was higher in the presence of *Nereis*, while in the absence of *Nereis* the abundance and contribution of β-AOB was significantly higher (Fig. 1.11, Tab. 5.4). In the coarse sediment, no difference between the treatments with and without worms could be detected. Total abundances were higher in the fine sediments.

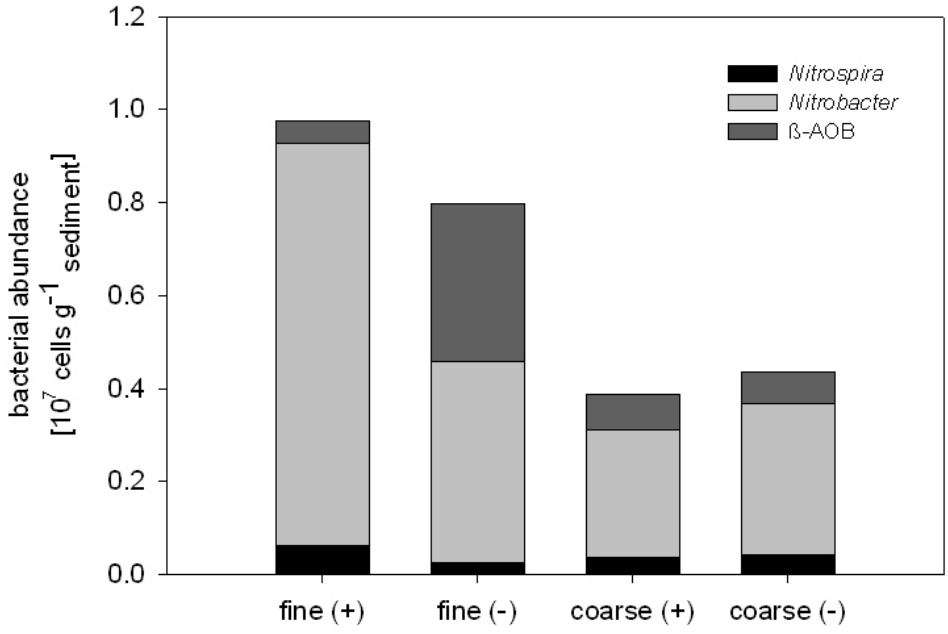

Fig. 1.11: Nitrifying bacteria as detected by FISH. (+) and (-) indicate the presence/absence of *Nereis diversicolor*.

In the fine sediment the results from the slurry assays show on average higher nitrification potentials in the treatments with worms (Fig. 5.3), although these differences are not statistically significant. The potential nitrite oxidation rates exceeded the ammonium oxidation potentials. In the coarse sediment, there were no

differences between the treatments with and without *Nereis*, but ammonium oxidation potentials exceeded nitrite oxidation potentials.

The higher abundances of bacteria can be explained by a higher surface area and the active water movement created by the worms (Fenchel 1996, Riisgard et al. 1996), which leads to an improved supply of oxygen and nutrients. This is especially important for the fine sediments, where otherwise due to the density this supply is mostly limited to diffusion, thus the effect is stronger here. Furthermore, Kristensen (1984) described nutrient recycling by bacteria, using ammonium excreeted by *Nereis virens* as a substrate. The predation pressure by *Nereis* might further keep the bacteria in an exponential growth phase and consequently enhance bacterial abundance.

Nitrifying bacteria were dominated by *Nitrobacter*, accounting for 50-89 % of the nitrifyers. Surprisingly, we found significantly higher abundances of β-AOB in the absence of worms in the fine sediments. This was unexpected considering the excretion of ammonium by the worms, and the resulting increased supply with substrate for these bacteria. Selective digestion is unlikely to be a reason, as both AOB and NOB are gram-negative bacteria, which are digested by *N. diversicolor* (Lucas & Bertru 1997). Therefore, grazing by the worms should not alter the proportions of the nitrifying community. The location of β-AOB might be a possible explanation , in a way that the β-AOB are more accessible for the worms.

Despite the higher numbers of nitrifying bacteria in the fine sediment with worms, the increase in nitrification potentials is not significantly. Yingst & Rhoads (1980) suggest that feeding by bioturbating invertebrates on the microbial community in their close vicinity keeps these bacteria in an active physiological state, which might be an explanation for the al least slightly elevated nitrification potentials in the presence of *N. diversicolor*. Surprisingly, in the coarse sediments the ammonium oxidation potential exceeded the nitrite oxidation potential. This is in contrast to natural sediments were higher nitrite oxidation potentials are commonly found (Schwoerbel 1999). Interactions of denitrification with other processes of the nitrogen cycle such as anammox could be an explanation. According to Dalsgaard et al. (2003) the requirements for anammox, the anaerobic bacterial oxidation of ammonia with nitrite, are nitrate rich waters and anoxic conditions. These conditions might occur within the flumes and may have reduced nutrients available for nitrite

oxidizing bacteria. The reduced nutrient availability could have led to nitrite oxidising bacteria at a low active physiological state. Alteration of this physiological state can only be achieved by increased nutrient concentrations over a certain time period and the time available for the slurry assays was too short to complete this change.

N. diversicolor did affect the total bacterial abundance as well as the abundance and taxonomic composition of nitrifying bacteria and hence the nitrification potentials. These effects did depend on the type of sediment. We suggest that the worm effect is stronger in the fine sediment, as the bioturbation effect is stronger here, were transport processes are more limited than in a coarse sediment with large interstitial spaces. This study focussed on the interaction between bacteria, especially nitrifying bacteria, and polychaetes. Further research should be conducted to reveal the factors controlling this interaction, and attention should be paid to other components of the benthic food web and how they interact with bioturbation and grazing by polychaetes and bacteria-catalyzed biogeochemical pathways.

1.7 Settling of fixed plankton ciliate samples

Beside the studies on nitrification and as a result of working in a group with a wider ecological scope, a fifth methodological study was conducted as part of this PhD thesis. It dealt with the sinking velocity of fixed ciliates from plankton samples. Settling is a widely used standard method for concentration of ciliates from plankton samples, where natural abundances are too low to achieve reliable results from counting unconcentrated samples. The sinking velocities of unfixed cells or particles such as phytoplankton organisms or marine snow have been addressed several times (e.g. Hamm 2002, Ptacnik et al. 2003, Peterson et al. 2005). For fixed samples the Utermöhl-method (Utermöhl 1958) is long known and used, but data regarding the duration of settling of the fixed samples rely mostly on experience or rough estimation. Only for phytoplankton at least some studies (Padisak et al. 2003 and literature cited therein) were conducted to achieve reliable sinking velocities, but for ciliates no data on settling times or sinking velocities were available so far. The

settling time is essential for the quality of the results, because if the time allowed is too short, the total abundance will be underestimated and the taxonomic composition will be skewed, as slowly sinking ciliates might be excluded. Unnecessarily long settling times lead to longer exposure to the fixative, which will consequently mean a loss of ciliate cells, and thus, an underestimation of the true abundance, too (Gifford & Caron 2000).

For the present study, settling times were measured empirically, and in addition a protocol was developed to determine the density (in terms of g ml^{-1}) of fixed ciliate cells to allow the theoretical calculation of the settling velocity using Stokes Law. Seven ciliate cultures were used, five freshwater species and two marine assemblages from the Baltic Sea and the Red Sea. These represented a broad spectrum of cell size (19-140 µm) as well as various cell shapes (Tab. 1.2). Sinking velocities were determined empirically using a sedimentation chamber on an inverted microscope and a semi-automatic imaging system. The ciliate cultures were fixed with glutardialdehyde (2 % f.c.). Then the chamber was filled with the ciliate culture and immediately placed on the inverted microscope. Every 15-30 seconds a micrograph, showing the ciliates that have sunk to the bottom of the counting chamber, was taken. Ciliates on the bottom of the chamber were counted on each picture until the number did not increase any more (see Fig. 6.3). The sinking velocity was calculated from the height of the chamber and the time until constancy.

For the calculation of the sinking velocities with Stokes Law, first the density (g ml^{-1}) of fixed ciliate cells had to be determined. For this we developed a protocol using a density gradient centrifugation. The ciliates were fed with indian ink to increase the visibility of the cells, thereafter the cells were fixed with glutardialdehyde (2 % f.c.). Other fixatives were considered, but due to their incompatibility with the Percoll used for the density gradient centrifugation method and because of the shrinkage effects (Leackey et al. 1994, Stoecker et al. 1994) which might affect the density of the cells, glutardialdehyde was chosen. Ciliates were concentrated via centrifugation and then applied to a density gradient. In a separate tube, density marker beads were also applied to the density gradient. After centrifugation the distances of the density marker beads from the Percoll surface were used to create a calibration curve, and ciliate density could be calculated from the distance from the Percoll surface.

Consequently, sinking velocities were calculated using a modification of Stokes Law (Vogel 1983), which uses the acceleration of gravity, radius of the sinking particle, density of the particle and the medium, viscosity and form resistance. To calculate the form resistance, we had to develop a formula which does not include the velocity of the sinking particle, as otherwise we would have had to use our empirically determined sinking velocities (for a detailed description of experimental setup, methods and the elaborate mathematical calculations see chapter 6.3). Sinking velocities were calculated for the freshwater species only, as the cell size in the marine assemblages was too diverse. Experimentally determined and calculated sinking velocities are presented in Tab. 1.2 and Fig. 1.12.

The settling rates of the marine ciliates were significantly lower than sinking of the freshwater species, even if the unusually low values of the Baltic Sea culture were not considered. This is caused by the smaller difference in density between sinking particle and medium, as the density of seawater is higher than the density of freshwater, while the density of the marine ciliates was in the range of the freshwater species. Though there was a significant correlation between ciliate density and empirical velocity, there were no correlations with cell size or form resistance. Cell shape was less important for the sinking velocities as the two species with the highest sinking velocities (*E. octocarinatus* 7.7 mm min^{-1}, *C. glaucoma* 9.4 mm min^{-1}) differed strongly in cell size and particularly in cell shape (Tab. 1.2). *Euplotes* is dorsoventrally flattened with a band of cilia, the membranell and several clusters of tightly arranged cilia, the cirri. This was expected to increase form resistance, while *Cyclidium* resembles a spheroid, which should have the lowest form resistance. The prominent membranelles and cirri at the edge of the cell might cause the high variance within the replicates of *E. octocarinatus*. These membranelles may strongly affect the orientation of the cell and therefore increase the settling time compared to spheroid shaped cells. This became clear comparing minimal and maximal settling times of *Cyclidium* and *Euplotes*, 8.3-10.7 and 5-75 mm min^{-1}, respectively. Additionally, the calculated settling time of *Euplotes* was even lower than the lowest empirical velocity. This indicates that there were factors affecting the settling velocity, which cannot be explained by the mathematical formulas. Not body shape but the appearance of appendages affected the settling velocity, which was not considered by Stokes law.

Table 1.2: Characteristics of the ciliate cultures, used for the studies. Calculated sinking velocity is based on Stokes equation (1) and is compared to the experimentally determined velocity. The salinities for the marine cultures are given, while FW indicates freshwater cultures.

Ciliates	Size (µm)	Cell shape	Salinity (‰)	calculated sinking velocity (mm min^{-1})	experimental sinking velocity (mm min^{-1})	Ciliate density (g ml^{-1})
Cyclidium glaucoma	23	spherical	FW	2.1	9.4	1.04
Tetrahymena pyriformis	60	ovoid	FW	2.4	2.4	1.02
Paramecium aurelia	140	ovoid-flattened	FW	5.3	4.5	1.08
Euplotes octocarinatus	100	flattened	FW	4.4	10.3	1.05
Colpidium colpoda	110	ovoid with nose	FW	2.8	7.7	1.02
Baltic Sea Large	38	mostly ovoid	16	n.d.	0.6	1.08
Baltic Sea Small	19	mostly ovoid	16	n.d.	0.5	1.08
Red Sea Large	20	ovoid and flattend	40	n.d.	1.7	1.05
Red Sea Small	52	ovoid and flattend	40	n.d.	2.7	1.05

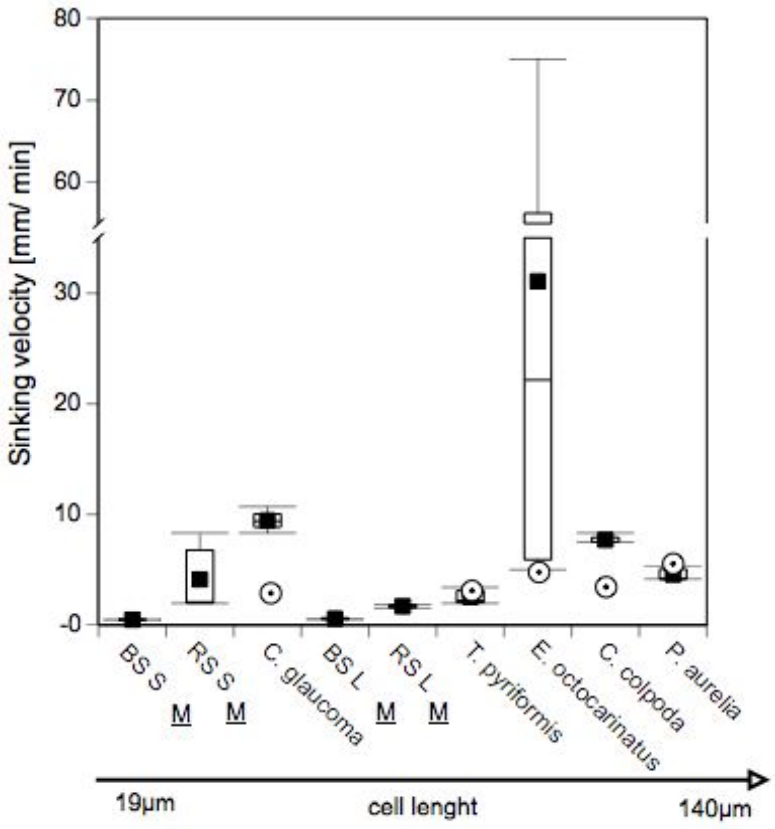

Fig. 1.12: Empirical (box-plots) and calculated (circles) sinking velocities arranged with increasing ciliate size from left to right. The calculated settling rates are given for the freshwater ciliates, but not for the marine cultures due to the diversity in the size structure. BS: Baltic Sea, RS: Red Sea, S: small, L: large, M: marine. The top, bottom, and line through the middle of the box correspond to the 75th percentile, 25th percentile, and 50th percentile (median), respectively. The whiskers on the bottom extend the 10th percentile (bottom decile) and top 90th percentile (top decile).

Therefore the empirical sinking velocities proved to be more reliable as the calculated ones, and we recommend to use the lowest empirical sinking velocities, 1.7 mm min^{-1} for marine and 2.4 mm min^{-1} for freshwater, when calculating the time necessary to settle a sample. These sinking velocities are much higher than expected, and this means that up to 95 % of time can be saved compared to the old, estimation-derived times that were used so far. As an example: concentration of ciliates in a 1 l graduated cylinder of 37 cm height will take 3.6 h for marine and 2.6 h for freshwater samples. Until now, settling times up to one week were often used for 1 l

cylinders. The methods for determining ciliate density and settling times both were established and can easily be repeated for other species. As there were significant differences in ciliate density between groups, this parameter should be measured for each species individually.

1.8 General conclusions

The main hypothesis of this PhD thesis was that ciliates have an impact on nitrification in aquatic sediments. The conclusions from the first three studies support this hypothesis. As a consistent and distinct trend in both marine and freshwater systems, we found higher abundances of nitrifying bacteria, higher nitrification potentials and higher nitrate concentrations. This was not always as significant as desirable, but this lack of statistical significance is likely due to the impossibility to design a control treatment completely without ciliates. Especially in the freshwater system, the low abundance and activity of nitrifying bacteria enhanced this problem. These studies need to be continued, as additional factors affecting nitrification directly or indirectly, have do be addressed (see chapter 1.9). Selective grazing as an important potential mechanism for the effect of ciliates on nitrification by selectively preying upon or against nitrifying bacteria could be ruled out. Furthermore, a comparison of the marine and the freshwater sediments reveals some insight. While the abiotic parameters (grain size, organic carbon content) of the marine and freshwater sediments used in our studies were quite similar, there were differences in the abundance of total bacteria and in the proportion and activity of nitrifying bacteria. Apart from the difference in salinity these contrasts are probably a consequence of the flowing water in rivers, where sediment is constantly relocated and gradients are less stable. The lower abundances of nitrifying bacteria in the fluvial sediments in our study are in contrast to the findings of Fischer & Pusch (2001) and Buesing & Marxen (2005), who identified riverine sediments as places of high bacterial production, similar to marine systems, and to Altmann et al. (2003), who found higher abundances of nitrifying bacteria in fluvial sediments. This contrast might be explained by the high spatial and temporal variability of

parameters such as discharge, flow velocity, bed load, substrate type, macrozoobenthos community and other biotic and abiotic factors in river systems, which our study could hardly depict.

In general, the benthic food webs in rivers have recieved less attention compared to marine sediments and data on benthic flagellates and ciliates are rare and contradictionary (Gücker & Fischer 2003 and literature cited herein). They go as far as the conclusions of Gücker & Fischer (2003), who question whether protists are able to control the benthic bacterial population by grazing at all. However, our results show that nitrifying bacteria were present and they indicate that nitrifyers were affected by ciliate abundance in our experiments.

Apart from the effects within the microbial food web, the fourth study from this dissertation showed that larger sediment infauna can also have an impact on nitrification and nitrifying bacteria. This effect is likely caused not only by the physical consequences of bioturbation, but also by grazing and defaecation of *Nereis diversicolor*. This goes along with the findings of Altmann et al. (2004b), although the bioturbator used in their study (insect larvae) was completely different to ours in size, grazing behaviour and type of bioturbation.

The results of the first four studies presented here show that trophic interactions within the benthic food web in aquatic sediments can have an impact on biogeochemical processes such as nitrification. This has rarely been recognized in research so far and is a new and innovative thought.

While not directly related to the other four studies, the fifth study is equally important. Settling is a widespread standard method for the enrichment ciliates from plankton samples, that urgently needed a review, as supported by our results. The settling times commonly used are much too long and hence might negatively affect the quality of the results, and are ineffective in terms of time management. Our new insights will not only help to save time, but also improve the reliability of the abundance and taxonomic data that will be determined with the application of this method in the future.

1.9 Outlook

The results of the experiments described here and the experimental design employed revealed further questions, which should be addressed in future research. For example, the experiments with bioturbation and the experiments with ciliates should be combined (i. e. treatments with worms and ciliates added cross-classified). This will allow further insight in how trophic interactions between bacteria, ciliates and meiofauna affect nitrification. Furthermore, this should not only be conducted in marine, but also in freshwater sediments, as Buesing & Marxen (2005) identified riverine sediments as places of high bacterial production, similar to marine systems, and Altmann et al. (2004b) and Marshall et al. (2004) already showed that bioturbation in rivers affects N-cycling. Other abiotic and biotic factors that might have an influence on the interaction of ciliates and nitrifying bacteria - such as temperature, flow velocity, organic carbon content, flagellate abundance and taxonomic composition or photosynthesis - need to be addressed in experiments. Another important continuation is the application of the questions and methods of this PhD study to field systems. The results so far are based only on laboratory studies, further investigations in the field are necessary to confirm the relationship between ciliates and nitrification. Seasonal and spatial aspects have to be taken into account. And the question remains: if ciliates can indeed have an impact on nitrification - and indeed they have - are other bacteria-catalyzed biogeochemical pathways affected by ciliates as well?

PART II

2. DO CILIATES HAVE AN EFFECT ON THE NITROGEN CYCLE THROUGH GRAZING ON NITRIFYING BACTERIA?

2.1 Abstract

Ciliated protists are important predators of bacteria in many aquatic habitats, including sediments. Since many biochemical transformations within the nitrogen cycle are catalyzed by bacteria, ciliates could have an indirect impact on the nitrogen cycle through selective grazing on nitrogen-transforming bacteria. As a case study, we examined ciliate grazing on nitrifying bacteria of the genera *Nitrosomonas* and *Nitrospira*. All experiments were designed as *in vitro*-experiments with cultures of different bacteria and ciliate species. The nitrifying bacteria used in our experiments were *Nitrosomonas europaea* and *Nitrospira moscoviensis*. The ciliates comprised of four species that are known as efficient bacterivores and common members of the protist community in aquatic systems: *Paramecium aurelia, Euplotes octocarinatus, Tetrahymena pyriformis* and *Cyclidium glaucoma*. Our experimental approach, using a combination of DAPI and FISH staining, was successful in allowing the observation of ingestion of specific bacteria and their detection within ciliate food vacuoles. However, the ciliates in this study showed no significant selective grazing. No food preferences for any bacterial taxon or any size class or morphotype were detected. Correlation with time between ciliate abundance and bacterial abundance or biovolume, using log transformed growth rates of ciliates and bacteria, showed no significant results. On the bacterial side, neither an active defence mechanism of the nitrifying bacteria against ciliate grazing, such as changes in morphology, nor competition for resources were observed. These results suggest that in our *in vitro*-experiments grazing by ciliates has no influence on abundance and growth of nitrifying bacteria.

2.2 Introduction

The role of bacteria in important biogeochemical cycles such as the nitrogen cycle has been well studied in the past in a wide range of aquatic systems (Hendricks 1996,

Daims et al. 2001). In the course of bacterial nitrification, ammonium originating from aerobic as well as anaerobic decomposition of organic matter, is oxidized into nitrate via nitrite. These transformation steps are carried out predominantly by two groups of chemolithoautotrophic bacteria and take place in oxic environments (such as oxic sediment layers) at neutral pH (Cébron et al. 2003). Ammonium-oxidizing bacteria (AOB) of the genera *Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosolobus* or *Nitrosovibrio* transform ammonium (NH_4^+) to nitrite (NO_2^-), and nitrite-oxidizing bacteria (NOB) of the genera *Nitrobacter, Nitrococcus, Nitrospina* or *Nitrospira* convert nitrite to nitrate (NO_3^-) (Spieck & Bock 1998). While the process of nitrification is well studied, little attention has been paid to the trophic factors controlling abundances and community structure of the bacteria involved (Alphei et al. 1996, Strauss & Dodds 1997). One of the peculiarities of nitrifying bacteria is the fact that – despite their important role in the biochemical cycling of matter in natural environments – their population sizes are usually very low (Altmann et al. 2003). This may partially be due to their low growth rates (Spieck & Bock 1998), but could theoretically also be caused by a high grazing vulnerability coupled with a food preference of the grazers present for these bacteria. While in pelagic systems small heterotrophic flagellates are considered to be the main bacterivores (Berninger et al. 1991) some ciliates can also be effective bacterial grazers (Šimek et al. 1994). This is especially true for sediments, where bacterial abundances per unit volume are considerably higher than those in the water column. However, even though sediments have been identified as habitats with high bacterial biomass and production (Mallory et al. 1983, Hendricks 1996) as well as with high numbers of benthic ciliates (Cleven 2004) only limited data are available to date on their trophic interactions (Kemp 1988, Diederichs et al. 2003). There is still a lack of knowledge about the role of ciliate bacterivory (Sherr & Sherr 2002) within the tophic interactions in general and concerning possible consequences for important biochemical cycles as the nitrogen transformations in detail. Lavrentyev et al. (1997) showed that ciliates do feed on nitrifying bacteria and suggest that protistan grazing may be a controlling factor for nitrification, but that study was conducted in a pelagic system and used fluorescently labeled nitrifying bacteria (FLNB). Selective feeding by bacterivorous ciliates and resulting effects on the bacterial community have also been reported (Fenchel 1980, Šimek et al. 1994) as well as the elimination of

relatively slow-growing bacteria through ciliated predators (Mallory et al. 1983). Methodological difficulties are part of the reasons for our lack of knowledge on the role of ciliates as grazers of nitrifying bacteria. For example, although the fluorescence-*in-situ*-hybridization (FISH) technique employed here (Rautenstrauss & Liehr 2002) is a powerful method for molecular studies and the observation of distinct bacterial taxa in different natural systems (Altmann et al. 2003, Ribblett et al. 2005), this technique had to be adapted in our study for the qualitative determination of bacteria consumed by ciliates for a better differentiation of single bacterial cells.

The focus of our study was (i) to detect ingested nitrifying bacteria within the food vacuoles of the bacterivorous ciliates *Paramecium aurelia, Euplotes octocarinatus, Tetrahymena pyriformis* and *Cyclidium glaucoma*, (ii) to learn about possible selection for or against nitrifying bacteria as food sources and (iii) to investigate food selectivity by the ciliates on the basis of bacterial size and morphology (cocci or rods were offered as food). Our overall aim was to determine whether ciliates have a direct effect on nitrifying bacteria in aquatic systems through their grazing on them.

2.3 Material & Methods

Working stocks

The utilized culture of *Paramecium aurelia* was acquired from CCAP (Culture collection of Algae and Protozoa, England); *Euplotes octocarinatus*, was obtained from the University of Stuttgart, Germany, and *Tetrahymena pyriformis* as well as *Cyclidium glaucoma* were isolated from the sandy sediment of the River Salzach near Salzburg, Austria. The organisms were cultivated in 50 ml culture flasks and fed once a week with 20 – 50 µl of a natural bacterial food mix, also extracted from Salzach sediments. *Paramecium* sp. and *Euplotes* sp. also occur in the River Salzach sediments. This assemblage of ciliates represented the natural size spectrum and different feeding mechanisms (Šimek et al. 1994, Eisenmann et al. 1998, Wilks & Sleigh 1998, Hausmann et al. 2003) of bacterivorous interstitial ciliates.

The ammonium-oxidizing *Nitrosomonas europaea* was grown at 27°C and the nitrite-oxidizing *Nitrospira moscoviensis* at 37°C (in the dark without shaking or

stirring). After they reached an abundance of approximately 10^5 cells ml^{-1}, cultures were stored at 20°C in the dark until use in the grazing experiments. Both cultures of nitrifying bacteria were obtained from the Biocenter Klein Flottbek, Hamburg, Germany (Ehrich et al. 1995). *Nitrosomonas europaea* and *Nitrospira moscoviensis* are gram-negative bacteria of approx. 0.5 µm in diameter: cell shape can depend on physiological state, microscopical observation during the experiments showed cells mostly coccoid (Ehrich et al. 1995).

Experimental design

The experiments were designed as *in vitro* experiments and conducted in three replicates at 20°C under natural light in centrifuge tubes of 50 ml volume. Incubation periods were 90 min ($t_0 - t_1$) and 240 min ($t_0 - t_2$). These intervals were chosen because preliminary studies showed that ciliate abundances stayed constant over this period. The different cultures of ciliates were fed with the "bacterial food mix" to a density of about $1*10^6$ to $1*10^7$ bacteria ml^{-1}. The nitrifying bacteria were added in an approximate quantitative ratio of 1:10. On average, $5*10^5$ bacteria ml^{-1} of nitrogen transforming bacteria were added at the start of the experiment. To stop the experiment, an equal volume of paraformaldehyde (PFA, 8% f.c.) was added. Samples were stored in the dark at 4°C for one hour, followed by concentration of the organisms on filters (2 ml for bacteria, white track-etch membrane filters, Whatman, pore size 0.2 µm and 4 ml for ciliates, membrane Millipore filters, pore size 1.2 µm). Due to the high abundances of bacteria in the "food mix", 500 µl sample were diluted in particle-free water and then concentrated onto filters. The filters were stored at -20°C until further processing. FISH protocols, followed by epifluorescence microscopy were used to detect ingested and free bacteria.

FISH (fluorescence *in situ* hybridization)

FISH is a successful method to investigate and detect ingested bacteria within the food vacuoles of protists (Diederichs et al. 2003). Probes Ntspa712, cNtspa712 (Daims et al. 2001) and Nso1225 (Mobarry et al. 1996) were used. For details on these oligonucleotides see www.microbial-ecology.de/probebase/ (Loy et al. 2003). They were labeled with cyanine dye (Cy3) and obtained from Thermo Hybaid Electron Corporation (Ulm, Germany). All probes were used in a concentration of

5 ng μl^{-1} (Diederichs et al. 2003). The hybridization and staining of food mix bacteria cells with DAPI (4'6-diamidino-2-phenylindole) followed the protocol by Pernthaler et al. (2001).

Epifluorescence microscopy

Stained cells were counted and analyzed by epifluorescence microscopy (Nikon Eclipse E800 microscope, equipped with a HBO-100 mercury burner) at 1000x magnification. Abundances were determined by counting DAPI-signals and CY3-signals using the Nikon UV-2a-filter set (EX 330-380 nm, BS 400 nm, LP 420 nm) and the CY3-filter-set (EX 510-560 nm, BS 580 nm, LP 590 nm), respectively. For analyzing ingested bacteria within the ciliates' food vacuoles, epifluorescence microscopy was supported by a Nikon Digital Camera DXM1200F and the PC-based image software Lucia Net (Version 1.16.5). Ciliate biovolume was estimated based on the geometrical shape of an ellipsoid. 10 % shrinkage (Stoecker et al., 1994) due to fixation was assumed.

Data Analysis

Statistics were carried out using SPSS software (Version 12.0, SPSS Inc.). Growth rates (μ) were calculated following the equation

$$\mu = \ln (N_t)/N_0) * 1/t \qquad (1)$$

where N_0 and N_t are the counted numbers of bacteria on a filter at t_0 and t_1 or t_2, respectively. It was postulated that there was no loss of bacteria abundance by other factors than ciliate grazing. t was specified as hours.

Grazing rate (g) was calculated as

$$g = \mu_{control} - \mu_{experiment} \qquad (2)$$

where $\mu_{control}$ is the growth rate of the set control group of corresponding bacteria or ciliates and $\mu_{experiment}$ is the growth rate in the experiment.

For calculating the ingestion rates (I) the geometric mean value was used as

$$N = (N_t - N_0)/(\ln N_t - \ln N_0) \quad (3)$$

because of the assumption that the increasing of cell numbers is exponential. N_0 is the cell number at t_0 and N_t at t_1 or t_2 respectively.
Ingestion rates (I) were calculated as

$$I = g * N_{prey} / N_{predator} \quad (4)$$

The ingestion rate indicates the number of ingested prey per time unit.
Clearance rates (C_c) considering the ciliate abundance were calculated as

$$C_c = g * 10^6 / N_{prey} \quad (5)$$

C_c is regarded as a measure of the volume (µl) of water which a ciliate clears of food type per time unit.
Clearance rates of ciliates for food-mix bacteria and nitrifying bacteria, respectively, were compared using Student's t-test (Sachs 2000). The calculation of relative clearance rates followed Chesson (1983). Pearson correlation analysis was carried out to test relationships between log-transformed growth rates of bacteria and ciliates (Köhler et al. 2002). A two-way ANOVA which included the grazing period (incubation time) was used to test ciliate biovolumes as well as ciliate and bacteria abundances. The general significance level was $\alpha = 0.05$.

2.4 Results

Ingestion rates

All ciliates tested in the grazing experiments ingested *N. europaea, N. moscoviensis* as well as the food-mix bacteria (Table 2.1). The ciliates *P. aurelia* and *T. pyriformis* showed similar ingestion patterns, with high ingestion rates during the first 90 min of the incubation period and a ≥ 50% reduction of their ingestion afterwards (between 90 and 240 min incubation). Mean ingestion of *P. aurelia* on food mix bacteria ranged from 285 (± 235 SD) bacteria $cell^{-1}h^{-1}$ (90 min incubation period) to

125 (± 78) bacteria cell^{-1} h^{-1} (240 min) and on *N. moscoviensis* from 56 (± 23) bacteria cell^{-1}h^{-1} (90 min) to 1 (± 2) bacteria cell^{-1}h^{-1} (240 min). The mean ingestion of *T. pyriformis* was slightly lower and ranged from 87 (± 73) bacteria cell^{-1}h^{-1} (90 min) to 17 (± 18) bacteria cell^{-1}h^{-1} (240 min) on food-mix bacteria, and from 13 (± 4) bacteria cell^{-1}h^{-1} (90 min) to 9 (± 1) bacteria cell^{-1}h^{-1} (240 min) on *N. moscoviensis*. The ingestion pattern of the ciliate species *E. octocarinatus* and *C. glaucoma* showed the opposite trend, with lower rates during the first 90 minutes of the incubation period and considerably higher rates thereafter (Table 2.1). The differences between the two periods were statistically significant (t-test, $p < 0.05$). Overall ingestion rates were similar for *E. octocarinatus* and *P. aurelia*, those obtained for *C. glaucoma* corresponded to the values obtained for *T. pyriformis*.

Table 2.1: Abundance [cell ml^{-1}] and biovolume [µm^3 cell^{-1}] of ciliates, ingestion rates (I) [bacteria cell^{-1} h^{-1}] (mean of 3 replicates ± 1 SD) and clearance rates (C) [nl cell^{-1} h^{-1}] of *P. aurelia, E. octocarinatus, T. pyriformis* and *C. glaucoma* on different bacterial food after grazing periods of 90 min and 240 min.

ciliate grazing period	ciliate abund. [cell ml^{-1}]	ciliate biovol. [µm^3 cell^{-1}]	food bacteria mix		*N. moscoviensis*		*N. europaea*	
			I	C	I	C	I	C
Paramecium aurelia								
t_1 – 90 min	3.14E+02	1.40E+05	285 ± 235	345 ± 162	56 ± 23	93 ± 14	120 ± 11	235 ± 548
t_2 – 240 min	3.71E+02	1.46E+05	125 ± 178	212 ± 232	1 ± 2	99 ± 49	9 ± 7	117 ± 390
Euplotes octocarinatus								
t_1 – 90 min	2.62E+02	3.64E+05	148 ± 112	138 ± 228	47 ± 7	71 ± 69	124 ± 54	233 ± 283
t_2 – 240 min	2.67E+02	4.54E+05	280 ± 67	213 ± 132	91 ± 23	72 ± 72	144 ± 225	362 ± 434
Tetrahymena pyriformis								
t_1 – 90 min	2.98E+03	3.86E+04	87 ± 73	68 ± 21	13 ± 4	15 ± 18	12 ± 74	12 ± 15
t_2 – 240 min	3.07E+03	3.77E+04	17 ± 18	34 ± 9	9 ± 1	17 ± 21	6 ± 14	16 ± 25
Cyclidium glaucoma								
t_1 – 90 min	3.62E+03	1.43E+03	34 ± 25	17 ± 36	2 ± 8	20 ± 53	4 ± 3	18 ± 10
t_2 – 240 min	3.19E+03	1.46E+03	143 ± 63	16 ± 23	13 ± 19	11 ± 58	8 ± 5	15 ± 23

P. aurelia and *T. pyriformis* fed on all bacteria offered to them, and there was a trend, though not statistically significant (t-test, $p > 0.05$), that they favoured cocci (Fig. 2.1). When *N. europaea* instead of *N. moscoviensis* was offered, the ingestion patterns described above remained the same. In accordance with the ingestion rates the clearance rates for the food mix bacteria of *P. aurelia* and *T. pyriformis* reached

their maximum after 90 min and decreased until 240 min. *E. octocarinatus* and *C. glaucoma* showed an opposite result, where clearance rates increased through the end of the incubation period of 240 min. The hourly clearance rates of each ciliate species on nitrifying bacteria showed no significant variations in the course of the grazing experiments. Corresponding clearance rates are also given in Table 2.1.

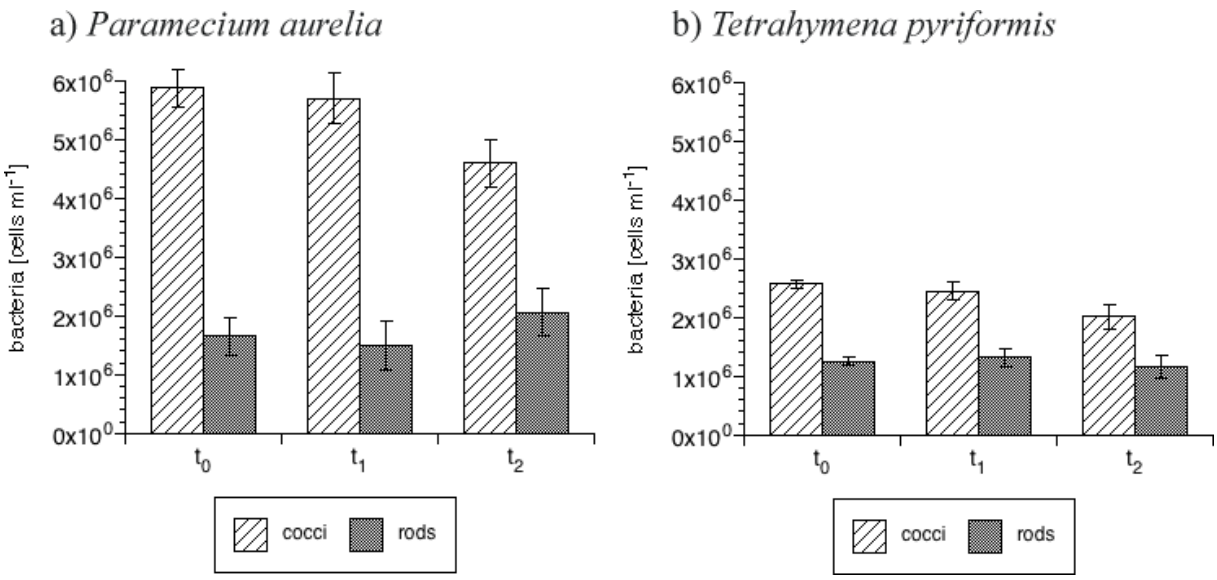

Fig. 2.1 : Total bacterial abundance in ciliate treatments: (**a**) *P. aurelia* (**b**) *T. pyriformis* after t_0 (0 min), t_1 (90 min) and t_2 (240 min) grazing periods; values are means of 3 replicates ± 1 SD.

Food preference

For calculating a possible preference for a certain food type, relative clearance rates following Chesson (1983) were compared. This is equivalent to the relative preference of the ciliate for each food type without considering the ciliate abundance. *P. aurelia, E. octocarinatus* and *T. pyriformis* showed no significant (t-test, $p > 0.05$) preference for any bacterial taxon in any experiment (Fig. 2.2). In contrast, *C. glaucoma* showed a trend for a preference for the food mix bacteria over the nitrifying forms, however, this was not statistically significant (t-test, $p > 0.05$). The relative clearance rates calculated from all experiments for both, *N. europaea* and *N. moscoviensis* also did not indicate any food preference of the ciliates.

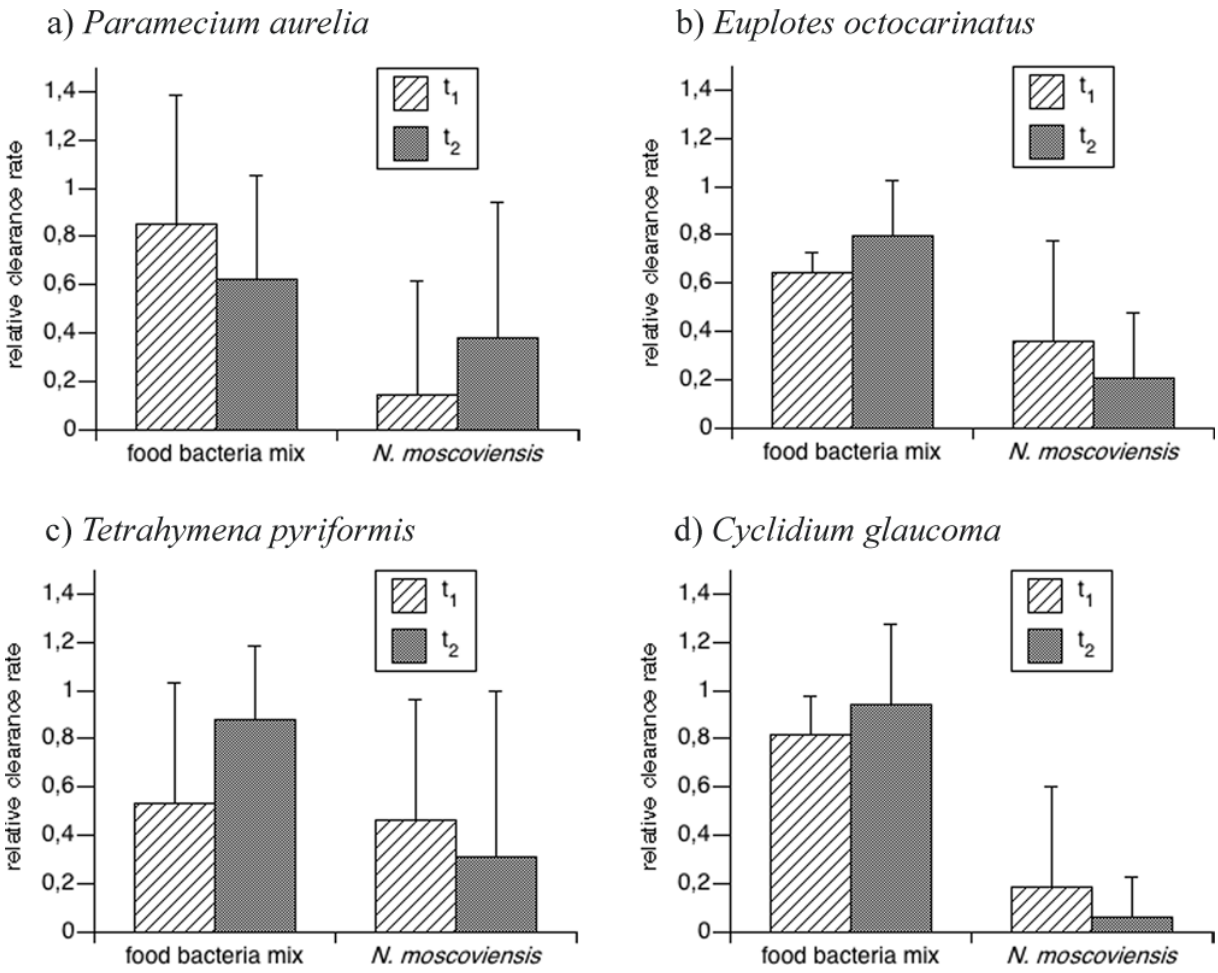

Fig. 2.2: Relative clearance rates (**a**) *P. aurelia* (**b**) *E. octocarinatus* (**c**) *T. pyriformis* (**d**) *C. glaucoma* on food bacteria mix and *N. moscoviensis*; values are means of 3 replicates ± 1 SD.

Detection of ingested nitrifying bacteria

The FISH technique allowed a qualitative detection of ingested nitrifying bacteria within the ciliates' food vacuoles. This approach worked well for all ciliate species used in our experiments. Concentration of PFA in the fixing solution was increased from 4% f.c. to 8% f.c. based on results of the preliminary studies. The higher concentrations of PFA resulted in a higher membrane permeability and ingested food bacteria were more clearly detectable by DAPI (Fig. 2.3a) and Cy3 (Fig. 2.3b) staining. In the time course of the grazing periods more and more food vacuoles became visible and the ingested nitrifying bacteria were distributed equally in all vacuoles. This implies a constant, though relatively low, grazing pressure on *N. moscoviensis* and *N. europaea* when offered as food.

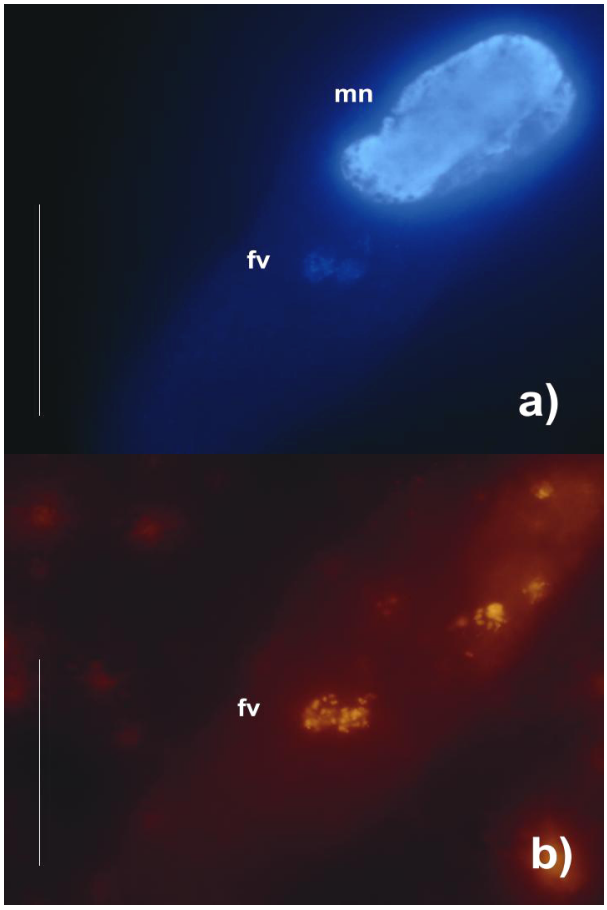

Fig. 2.3: *P. aurelia* with ingested *N. moscoviensis* (**a**) DAPI and (**b**) Cy3 stained. 1000x magnification; mn – macronucleus, fv – food vacuole. Scale bars: 50 µm.

2.5 Discussion

Natural, diverse ciliate communities consist of species of different feeding types (Fenchel 1986), resulting in differences regarding their food preference (concerning size, morphology, chemical "taste", way of movement of their food, etc.) (Jezbera et al., 2005; Jürgens & DeMott 1995). Furthermore, trophic relationships are influenced by a variety of abiotic (temperature, pH, light regime, flow, etc.) and biotic factors (community composition, food concentration, etc). Therefore, the correct interpretation of results from grazing experiments depends on knowledge of a wide range of factors. Selective feeding by protists can have a strong effect on the community composition of their prey, in our case on the abundance of single bacterial species within the offered bacterial species mixture. Consequently, selective

feeding e.g. on nitrifying bacteria could have an influence on nitrification and on the efficiency of the various nitrogen transformation steps. With regard to these possible consequences the grazing effect of different bacterivorous ciliates on nitrifying bacteria was investigated in the present study.

Detection of ingested nitrifying bacteria

The detection of ingested nitrifying bacteria within the food vacuoles of the ciliates used in this study was one of our aims. Due to the great significance of bacterially mediated N-transformations in natural systems, this question is of high ecological relevance. We were successful in applying this new technique (Diederichs et al. 2003), which opens great potential for detailed grazing studies in the future. In particular, the detection of ingested nitrifying bacteria was possible (Fig. 2.3). The ingested bacteria were stained with a combination of DAPI and Cy3; all ciliates ingested *N. europaea* and *N. moscoviensis*, and nitrifying bacteria could be distinguished from the other "food bacteria". However, the bacteria were too densely packed or already in a state of digestion, that it was not possible to count individual bacteria inside food vacuoles, although up to 450 ciliate cells per replicate were investigated. Thus we were unable to determine the uptake rates directly from counts of ingested nitrifying bacteria. Based on size of bacteria, most filled food vacuoles contained numerous bacteria. Consequently, uptake rates had to be calculated from abundances of nitrifying bacteria. A rough estimation of the number of ingested cells by using the ratio between the volume of the ciliate and the volume of the food vacuole would have been possible (Eisenmann et al. 1998) but was not the scope of this study.

FISH is a powerful method for investigation of ingested bacteria, but in order to carry out successful quantitative studies it is necessary to improve the technique, including the microscopical analysis. Food vacuoles are three-dimensional structures, and two-dimensional microscopical examination is not always sufficient for the enumeration of ingested particles. The use of laser scanning microscopes may be a solution here.

Ingestion rates

All ciliates investigated in our study were feeding efficiently on the bacterial food offered, which is supported by their positive growth rates. All ciliates also ingested the bacteria in large quantities, in most cases reaching almost the maximum values recorded in the literature. Furthermore, our combination technique of DAPI and FISH confirmed that all ciliates ingested the nitrifying bacteria *N. moscoviensis* and *N. europaea*.

N. moscoviensis and *N. europaea* appear in cocci of the size range from 0.4 to 0.5 µm diameter, equivalent to a cell volume of approx. 0.524 μm^3 $cell^{-1}$ (Ehrich et al. 1995). This puts them into a size class especially relevant as food for small ciliates, such as *C. glaucoma* and *T. pyriformis* (Fenchel 1986, Šimek et al. 1994, Posch et al. 2001). Both ciliate species are well known as bacterivores. *C. glaucoma*'s bacterial uptake remains almost linear at particle concentrations of up to 10^7 ml^{-1} (Fenchel 1986), and for *T. pyriformis* ingestion rates of up to 100 % of their body volume per hour are recorded (Eisenmann et al. 1998). In our study, *T. pyriformis* ingested bacteria equivalent to 75 % of their own volume per hour, while rates for *C. glaucoma* reached values of 97 % (on food mix bacteria and *N. moscoviensis* combined). However, despite these high ingestion rates no significant decrease of bacteria of defined size classes was recorded during our grazing periods (t-test, $p > 0.05$). Consequently, abundances and biovolumes of the bacterial community in our experiments were not noticeably affected. Larger ciliates typically have the capacity to ingest 10 – 30 % of their own cell volume per hour (Fenchel 1980). This was confirmed in our experiments. *P. aurelia* took up approximately 397 μm^3 bacteria h^{-1} which corresponds to 44 % of its cell volume. However, this grazing activity caused a non significant (t-test, $p > 0.05$) decrease among the bacterial mix and had no effect on total biovolume of the bacterial community. *Euplotes octocarinatus* was feeding constantly on bacteria throughout each treatment but the rates differed between the two incubation periods. The absolute ingestion rates of *E. octocarinatus* and *P. aurelia* on *N. moscoviensis* were similar. In addition to total ingestion rates, clearance rates, i. e. the volume of water a ciliate clears of food particles per unit time, could be calculated. The rates calculated from our experiments were equivalent (*E. octocarinatus*) or slightly lower (*P. aurelia* and *T. pyriformis*) than those recorded by Fenchel (1980).

Food preference

A central aim of our study was to establish whether selective grazing pressure on nitrifying bacteria might affect their population dynamics and consequently nitrification rates in natural systems. In all our experiments, with all grazers, ingestion rates for nitrifying bacteria were even lower than those for the food mix bacteria. This does not just refer to absolute ingestion rates, but is also valid on a relative scale, taking the ratio of nitrifying bacteria to food mix bacteria in our experiments (approx. 1:10) into account. None of the ciliates tested in our study showed selective grazing, only *C. glaucoma* had a slight, though not statistically significant (t-test, $p > 0.05$) preference against nitrifying bacteria. Consequently, ciliate grazing had no positive or negative effect on the abundances of nitrifying bacteria. Given that in natural environments, such as stream sediments, where the contribution of nitrifying bacteria to the entire bacterial community is even lower (approx. 0.16%; Altmann et al. 2004a), factors other than grazing appear to be responsible for their low population sizes, low growth rates and long doubling times (at least 12 hours, even under optimal nitrite and temperature conditions; Ehrich et al. 1995).

Conclusions

The results of our laboratory experiments imply that nitrifying bacteria did not underlie disproportionately high or low grazing pressure by a diverse ciliate community (*P. aurelia, E. octocarinatus, T. pyriformis* and *C. glaucoma*), and that the ciliates did not influence the species composition or the composition of morphotypes within the bacterial community. Our results further imply that the ciliates do not have a direct effect on bacterial nitrogen transformations and on the nitrification within aquatic systems. Ingested bacteria were detected within the ciliates' food vacuoles, but the rates measured were too low to infer a significant reduction of these bacteria through grazing. Further, no quantitative interpretation of food vacuole contents was possible. Nitrite- and ammonium transforming bacteria could be detected within the food vacuoles of the ciliates using a combination of DAPI and FISH marking techniques, however, we still see a need for further improvement of the approaches and the methods used in our study. The FISH

technology has to be improved for quantitative registration of consumed bacteria by protistan grazers from natural systems. In addition, experiments using ammonium-oxidizing and nitrite-oxidizing bacteria as potential food are still lacking, in order to draw conclusions on possible effects on the whole nitrogen cycle in natural systems. It would further be desirable to apply our approach to natural systems.

3. THE IMPACT OF CILIATES ON NITRIFICATION IN SEDIMENTS OF THE RIVER SALZACH (AUSTRIA)

3.1 Abstract

Rivers are of great biogeochemical importance for the transport and processing of matter. Within the river, sediments play an essential role as location of biogeochemical processes, such as nitrification, due to the steep gradients at the water-sediment-interface, their high bacterial population and their connection to the groundwater and the riparian zone. While nitrification is well studied in terms of autecological studies on the relevant nitrifying bacteria, the catalyzing organisms have only been rarely regarded as part of a food web. This study focussed on the interaction between nitrifying bacteria and ciliates. Ciliates are important bacterivores in sediments. We designed an experimental study with natural sediments transferred to laboratory flumes, where the ciliate abundance was manipulated. The sediments were either untreated, autoclaved or frozen to achieve different compositions of inhabiting fauna and nutrient levels. Total bacteria, nitrifying bacteria, flagellates and ciliates were counted. Nitrification potentials and *in situ* concentrations of ammonium and nitrate were measured with slurry assays and microsensors, respectively. The results showed that the abundance of ammonium-oxidizing bacteria increased in treatments with high ciliate abundance. The contribution of nitrifying bacteria to the total bacterial abundance and their activity, i. e. the nitrification potential of the sediments, were very low in our fluvial sediments from the River Salzach, Austria, when compared to other systems such as marine sediments.

3.2 Introduction

Rivers contain only 0,0001% of the total water on earth (Wetzel 2001). In spite of these low quantities, running waters are of enormous geological significance, as they move large amounts of dissolved and particulate matter from the land to the sea, and

this matter is subject to biogeochemical processes during the transport. Nitrogen and its compounds - essential nutrients for all organisms and responsible for environmental problems such as eutrophication - are among the most important elements transported and processed in a river. Among the biogeochemical processes within the N-cycle, this study focussed on nitrification. Nitrification is the sequential oxidation of ammonium (NH_4^+) to nitrate (NO_3^-) via nitrite (NO_2^-), and it is predominantly accomplished by chemolithotrophic bacteria. The first step is the ammonium oxidation, which proceeds by a series of oxidation stages through hydroxylamine and pyruvic oxime to nitrous acid. This transformation is catalyzed by bacteria, which are largely confined to the genus *Nitrosomonas* (Nitrobacteriaceae, order Pseudomonadales), although *Nitrosococcus, Nitrosospira, Nitrosolobus, Nitrosovibrio* and several other taxa, including methane-oxidizing bacteria and crenarchaeota, are also known to be capable of this process (Wetzel 2001 and literature cited herein, Könnecke et al. 2005). The second step is the nitrite oxidation. The bacteria catalyzing this step are *Nitrobacter, Nitrospira, Nitrospina* and *Nitrococcus*. Nitrification is an aerobic process and requires a pH close to neutral. It does not only take place in the water column, but also in the sediments, were it is spatially closely coupled to other biogeochemical processes due to the steep gradients at the sediment/water interface, and where bacterial abundances are higher than in the water column. Only recently, sandy fluvial sediments have been identified as places of strong microbial activity (Hendricks 1996, Buesing & Marxen 2005), similar to marine sediments. Besides by bacteria, the interstitial system of a sandy sediment is also inhabited by a broad variety of other organisms, such as flagellates, ciliates, gastrotrichs, algae, nematods and other benthic meiofauna, forming a benthic food web (e.g. Epstein 1997a, Schmid-Araya & Schmid 2000). While in pelagic systems heterotrophic flagellates are considered to be the main bacterial grazers (Berninger et al. 1991), in the benthic food web ciliates are important bacterivores (Kemp 1988, Dietrich & Arndt 2000). Ciliates belong to the protists and are a very heterogeneous group regarding their metabolism, feeding mechanism, locomotion, and habitat preferences (Hausmann & Bradbury 1996). They occur not only in aquatic environments, but also in soils and as parasites. Interstitial ciliates feed mainly on bacteria, flagellates, other ciliates and algae and

are adapted to their environment in several complex ways (Berninger & Epstein 1995, Wickham et al. 2000).

Many autecological studies on nitrifying bacteria have been conducted, but despite their importance, these prokaryotes have only rarely been looked at as a part of a food web, in which they have to compete for substrates and nutrients and in which they might be prey to other organisms (Verhagen & Laanbroeck 1992), particularly to ciliates (Lavrentyev et al. 1997, Strauss & Dodds 1997). Prast et al. (2007) demonstrated for marine sediments that ciliates do affect the abundance, taxonomic composition and activity of nitrifying bacteria, concluding that this effect is caused by increased nutrient supply due to a stimulation of bacterial production through ciliate grazing and consequent nutrient recycling and/or an increased water flow in the sediments because of ciliate activity. In addition, Neubacher et al. (2007) showed that nitrifying bacteria are ingested by benthic ciliates, but there is no prey selection for or against them.

The nitrogen cycle in river sediments in general (e. g. Kemp & Dodds 2002, Cavanaugh et al. 2006) and nitrification in fluvial sediments in particular (e. g. Strauss & Lamberti 2000, Strauss 2002, Strauss et al. 2002, Strauss et al. 2004) has been subject to research before, but these studies did not take the interaction between nitrifying bacteria and ciliates into account, which was the focus of this study. Our aim was to find out whether a change in ciliate abundance would affect the activity and composition of nitrifying bacteria in fluvial sediments.

3.3 Material & Methods

Experimental setup

Experiments were conducted in six laboratory flumes (length: 56 cm, width: 9 cm, height: 8 cm, depth of sediment layer ~4 cm; Fig. 3.1). This allowed a control of basic parameters and unpredictable environmental influences could be excluded. As a model for fluvial sediments, natural sediments were collected from the River Salzach in Elsbethen-Glasenbach (near Salzburg, Austria; 47° 46′ 02′′N, 13° 46′ 29′′ E) on October 21, 2005 (t_0), from the upper 10 cm, close to the river bank.

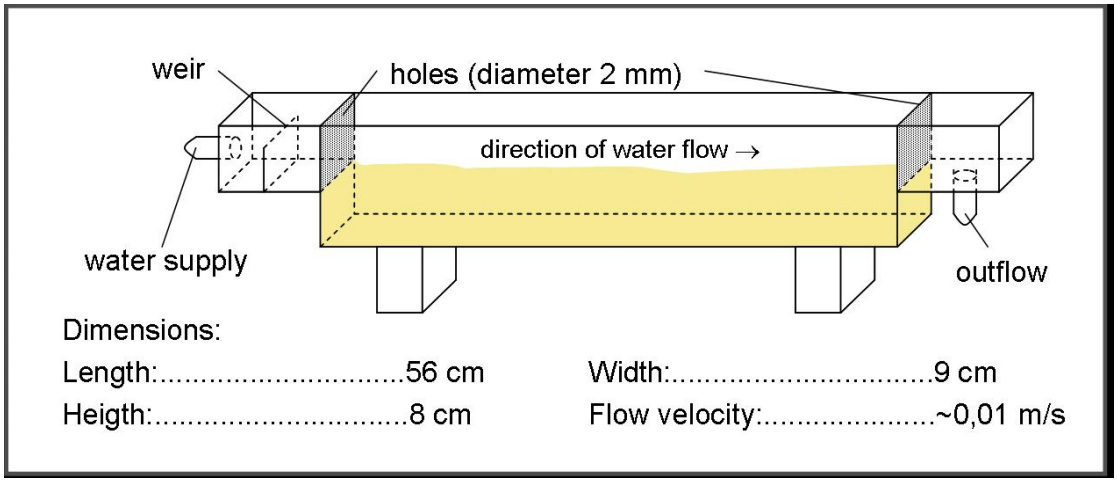

Fig. 3.1: Dimensions of the flumes, which were used for the experiments.

Sediments were treated in three ways: for the first treatment, sediments were autoclaved to kill all natural organisms from the sediments and to release nutrients. For the second treatment, unmanipulated sediments were transfered into the flumes. For the third treatment, sediments were frozen and unfrozen three times over a period of ten days. Freezing kills meiofauna, but protists and bacteria survive this procedure. Sediments were then placed for one week in the flumes prior to the experiments to allow vertical gradients to re-establish. Water flow velocity in the flumes was ~0.01 m s^{-1} with tap water, temperature was adjusted to 20 °C, each flume had an independent water circuit with a reservoir of 15 l, pump, aeration and heating. Two flumes were used per treatment (Tab. 3.1). All flumes were kept in the dark to avoid effects of photosynthesis.

After one week (t_1), a ciliate cocktail consisting of four ciliate species (Tab. 3.2) was added to one flume per treatment (+Cil) at a final density of 595 cells cm^3 of sediment for the flumes 1 and 3, and 1062 cells cm^3 of sediment for the flume 5, respectively. This assemblage of ciliates represented the natural size spectrum and different feeding mechanisms of bacterivorous interstitial ciliates (Šimek et al. 1994, Eisenmann et al. 1998, Wilks & Sleigh 1998, Hausmann et al. 2003). The other flumes recieved a similar amount of 1.2 µm-filtered culture cocktail as control, but without ciliates (-Cil). The sediments were then incubated for three days, and then the samplings and measurements were conducted (t_2).

Tab. 3.1: Treatment of sediment and flume numbers.

	+Cil	-Cil
autoclaved	1	2
natural	3	4
frozen	5	6

Tab. 3.2: Ciliate cocktail added to the flumes.

Species	Origin of culture	Flume 1, 3 [ind./cm^3]	Flume 5 [ind./cm^3]
Euplotes octocarinatus	University Stuttgart, Germany	4	8
Tetrahymena pyriformis	isolated from River Salzach	305	479
Paramecium aurelia	CCAP, Widermere, England	45	166
Cyclidium glaucoma	isolated from River Salzach	241	409
total		595	1062

The ciliates were cultured in a 1:1 mixture of Volvic® mineral water and SMB-medium (1.5 mM NaCl, 0.05 mM KCl, 0.4 mM $CaCl_2 * 2\ H_2O$, 0.05 mM $MgCl_2 * 6\ H_2O$, 0.05 mM $MgSO_4 * 7\ H_2O$, 2.0 mM sodium phosphate buffer, pH 6.8; Miyake 1981) in the dark at 20 °C with autoclaved wheat grains as carbon source. No nitrifying bacteria were found among the food bacteria in the cultures. Some ciliates in low natural abundances were still present in the –Cil treatment, 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.

Sampling procedures

All samples were taken with a sediment corer (10ml plastic syringe with cut-off tip, diameter 16 mm). Bacteria, flagellate and ciliate samples were transferred into preweighted 50 ml plastic centrifugation tubes and immedeately fixed with glutardialdehyde (GA; 2 % f.c.). Samples for nitrifying bacteria were transferred into 50 ml centrifugation tubes as well and were fixed with paraformaldehyde (4 % f.c.). 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). Samples for abiotic parameters, such as porosity, ash free dry weight and grain sizes, were collected and stored in 50 ml centrifugation tubes until further processing. Three replicates per flume were prepared for all samples.

Bacterial, flagellate and ciliate counts

Numbers of total bacterial and flagellate abundances were aquired using the DAPI method (Porter & Feig 1980). Samples were sonicated (Labsonic M, puls: 0.9, amplitude: 60 %, 60 s) in order to separate attached bacteria from sediment particles and break up bacterial aggregates. For extraction of bacteria and flagellates, samples were resuspended in sterile tapwater with 2 % GA and gently shaken by hand for 60 seconds. Afterwards, 3.5 ml of supernatant were immediately pipetted off into a 50 ml centrifuge tube. To achieve a quantitative extraction, this washing procedure was repeated at least five times per sample. 200 or 250 µl of the supernatant diluted with 2 ml of particle-free tapwater were then filtered onto a black 0.2 µm polycarbonate filter (Nuclepore) for bacterial counts or onto a 0.8 µm-filter (Nuclepore) for flagellate counts, respectively.

The NOB *Nitrospira* and *Nitrobacter* and the ammonium-oxidizing β-proteobacteria (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) for β-AOB, respectively (for details on these probes see probeBase; Loy et al. 2003). All probes were labeled with the fluorescent dye Cy3. Hybridization procedure followed a protocol described before by Pernthaler et al. (2001). Washing and filtration were identical to the procedure 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 seven times with 5 ml of sterile tap water with 2 % GA to remove the 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), counted and taxonomically identified using a silver staining method which stains nuclei, cilia and ciliary basal bodies (Montagnes

& Lynn 1987, Skibbe 1994). All counts were conducted with a Nikon Eclipse E800 microscope.

Microsensor measurements

Oxygen microprofiles were measured using a Clark-type oxygen microelectrode (Revsbech 1989), which was connected to a PA-2000 picoamperemeter (Unisense). For the *in situ* measurements of NH_4^+- and NO_3^--concentrations, liquid ion exchanger (LIX)-type microelectrodes were used (DeBeer et al. 1997), connected to a ME02020 millivoltmeter (MasCom). Picoamperemeter and millivoltmeter were connected to a computer via an ADC-101 analog/digital converter (Pico). The microsensors were positioned with a MMS motorized micromanipulator system (Unisense). The software Profix 3.0 (Unisense) was used for controling the motorized micromanipulator (with a MC-232 controller, Unisense) and recording of data.

Nitrification potential (slurry assay)

Sediment samples were slurried (~6.5 g sediment + 100 ml of 0.2 µm-filtered SMB-medium) and transferred into 200 ml Erlenmeyer flasks. Each flask was amended with NH_4^+ (50 µM) and $NaClO_3$ (20 mM) for estimation of NH_4^+-oxidation rates or with NO_2^- (25 µM) and allylthiourea (ATU; 10 mg/l) for NO_2^--oxidation rates, respectively. $NaClO_3$ and ATU are inhibitors for the respective complementary step of nitrification. The Erlenmeyer flasks were aerated and stirred. Samples were taken from each flask after 0, 1.5, 5 and 22 h (t_0, t_1, t_2, t_3). Prior to sampling, stirring and aeration were stopped for five minutes. Samples were centrifuged for 5 minutes (Heraeus Multifuge 4KR, 4400 rpm) to remove remaining sediment particles. NO_2^- concentrations were measured photometrically using a Dr. Lange Xion 500 photometer and Merck Spectroquant NO_2^- test kit 1.14776.0001 (Dollhopf et al. 2005).

Abiotic parameters

Dry weight, ashfree dry weight, porosity (calculated according to Hölting 1996) and grain sizes (Retsch AS 200 basic wet sieving machine) were determined. All results given in g^{-1} refer to dry weight. SigmaXL (Version 2000) and SPSS (Version 11.0) software were used for statistical analysis.

3.4 Results

Abiotic parameters

Sediment grain size distribution, porosity and weight loss on ignition are presented in Tab. 3.3. With the main fraction of sediment grains between 0.49 and 0.063 mm and an ignition loss of 1.74 % the sediments represented a typical sandy sediment. Concentrations of NH_4^+, NO_2^- and NO_3^- from the water in the flumes are shown in Tab. 3.4. NH_4^+ concentrations showed no differences between the treatments, while for NO_2^- the concentrations between flumes 1 and 2 as well as 5 and 6 were significantly different (ANOVA, $p<0.05$). NO_3^- concentrations showed significant differences between the +Cil and –Cil treatments in the autoclaved and natural sediments, with higher values in the –Cil treatments (ANOVA, $p<0.05$).

Tab. 3.3: Abiotic sediment characteristics (n=6).

grain size [mm]	% portion
>0.5	2.7 (± 1.6)
0.49-0.125	37.7 (± 8.6)
0.125-0.063	40.1 (± 2.0)
0.063-0.025	15.1 (± 5.2)
<0.025	4.3 (± 4.2)
	Mean (± SD)
Porosity	37.23 (± 2.11)
Sediment ignition loss [%]	1.74 (± 0.20)

Tab. 3.4: Concentrations of NH_4^+, NO_2^- and NO_3^- from the flumes (n=3).

Flume	1	2	3	4	5	6
Treatment	+Cil autocl.	-Cil autocl.	+Cil nat.	-Cil nat.	+Cil froz.	-Cil froz.
NH_4^+-N [mg/l]	0.099 (±0.023)	0.101 (±0.002)	0.073 (±0.008)	0.066 (±0.006)	0.078 (±0.004)	0.060 (±0.007)
NO_2^--N [mg/l]	0.094 (±0.004)	0.208 (±0.012)	0.074 (±0.004)	0.093 (±0.009)	0.222 (±0.007)	0.044 (±0.006)
NO_3^--N [mg/l]	0.567 (±0.180)	0.984 (±0.092)	0.393 (±0.148)	0.935 (±0.019)	1.333 (±0.076)	1.128 (±0.066)

Microsensor profiles

All profiles show the mean of three (in some cases four) individual profiles that were measured in different spots within the flume. The oxygen profiles showed that the oxic zone of the sediments in the flumes was limited to the upper 4-5 mm. A typical oxygen profile is presented in Fig. 3.2.

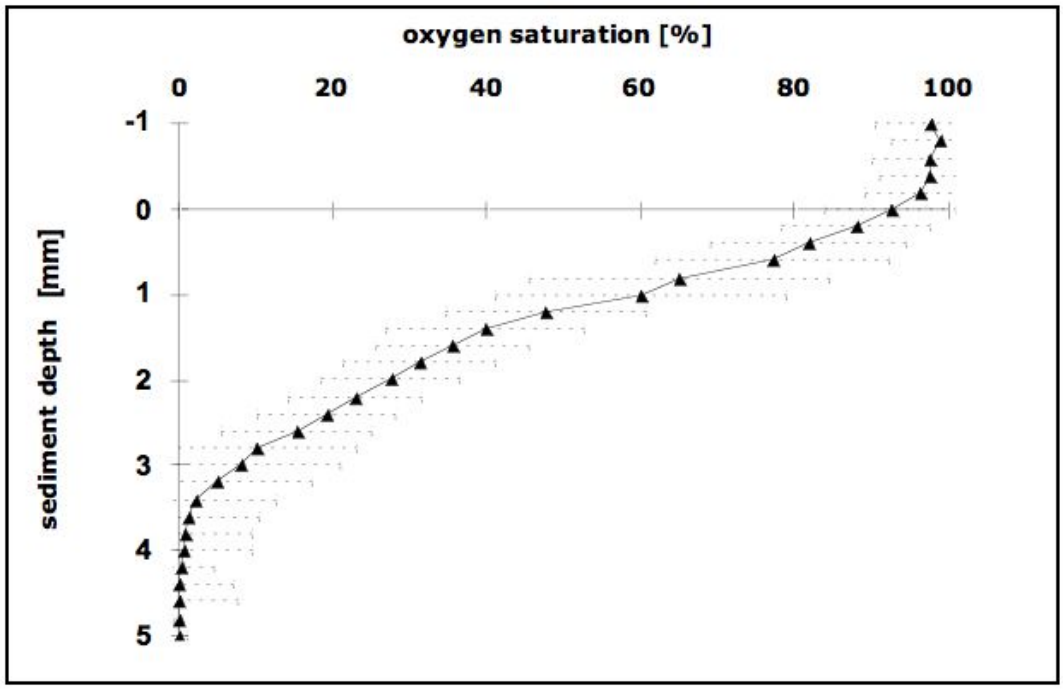

Fig. 3.2: Typical oxygen profile from the sediments in the flumes. Error bars show ± 1 standard deviation, n = 3.

Nitrate- and ammonium-profiles from the sediments in the experimental flumes are presented in Fig. 3.3. Ammonium profiles show a constant concentration over the oxic zone or a slight increase towards the anoxic zone. Mean concentrations are different between the +Cil and –Cil flumes for the autoclaved and the natural treatments (ANOVA, $p < 0.001$), with higher values for the –Cil flume with the autoclaved sediment and the +Cil flume for the natural sediment. Nitrate concentrations in the flumes with the autoclaved sediments were very low and showed no difference between the +Cil and –Cil treatment, but mean concentrations were significantly different from all other flumes (ANOVA, $p < 0.001$). In the natural treatment, concentrations were higher, but also showed no difference between the +Cil and –Cil treatment. Mean concentrations were significantly different from the concentrations in the autoclaved sediments and from the sediments that had been

subject to freezing (furtheron referred to as the „frozen“ treatment) (ANOVA, $p < 0.001$). In the frozen sediment, nitrate concentrations were highest, and the mean concentration in the +Cil flume was significantly higher (ANOVA, $p < 0.001$) in the +Cil treatment.

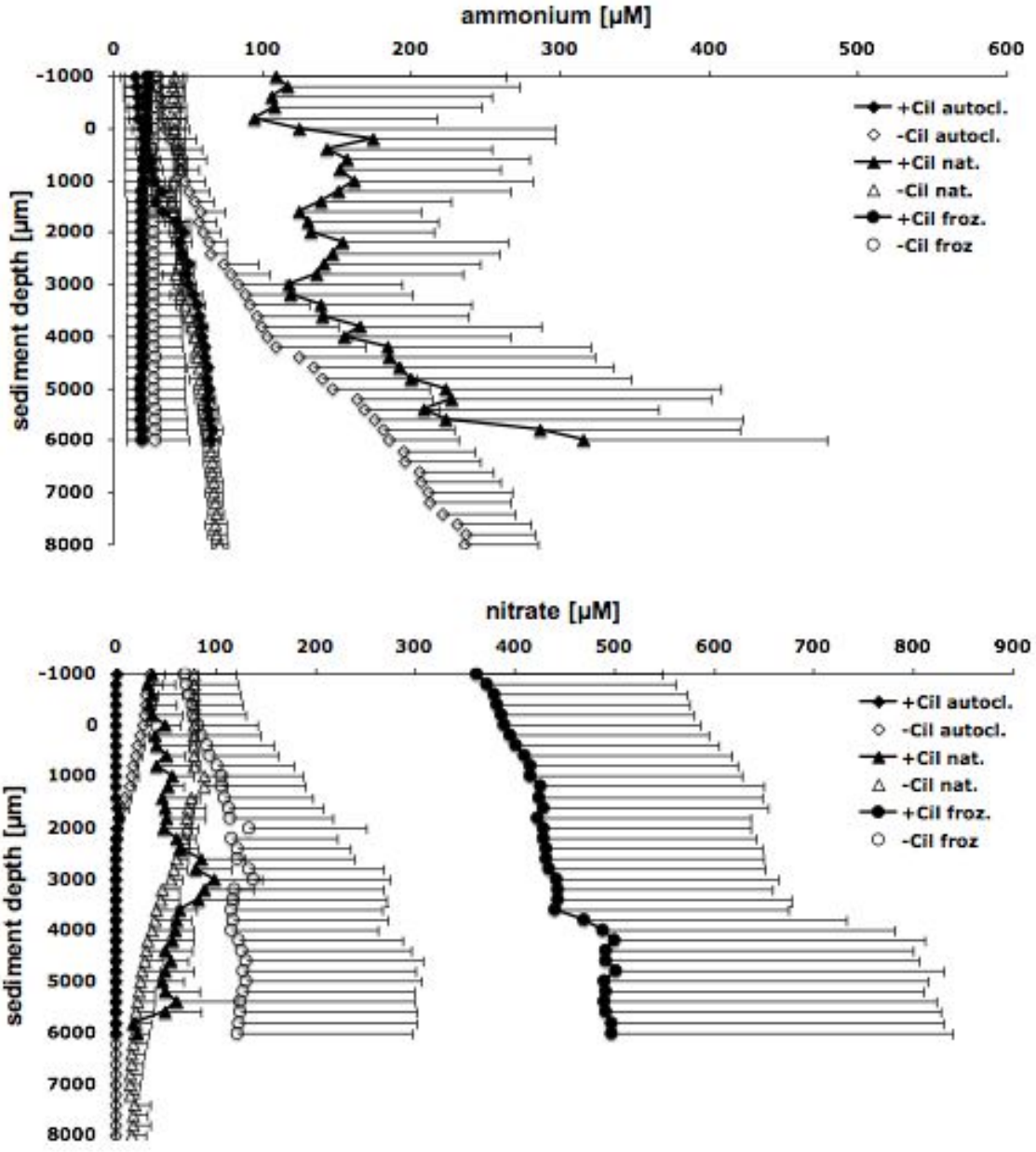

Fig. 3.3: Ammonium- and nitrate-profiles from the sediments in the experimental flumes. Error bars show + or - 1 standard deviation, only one side is shown for better clearance of the figure. Depth = 0 refers to the sediment surface, n = 3.

Nitrification potentials

Nitrification potentials were calculated from the change in nitrite concentration over time and the weight of the sediment sample per Erlenmeyer flask. No nitrification potential could be measured in the sediments in the experimental flumes after the incubation period. The results of the measurements for the total period (t_0-t_3) are presented in Fig. 3.4, but this reflects only the background noise of the photometric measurements, including even negative values. No nitrification potential could be detected. All results are listed in Tab. 3.5 in detail.

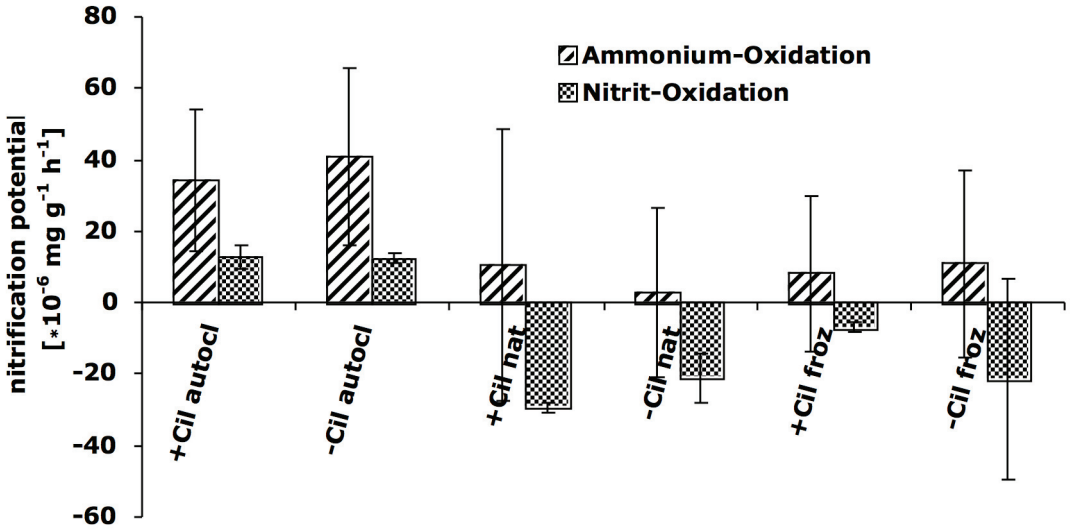

Fig. 3.4: Nitrification potentials t_0-t_3 of the sediments in the experimental flumes at the end of the incubation period. The measured potentials are all very low and are essentially „0“, the values more represent the background noise. Error bars show ± 1 standard deviation

Tab. 3.5: Nitrification potentials of the sediments [$*10^{-6}$ mg g^{-1} h^{-1}] in the experimental flumes at the end of the incubation period.

	Treatment	Replicate	t_0-t_1	t_1-t_2	t_2-t_3	t_0-t_3
ammonium oxidation	+Cil autoclaved	1.1	429	-113	56	54
		1.2	302	-1300	221	34
		1.3	360	-243	30	15
	-Cil autoclaved	2.1	511	-104	33	42
		2.2	324	-65	70	65
		2.3	411	-87	3	15
	+Cil natural	3.1	441	-175	63	53
		3.2	165	-103	3	-1
		3.3	236	-78	-32	-21
	-Cil natural	4.1	513	-89	-29	-4
		4.2	351	-92	-33	-17
		4.3	382	5	9	29
	+Cil frozen	5.1	33	-85	7	-3
		5.2	-45	52	38	33
		5.3	25	-66	0	-6
	-Cil frozen	5.1	271	87	20	41
		6.2	53	21	-16	-7
		6.3	177	-99	0	-2
nitrite oxidation	+Cil autoclaved	1.1	-57	137	-4	16
		1.2	27	111	7	9
		1.3	-36	119	-1	13
	-Cil autoclaved	2.1	28	93	0	13
		2.2	37	90	-1	14
		2.3	-20	99	0	11
	+Cil natural	3.1	-94	89	46	-31
		3.2	-67	83	46	-29
		3.3	-31	82	47	-28
	-Cil natural	4.1	-28	61	36	-22
		4.2	-44	81	26	-13
		4.3	-10	64	44	-27
	+Cil frozen	5.1	-25	7	9	-8
		5.2	0	4	8	-5
		5.3	-18	-58	-1	-7
	-Cil frozen	5.1	0	-14	5	-6
		6.2	9	-10	5	-5
		6.3	5	-35	64	-54

Bacterial & flagellate abundances

Abundances of total and nitrifying bacteria are presented in Fig. 3.5. Total bacterial abundances ranged from $1.36 * 10^8$ to $2.83 * 10^8$ cells g^{-1}. There were no differences between the treatments (ANOVA, $p>0.05$). Together, the three investigated nitrifying bacteria accounted for 0.012 to 0.030 % of the total bacterial abundance. While the nitrite-oxidizing bacteria (*Nitrobacter* and *Nitrospira*) showed no differences between the +Cil and –Cil treatments, the ammonium-oxidizing *Nitrosomonas* were significantly more numerous in the +Cil treatments (t-test, $p=0.039$, 0.005 and 0.004, respectively). Abundances of NOB exceeded those of AOB. Total flagellate abundances ranged from $2.4 * 10^5$ to $3.6 * 10^6$ cells g^{-1}, which corresponds to biovolumes of $5.2 * 10^6$ to $2.7 * 10^7$ μm^3 g^{-1} (Fig. 3.6). The contribution of autotrophs was between 5 and 63 % in terms of abundance, but between 23 and 83 % in terms of biovolume. No significant difference between the corrsponding +Cil and –Cil treatments was found (ANOVA, $p>0.05$), but the abundance in the –Cil frozen treatment was significantly higher than the abundances in the autoclaved and natural sediments.

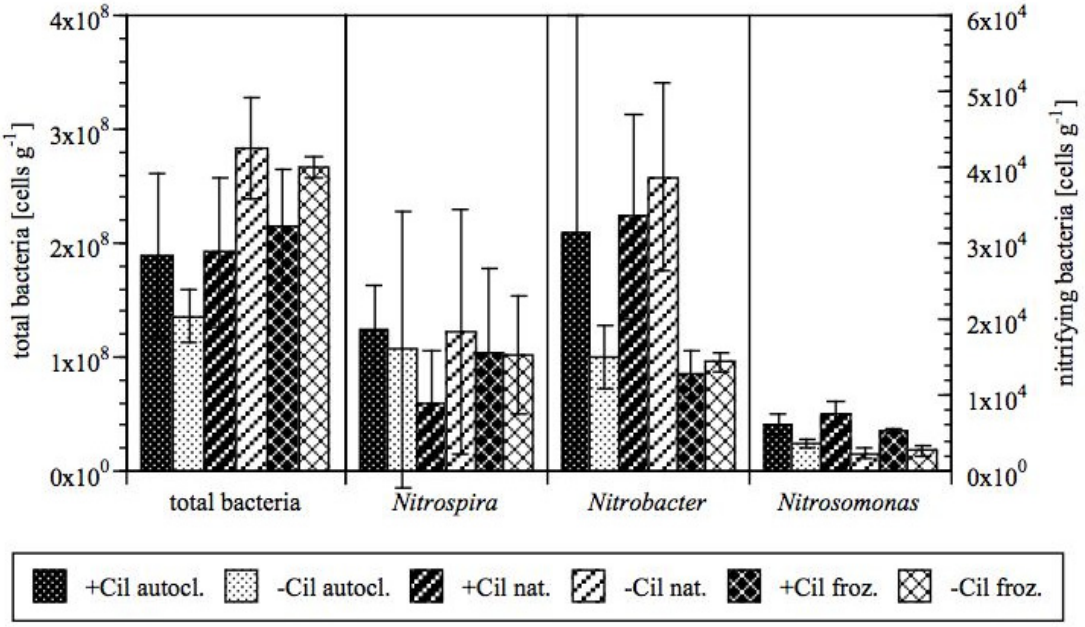

Fig. 3.5: Total bacterial abundances and abundances of the investigated nitrifying bacteria after the incubation period. Similar patterns indicate the autoclaved, natural and frozen sediments, the dark pattern corrsponds to the +Cil treatment, the lighter pattern refers to the –Cil tretament. Error bars show ± 1 standard deviation.

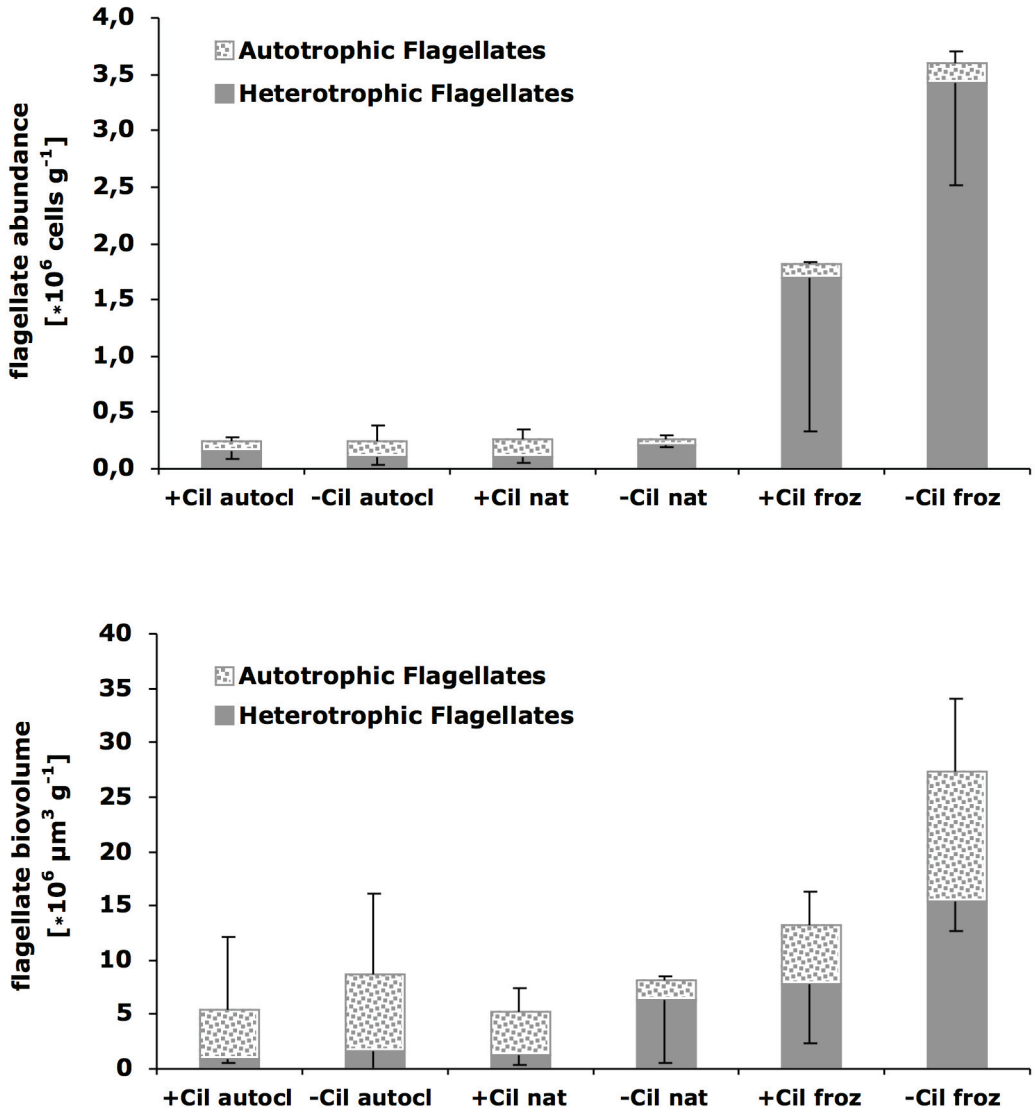

Fig. 3.6: Abundances and biovolumes of flagellates in the exerimental flumes at the end of the incubation period. Error bars show ± 1 standard deviation.

Ciliate counts

Despite the shaking procedure and the subsequent density gradient centrifugation, it was not possible to separate enough sediment particles from the ciliates. Thus, the sample volume that could be filtered onto a 1.2 µm celluloseactetate filter for the QPS method was very low before the filter was clogged, limiting the number of ciliate cells on the filter. There were only about three to five ciliate cells on a filter for the +Cil treatments and only zero to two on the –Cil treatments. Furthermore, the

high density of sediment particles on the filter made identification of the ciliates impossible. However, the numbers indicated that natural ciliate abundances were very low, but it did not allow a reliable count of ciliates or the determination of their taxonomic composition.

3.5 Discussion

The question of the study was whether an increase of ciliate abundance would lead to a change in the activity, community structure and abundance of nitrifying bacteria. Despite the low ciliate abundances in the experimental flumes, ciliate abundance did have a significant impact on the abundance of ammonium-oxidizing bacteria (*Nitrosomonas*), with higher abundances in the +Cil flumes for all three sediment treatments. This does not take into account the ammonium-oxidizing crenarcheota, which were discribed only recently during the course of our study (Könnecke et al. 2005). For the investigated nitrite-oxidizing bacteria, no trend could be observed. Other nitrifying bacteria (e.g. *Nitrospina* and *Nitrococcus*) might have been present, but are unlikely to be numerically important (Watson et al. 1981). The contribution of the nitrifying bacteria to the total bacterial abundances was very low compared to other freshwater sediments (Altmann et al. 2003). Total bacterial abundances were well within the range reported for other fluvial sediments (e.g. Gücker & Fischer 2003).

Although riverine sediments have been identified as places of high bacterial production similar to marine systems (Buesing & Marxen 2005), there was no detectable nitrification potential in the sediments used in our study, i.e. no activity of nitrifying bacteria. As the concentrations of ammonium, nitrite and nitrate were in the range reported for other fluvial sediments (Cleven & Meyer 2003), this can not be explained by a lack of substrate supply. Beside the low abundances of nitrifying bacteria, they were obviously in a state of low metabolic activity. Only in the frozen sediments, the nitrate concentration in the +Cil flume was significantly higher than in the –Cil flume, indicating higher nitrification rates with enhanced ciliate abundance. Surprisingly, our treatments of the sediments – autoclaved, natural or frozen – did

not affect the biotic or abiotic parameters measured in our study as strongly as we expected. Nitrate microprofiles for the autoclaved sediment were significantly different from all other flumes. The very low concentrations together with abundances of bacteria and flagellates, that were similar to the natural flumes, indicate that all nutrients released by autoclaving were retransformed into biomass during the incubation period. Total bacterial abundances were all in the same range, no statistical difference could be detected, neither between the +Cil and –Cil flumes of one treatment nor between the treatments. Ammonium-oxidizing bacteria showed a difference between the +Cil and –Cil flumes for all treatments, but no difference between the treatments. No differences were found for the nitrite-oxidizing bacteria. There was no special inoculation after autoclaving the sediments, but the flumes were filled with natural sediment before and were not sterilized. Nevertheless the bacterial population was back to an abundance similar to the untreated, natural sediment within the incubation period. At least for the nitrifying bacteria, there was no change in community structure. Flagellate abundances were highest in the frozen sediment, but this difference was not significant, too. As indicated by the large portion of biovolume, 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 a top-down control of benthic bacteria by heterotrophic flagellates seems unlikely (Dietrich & Arndt 2000 and literature cited therein).

Conclusion & Outlook

Despite the low abundances of ciliates, the low contribution of nitrifying bacteria and the very low nitrification potentials, we found two indications for enhanced nitrification in the presence of increased ciliate abundances. These were the higher abundances of *Nitrosomonas* in all treatments and the higher nitrate concentrations in the frozen sediment. This is consistent with the findings of Prast et al. (2007) in marine sediments. The increase in abundance and the higher nitrate concentrations as a result of increased nitrification can be explained with two effects by the ciliates. Ciliate digestion of food leads to an ammonium excretion of 0.25 – 2 $\mu g\, N\, h^{-1}\, mg^{-1}$ dryweight (Ferrier-Pagès & Rassoulzadegan 1994), which contributes to an

improved substrate availability for nitrifiers in the presence of ciliates. The movement and filtration activity of ciliates enhances the transport of solutes such as NH_4^+, NO_2^-, NO_3^- and O_2 through the interstitial spaces in the sediment (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 mostly limited to diffusion otherwise (Huettel & Webster 2001).

Furthermore, as a consequence of these results more questions have to be addressed in future studies. The interaction of ciliates and nitrifying bacteria might be influenced by factors that were not manipulated in our laboratory study. Seasonal effects need to be adressed. The results of a similar experiment with sediments collected in spring (with much higher ciliate abundances) showed that the enhancement of ciliate abundance led to significantly higher nitrate concentrations in the sediment (Prast, unbublished data), both in natural and frozen treatments. Furtheron, the role of flow velocity needs to be investigated. The effectiveness of sorption and transport processes into sediments, and thus the importance of the contribution of sediments to the total balance of matter in a river and the rivers self-purification ability, also greatly depends on the flow characteristics such as the hydraulic exchange between water and the hyporheic zone (diffusive vs. advective transport) or the boundary layer (laminar vs. turbulent flow) (Hunt & Parry 1998, Battin 2000, Huettel & Webster 2001, Battin et al. 2003, Fischer et al. 2003, Huettel et al. 2003, Fischer et al. 2005). This might also have an impact on the activity, composition and distribution of bacteria and ciliates. In our study, flow velocity was constant in all flumes and very purposely low to avoid advective transport in order to keep the experimental system as simple and controllable as possible, but further studies with varying flow velocities should be conducted. Also the abiotic sediment characteristics might be of importance. Bischoff & Prast (subm.) showed that the grain size of the sediment has an impact on nitrification in bioturbated sediments, this might also affect the interaction of ciliates with nitrification. Organic carbon content of the sediment is likely another important factor (Strauss & Lamberti 2000). Bioturbation by benthic organisms can be a major process in river habitats. Mermillod-Blondin et al. (2003, 2004) showed that aerobic (oxygen consumption) and anaerobic (denitrification and fermentative decomposition of organic matter)

microbial processes in the sediment were stimulated in the presence of bioturbators, which might be a further factor controlling the nitrifying bacteria – ciliates interaction.

4. IMPACT OF CILIATES ON NITRIFICATION AND NITRIFYING BACTERIA IN BALTIC SEA SEDIMENTS

4.1 Abstract

Nitrification in aquatic sediments is catalyzed by bacteria. While many autecological studies on these bacteria have been published, only rarely these bacteria have been regarded as part of the benthic microbial food web. Ciliates are important as grazers on bacteria, but also for remineralization of matter. We tested the hypothesis that ciliates can have an impact on 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.

4.2 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 two functionally different groups of proteobacteria: ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) (Spiek & Bock 1998) as well archaea (Könnecke et al. 2005) The latter were only recently discovered and not taken into consideration in this study, but might be quantitatively important. Ciliates are important bacterial grazers in aquatic systems (Sherr & Sherr 2002), including sediments (Epstein 1997b, Cleven 2004), with a wide array of feeding strategies. Grazing pressure of protists can have a large impact on 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.

4.3 Material and Methods

Experimental Setup

Experiments were conducted in two laboratory flumes (length: 32.4 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, approx. 54°21´N 10°09´E), close to the shoreline, sieved through a 2 mm sieve and incubated in the flumes for 40 days prior to the experiments in order to let vertical gradients re-establish. Water circulation was ~10 l h^{-1} with artificial sea water (salinity 26 ‰, temperature 20 °C), each flume had an independent water circuit. Illumination was 12/12 hours 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 (list of species found in Tab. 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 (+ ciliates treatment, +Cil). The ciliates were isolated six weeks prior to the experiments and were cultured in artificial sea water (salinity 24 ‰) in the dark at 20 °C with autoclaved wheat grains as carbon source. No nitrifying bacteria were found among

the food bacteria in the cultures. An identical volume of 1.2 µm-filtered ciliate cocktail was added to the other flume as a control (- ciliates treatment, -Cil). Some ciliates in low natural abundances were still present in the –Cil treatment, 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 three days.

Sampling procedure

All samples were taken with a sediment corer (diameter 16 mm). Bacteria, flagellate and ciliate samples were transferred into preweighted 50 ml plastic centrifugation tubes and were fixed with glutardialdehyde (GA; 2 % f.c.). Samples for nitrifying bacteria were transferred into 50 ml centrifugation tubes as well and were fixed with paraformaldehyde (4 % f.c.). For each group three 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 slurry assay (see below) with six 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 (six independent replicates per flume).

Enumeration of bacteria, flagellates and ciliates

Numbers for total bacterial and flagellate abundances were aquired using epifluorescence microscopy. Fixed samples were sonicated (Labsonic M, puls: 0.9, amplitude: 60 %, 60 sec.) in order to separate attached bacteria from sediment particles. For extraction of bacteria and flagellates, samples were then resuspended in sterile artificial seawater with 2 % GA and gently shaken by hand for 60 seconds. Afterwards, 3.5 ml of supernatant was immediately pipetted off into a 50 ml centrifuge tube. To achive a quantitative extraction, this washing procedure was repeated at least five times per sample. 200 or 250 µl of the supernatant were diluted with 1800 or 1750 µl of particle-free artificial sea water, stained with DAPI (Porter & Feig 1980), and then filtered onto a black 0.2 µm-filter (Nuclepore) for bacterial counts or onto a 0,8 µm-filter (Nuclepore) for flagellate counts, respectively.

The NOB *Nitrospira* and *Nitrobacter* and the ammonium-oxidizing β-Proteobacteria (β-AOB) were detected with fluorescence-*in-situ*-hybridization (FISH). Probes

Ntspa 712 and cNtspa712 (Daims et al. 2001), NIT3 (Wagner et al. 1996) were used for NOB and Nso1225 (Mobarry et al. 1996) for β-AOB, respectively (for details on these oligonucleotide probes see probeBase; (Loy et al. 2003). All probes were labeled with the fluorescent dye Cy3. Hybridization procedure followed a protocol described before by Pernthaler et al. (2001). Washing and filtration was identical to the procedure 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 seven times with 5 ml of sterile artificial sea water 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) and counted and taxonomically identified using a silver staining method which stains nuclei, cilia and ciliary basal bodies (Skibbe 1994). All counts were conducted with a Nikon Eclipse E800 microscope at 1000x magnification for bacteria and ciliates and 400x-1000x 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) for NO_2^--oxidation rates, respectively. $NaClO_3$ and ATU are inhibitors for the 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 continously. Sub-samples were taken from each flask after 0, 1.5, 5 and 22 h (t_0, t_1, t_2, t_3). Prior to sampling stirring and aeration were stopped for five minutes. Samples were centrifuged for 5 minutes (Heraeus Labofuge 200, 5000 rpm) 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 Stief pers. comm.). Nitrification potentials were calculated from the change in NO_2^- concentration and time.

Abiotic parameters

Temperature, pH, oxygen saturation and salinity in the water circuit were measured with a multiprobe (WTW Multimeter 350i; Table 4.1), 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, ashfree dry weight, porosity (calculated according to Hölting 1996) and grain size distribution were determined (Retsch AS 200 basic wet sieving machine; Table 4.2). All results given in g^{-1} 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). Due to the salinity of the system this was not possible.

4.4 Results

Table 4.1: Abiotic parameters in the different treatments in the experimental flumes. pH, O_2, temperature and salinity were measured with a handheld WTW Multi 350i, using a ConOX and a SenTix 41 probe. Nutrients were measured photometrically with a Bran + Luebbe AA3 autoanalyzer.

	+ Ciliates	- Ciliates
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 4.2: Sediment grain size distribution in the experimental flumes.

grain size [mm]	% 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)

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. 4.1). Abundances of the three investigated types of nitrifying bacteria *Nitrosomonas*, *Nitrospira* and *Nitrobacter* were always lower in the treatments without ciliates. 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 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, and was 54 and 60 % in terms of total flagellate abundance, but 84 and 91 % of the biovolume.

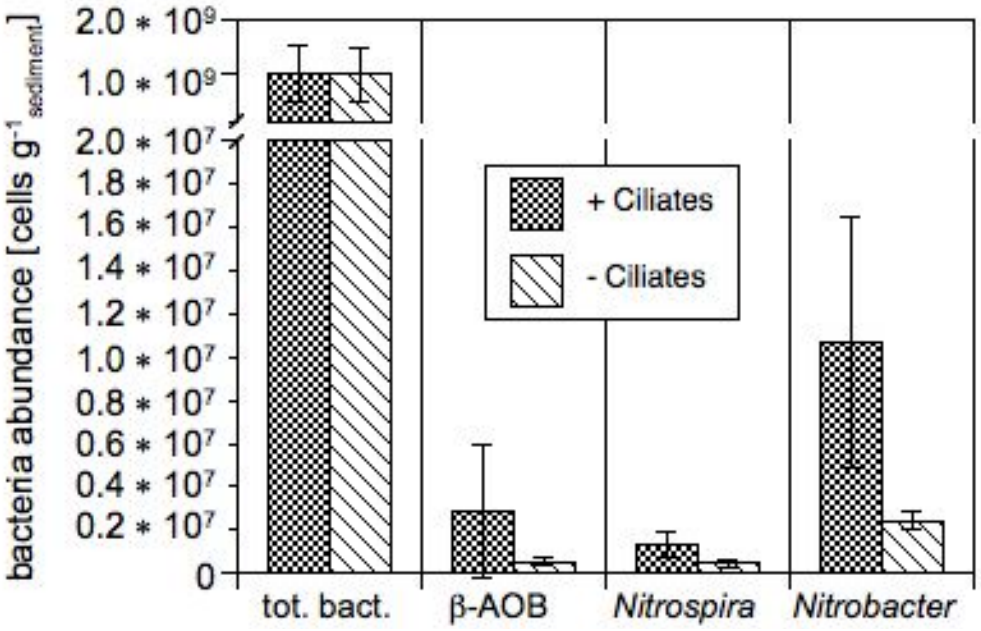

Figure 4.1: Bacterial abundances in the experimental sediments after the incubation period (error bars: ± 1 SD).

Ciliates

Ciliate abundance in the +Cil treatment was 1478 (± 578) cells g^{-1}, i. e. 52 % of the ciliates initally added to the sediment were recovered. The abundance in the -Cil treatment was 189 (± 120) cells g^{-1}, which is significantly lower (t-test, $p = 0.006$), and 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. 4.2) and was dominated by *Metacystis* spp. and *Euplotes* spp. Together these two 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 (Table 4.3).

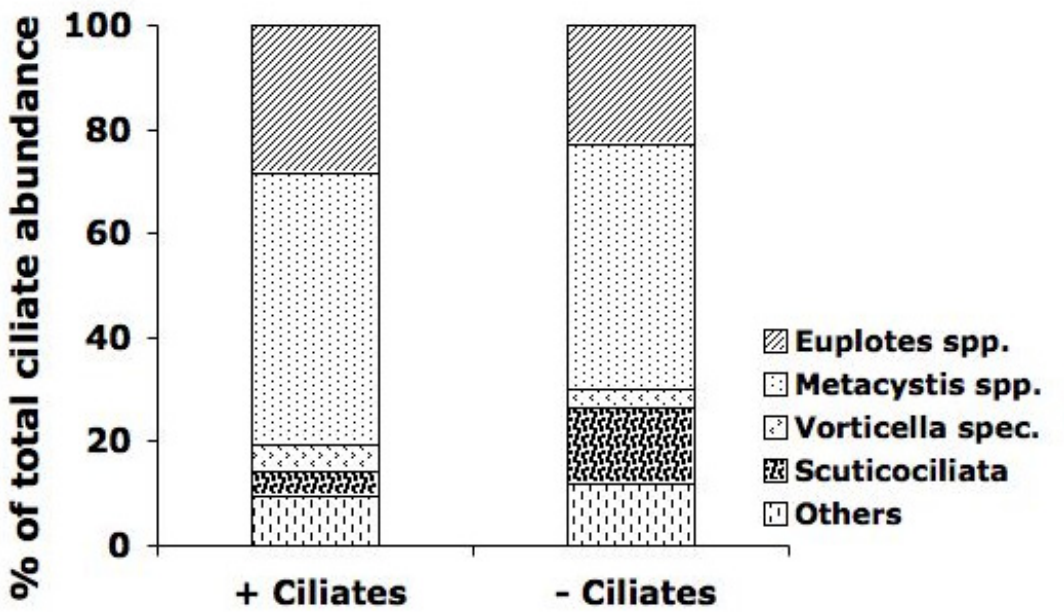

Figure 4.2: Taxonomic composition of ciliates in the experimental flumes after the three day incubation period.

Table 4.3: Taxonomic composition of ciliate community [% of total abundance]. Total abundances were 1478 (± 578) cells g^{-1} in the +Ciliates treatment and 189 (± 120) cells g^{-1} in the -Ciliates treatment.

	+ Ciliates	- Ciliates
Euplotes spp.	28.5 (± 11.8)	22.8 (± 8.6)
Aspidisca spec.	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 spec.	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 spec.	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)

Nitrification potential

Nitrification potentials ranging from 0.08 to 5.10 µg N g^{-1} h^{-1} were measured in the different treatments (Fig. 4.3). In the –Cil treatment the rates were generally lower than in the +Cil treatment (except for nitrite oxidation t_1-t_2). The difference was significant for ammonium oxidation t_1-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-t_1, t_1-t_2 and t_2-$_3$, 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-t_1 caused a depletion of the substrates and consequently the rates measured in the intervals t_1-t_2 and t_2-t_3 were much lower and not included in our further analysis.

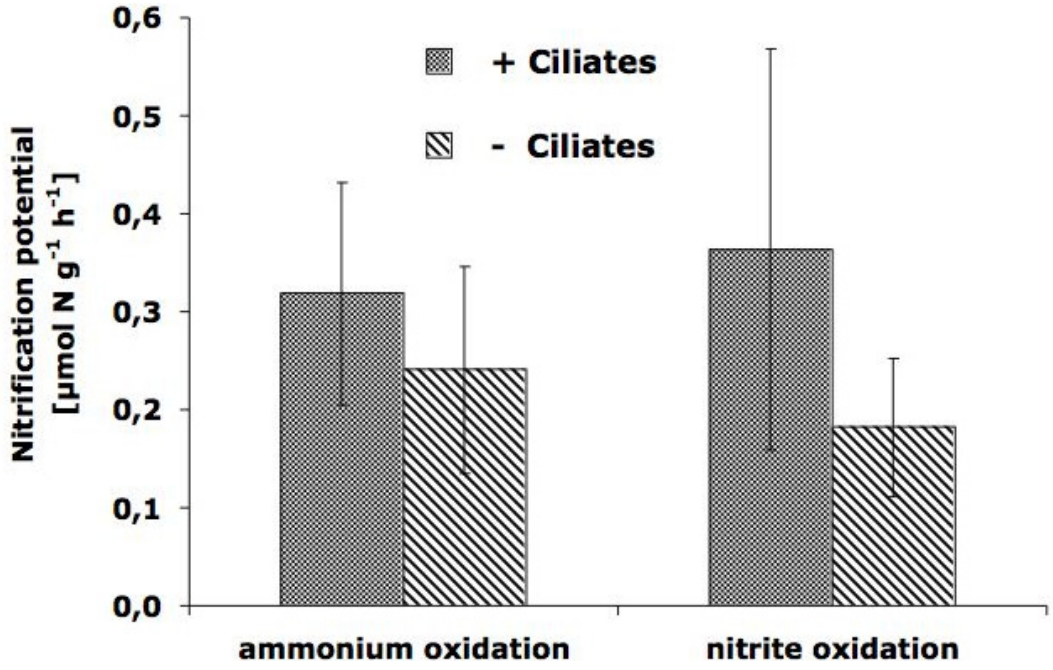

Figure 4.3: Nitrification potentials of the sediments in the experimental flumes after the incubation period, as determined with the slurry assays. Rates from t_0-t_1 are shown. Due to the high rates the substrates in the slurry assay were consumed very quickly and the rates from t_2 and t_3 were not used for further analysis (t_1=1.5 h; error bars: ± 1 SD).

4.5 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 and Dodds (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. subm.) can be excluded. We conclude that the presence of ciliates, especially in high abundances, enhances transport procesess 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 – 2 µg N h^{-1} mg^{-1} dryweight (Ferrier-Pagès & Rassoulzadegan 1994), which further contributes to an improved substrate availability for nitrifiers in the presence of ciliates. Lavrentyev et al. (1997) showed that in a planktonic systems ciliate grazing pressure will lead to 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 the treatments with or without ciliates, indicating that grazing pressure from ciliates was rather low or that grazing losses of bacteria and flagellates were compensated by increased growth, accompanied by a change in community composition. As indicated by the large portion of biovolume, flagellate community was dominated by large phototrophic flagellates, heterotrophic flagellates were rather small in size. Other studies have shown that – in opposition to planktonic systems – in sediments ciliates might be more important as bacterial grazers than heterotrophic flagellates and a top-down conrtol 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. Besides bacteria and flagellates, diatoms were found in our systems. Presumably they were too large to serve as prey for the ciliate

species in our sediments. As the ciliate abundance was eightfold increased after the incubation period in the +Cil treatment, we assume that ciliate grazing pressure on bacteria and flagellates was rather high. This is consistent with the ciliate community composition, 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 substrate for nitrification is the main excretion product of ciliates (Ferrier-Pagès & Rassoulzadegan 1994). Another explanation for the increase in nitrification potentials and 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 mostly limited to diffusion otherwise (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. Furtheron, it cannot be completely ruled out that the use of other ciliates 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. 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 impact on the nutrient level and can be ruled out as a source of the increased nutrient supply in the +Cil treatments.

Total bacterial abundances were well within the range of abundances reported for other marine and freshwater sediments (Llobet-Brossa et al. 1998, Kuwae & Hosokawa 1999, Altmann et al. 2004a). In some studies bacteria of the genus *Nitrospira* have been identified as the dominating nitrite oxidizing bacteria (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 by more than fivefold in the –Cil treatment and eightfold in the +Cil treatment. 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 the experiments a new ammonia-oxidizing crenarchaeota was discribed (Könneke et al. 2005). Future experiments should consider also the presence and abundance of this new type of ammonia-oxidizing microorganisms, which might show a different reaction to ciliate grazing.

Conclusions

Ciliates have an impact on the abundance of nitrifying bacteria in sediments and hence on the nitrification potential. The reason for the increased abundances of nitrifyers and nitrification potentials in the presence of ciliates probably is nitrogen recycling, that leads to an enhanced nitrogen availability for nitrifying bacteria. This indicates that nitrifying bacteria are rather subject to bottom-up control by substrate availability than top-down controlled by ciliate grazing. Ciliate grazing did not negatively affect the nitrifying bacteria, although Lavrentyev et al. (1997) and Neubacher et al. (subm.) showed that they are ingested by ciliates. Another possible

explanation for the increased abundances of nitrifying bacteria and nitrification potentials in the +Cil treatments might be an increased transport of substrates and oxygen in the sediments due to movement and filtration activity 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,too.

5. IMPACT OF *NEREIS DIVERSICOLOR* (O.F. MUELLER, 1776) ON NITRIFICATION AND NITRIFYING BACTERIA IN TWO TYPES OF SEDIMENTS

5.1 Abstract

Nitrification is a microbial process which is catalyzed by bacteria. While numerous autecological studies on these nitrifying bacteria are published, only few publications consider bacteria involved in the nitrification process as an important part of the benthic microbial food web. We tested the hypothesis that the polychaete *Nereis diversicolor*, described as an organism able to feed on bacteria, might influence the nitrification potential. The effects can be direct by actively changing the bacterial community or indirect by bioturbation and therefore enlarging the surface layer and supplying substrate to the bacteria. Experiments with two types of sediments in laboratory flumes, with and without the addition of *N. diversicolor*, were conducted. Higher bacterial abundances in the presence of worms and changes in nitrifying bacteria between treatments for the fine sediment were found. The grain size distribution of the sediment seems to have an impact on the nitrification potential and abundance of bacteria.

5.2 Introduction

The nitrogen cycle is one of the most important biogeochemical cycles as nitrogen is an essential nutrient for all organisms. Various types of prokaryotes are responsible for transformations within the nitrogen cycle such as nitrogen fixation, nitrification, nitrate reduction or denitrification (Ramaiah 2005). A lot of studies dealing with these bacteria have been conducted in the past, but only few considered them as part of a food web, in which these bacteria have to compete for nutrients/substrates with other organisms, such as benthic microalgae or ammonia oxidizing crenarchaeota (Risgaard-Petersen et al. 2004; Koenneke et al. 2005), or in which they might be a prey for other animals (Verhagen & Laanbroek 1992).

This study focussed on nitrifying bacteria in sediments of the Baltic Sea and their interaction with a benthic macroinvertebrate, the polychaete *N. diversicolor*. Nitrification is the sequential oxidation of ammonium (NH_4^+) to nitrite (NO_2^-) and nitrate (NO_3^-) and is predominantly accomplished by chemolithotrophic bacteria under oxic conditions. This process is a dominant metabolic pathway in the water column and the upper layer of aquatic sediments. The nitrifying bacteria consist of two functionally different groups of proteobacteria: ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) (Spiek & Bock 1998). The ammonia oxidizing crenarchaeota (Koennecke et al. 2005) were only discovered during the course of this study and, thus, were not taken into account, but they should be considered in further studies.

Deposit feeding organisms, such as polychaetes are described as important bacterial grazers in aquatic sediments (Grossmann & Reichardt 1991; Sherr and Sherr 2002; Lucas et al. 2003). Plante et al. (1989) concluded that grazing pressure of polychaetes had an effect on bacterial activity and alternates the community structure in the sediment. Hence polychaete grazing might also affect the metabolic pathways in which bacteria play an essential role. The aim of this study was to find out whether an in-fauna macroinvertebrate could have an impact on the nitrification in Baltic Sea sediments by grazing pressure and / or bioturbation. A laboratory experiment was conducted using flumes with natural sediments with and without the addition of *N. diversicolor* to analyze the effects on the abundance and taxonomic composition of nitrifying bacteria in the sediments and their nitrification potential. This polychaete species was selected because it is known as an important bioturbator of shallow sediments and it is an indigenous species of the sediments that were used for the experiment. *N. diversicolor* inhabits sediments such as sandy mud or gravel and is able to adapt to a wide variety of environmental conditions, including salinity, temperature and oxygen availability.

5.3 Material and Methods

Experimental Setup

Experiments were conducted in four laboratory flumes (length: 32.4 cm, width: 17.4 cm, height: 18.7 cm, depth of sediment layer ~7.0 cm). Natural sediments were collected from the Baltic Sea (Bay of Kiel, approx. 54°21´N 10°09´E) and after cleaning and drying for 48 hours at room temperature incubated in the flumes for 30 days prior to the stocking of the worms. Two types of sediment were used. Sediment 1, the "fine sediment", had a grain size of 0 – 2 mm and sediment 2, the "coarse sediment", had a grain size of 0 – 4 mm (Tab. 5.1). Water replacement per flume was ~10 l h^{-1} with artificial sea water (salinity ~29 psu, temperature ~20 °C). All flumes were connected to form a closed recirculating system which also included a basin for water collection, a biofiltration unit and a pump for water circulation. Light regime was 12:12 h light/dark with artificial light (~25 µE $m^{-2}s^{-1}$).

Tab. 5.1: Grain size distribution of both sediment types. Numbers display means ± standard deviation (n = 6). For the fine sediment the major fraction was in the range of 0.49 and 0.125 mm, whereas for the coarse sediment the largest part exceeded 0.5 mm.

Grain size [mm]	*% portion*	
	fine sediment	coarse sediment
> 0.5	32.2 (± 3.7)	96.6 (± 0.9)
0.49 – 0.125	63.1 (± 3.4)	0.4 (± 0.3)
0.125 – 0.063	1.1 (± 0.6)	0.0 (± 0.0)
0.063 – 0.025	0.3 (± 0.3)	0.1 (± 0.0)
< 0.025	3.3 (± 0.7)	2.9 (± 0.6)

All worms used during this experiment were first generation offspring, reared from wild caught individuals, in a laboratory recirculating system. They were chosen randomly and transferred to one flume of each sediment type. The flumes stocked with worms were referred to as +worm treatments. The second flume of each sediment type was used as a control in the absence of *N. diversicolor* and these flumes were referred to as –worm treatments. Both treatments with worms were stocked 40 days prior to the measurements with 30 individuals per flume resulting in calculated initial densities of approx. 1000 individuals m^{-2}, which is according to Davey (1994) close to natural densities of adult *N. diversicolor*.

Sampling procedure

All samples of the fine sediment were taken with a plastic sediment corer (∅ 1.6 cm, total length 8.2 cm) by drilling the corer into the sediment, sealing the upper end and pulling the filled corer out of the sediment. Samples for total prokaryotic abundance were transferred into pre-weighed 50 ml centrifugation tubes and were fixed with glutardialdehyde (GA; 2% f.c.). Samples for the analysis of nitrifying bacteria were transferred into a second set of 50 ml centrifugation tubes and were fixed with paraformaldehyde (4% f.c.). From each of the four flumes used for the experiment three samples from different locations per flume were collected. During the complete acclimatisation phase no worm mortality was observed. Sampling locations were selected randomly not considering the location of worm burrow entrances assuming a complex net of burrows within the entire sediments as described by Davey (1994).

Samples from the "fine sediment" for the determination of the nitrification potential were collected with the same type of corer but transferred directly into Erlenmeyer flasks used for a slurry assay (see below) with six replicates per flume. Samples from the "coarse sediment" were collected with a spoon and processed analogously to the samples from the fine sediment. The change of sampling procedure was necessary because it was not possible to collect sufficient amounts of coarse sediment with the sediment corer used for the fine sediment. Samples collected with the spoon included all depth horizons of the sediment.

All samples for the determination of abiotic parameters such as porosity, ash free dry weight and grain size were also collected with a spoon. Wet weights of sample material were recorded before the sediment samples were dried at 60 ± 5 °C for 24 hours. Six replicates per flume were sampled.

Prokaryote counts

Numbers for total bacterial abundances were obtained using the DAPI-method (Porter & Feig 1980). Samples were sonicated in order to remove attached bacteria from sediment grains (Labsonic M, puls: 0.9, amplitude: 60%, 60 s). Samples were resuspended in sterile artificial seawater with 2% GA and gently shaken by hand for 60 seconds. Afterwards, 3.5 ml of supernatant were immediately pipetted off into a 50 ml centrifuge tube. To achieve a quantitative bacteria removal from particles, this washing procedure was repeated at least five times per sample. 200 or 250 µl of the

supernatant, diluted in 2 ml of particle free artificial seawater, were then filtered onto a black 0.2 µm-filter (Nuclepore) for prokaryotic counts.

The NOB *Nitrospira* sp. and *Nitrobacter* sp. as well as the ammonium oxidizing β-proteobacteria (β-AOB) were detected with fluorescence-*in-situ*-hybridization (FISH). Probes Ntspa712 and cNtspa712 (Daims et al. 2001), NIT3 (Wagner et al. 1996) were used for NOB and Nso1225 (Mobarry et al. 1996) for β-AOB, respectively (for details on these oligonucleotide probes see probeBase; (Loy et al. 2003)). All probes were labeled with Cy3. Hybridization procedure followed the protocol by Pernthaler et al. (2001). Washing and filtration was identical to the procedure applied for the DAPI-method except that white 0.2 µm-filters (Nuclepore) were used. All counts were conducted with a Nikon Eclipse E800 microscope.

Nitrification potential (Slurry assay)

Ammonia oxidizing potential and nitrite oxidizing potential were analyzed by performing two different approaches. A total number of twelve Erlenmeyer flasks for each oxidizing approach were used. This total number was divided into different groups such as sediment types, “fine” and “coarse”, and sediments stocked with worms and sediments without worms. For each set three replicates were analyzed for each sampling time.

Sediment samples were slurried by adding filtered artificial seawater (~6.5 g sediment + 100 ml 0.2 µm-filtered artificial seawater per flask) and placed into 200 ml Erlenmeyer flasks. All flasks of the ammonia oxidizing group were supplied with NH_4^+ (50 µM) and $NaClO_3$ (20 mM) for analyzing the NH_4^+-oxidation rates. All flasks of the nitrite oxidizing group were supplied with NO_2^- (25 µM) and allylthiourea (10.0 mg/l) for analyzing the NO_2^--oxidation rates. The Erlenmeyer flasks were aerated via an air pump and stirred with a magnetic stirrer. Samples were taken out of each flask after 0, 1.5, 5 and 22 h (t_0, t_1, t_2, t_3). Prior to collecting one sample of 10 ml per flask by using a pipette, stirring and aeration were stopped for five minutes. Samples were centrifuged for five minutes (Heraeus Labofuge 200, 5000 rpm) to remove remaining sediment particles. NO_2^- concentrations were measured photometrically using a Bran + Luebbe AutoAnalyzer (AA3) (Dollhopf et al. (2005), Stief pers. comm.).

Abiotic parameters

Temperature, pH, oxygen saturation and salinity in the water circuit were measured with a multiprobe (WTW Multimeter 350i; Tab. 5.2), NO_2^- *in situ* concentrations were measured photometrically (Bran+Luebbe, AutoAnalyzer AA3). Dry weight, total organic matter (TOM), porosity (calculated according to Hölting 1996) and grain sizes were determined (Retsch AS 200 basic wet sieving machine). All results given in g^{-1} refer to dry weight. SigmaXL (Version 2000) and SPSS (Version 11.0) software were used for statistical analysis. Data were tested for normality (Kolmogorov-Smirnov test) and for homogeneity of variances (Levene's test) prior to analysis. t-tests and analyses of variances (ANOVA), followed by Tukeys HSD posthoc tests were performed with the data.

5.4 Results

Abiotic parameters

Except sediment porosity all other abiotic parameters did not differ significantly between treatments (Tab. 5.2).

Tab. 5.2: Abiotic parameters (means ± SD) measured *in situ* within the experimental flumes. For pH, oxygen saturation, temperature and salinity n = 5, the number of replicates for sediment porosity and TOM was n = 3.

	fine sediment		*coarse sediment*	
	+ worms	- worms	+ worms	- worms
pH	8.15 ± 0.09	8.18 ± 0.06	8.18 ± 0.05	8.19 ± 0.05
oxygen saturation [%]	83.0 ± 5.8	82.8 ± 5.8	88.3 ± 3.4	88.5 ± 3.6
Temperature [°C]	19.4 ± 0.3	19.4 ± 0.3	19.4 ± 0.3	19.4 ± 0.3
Salinity [psu]	29.5 ± 0.6	29.5 ± 0.6	29.5 ± 0.6	29.5 ± 0.6
Sediment porosity	24.4 ± 0.7	25.5 ± 0.6	18.4 ± 1.4	19.1 ± 2.7
TOM [%]	0.22 ± 0.06	0.20 ± 0.03	0.32 ± 0.04	0.37 ± 0.05

Bacteria

Over all treatments, total bacterial abundances ranged from 9.4 x 10^7 to 2.5 x 10^8 cells g^{-1}. Numbers differed significantly between treatments for the fine sediment with and without worms (Tab. 5.3; Fig. 5.1). The bacterial abundance for the treatment with worms present was on average almost twice as high as the abundance in the treatment without worms. For the coarse sediment the difference between treatments was not statistically significant. For this sediment in the +worm treatment one bacterial count showed lower values compared to the other two counts. Comparing the two types of sediment bacterial abundance differed significantly in the absence of polychaetes (Tab. 5.3; Fig. 5.1) showing higher values for the coarse sediment.

Tab. 5.3: t-test results from total bacterial abundances in the two types of sediment with and without worms.

	Levene's test		*t-test*		
	F	Sig.	*df*	t	Sig. (2-tailed)
fine sediment					
+worms vs. –worms	5.778	0.074	4	6.651	**0.030**
coarse sediment					
+worms vs. –worms	8.388	0.044	4	0.649	0.552
worms present					
fine sediment vs. coarse sediment	5.489	0.079	4	0.362	0.735
worms absent					
fine sediment vs. coarse sediment	0.136	0.731	4	-7.616	**0.002**

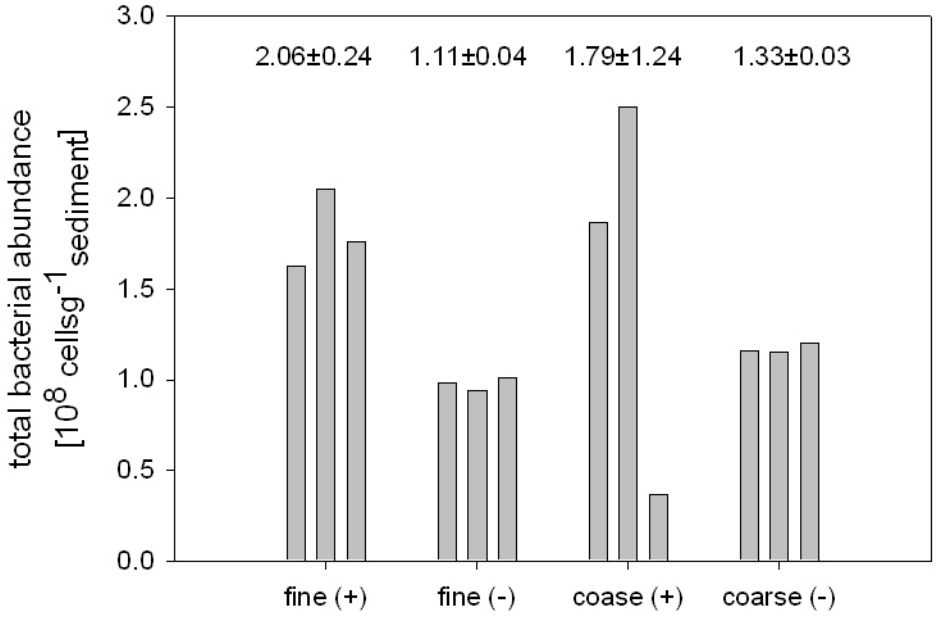

Fig. 5.1: Total bacterial abundances obtained from all treatments. Fine and coarse represent the type of sediment used during the experiment; (+) and (-) represent the presence or absence of *N. diversicolor*. Bars refer to the abundances counted for each replicate. Figures displayed above the bars represent the means ± standard deviations of the corresponding treatment.

Abundances of the three investigated types of nitrifying bacteria (*Nitrospira* sp., *Nitrobacter* sp. and β-AOB) differed among treatments: In the fine sediment abundances were lower in the treatment without polychaetes but in the coarse sediment abundances were higher in the treatment without polychaetes. Combined the investigated nitrifying bacteria contributed 4.65 and 3.80% of total bacteria in both treatments of the fine sediment. For the coarse sediment they accounted for 1.85 and 2.08% in both treatments, respectively. The abundances of the three types of nitrifying bacteria differed within the treatments (Fig. 5.2) and for *Nitrospira* in the fine sediment this difference was statistically significant (Tab. 5.4; Fig. 5.2). The total abundance of *Nitrospira* sp. increased in the presence of *N. diversicolor*, as well as the relative abundance did. The total and relative abundance of *Nitrobacter* sp. doubled in the presence of the worms. The situation for β-AOB was different. In the absence of the worms total and relative abundance of these bacteria were significantly higher (Tab. 5.4; Fig. 5.2). For the coarse sediment no differences were detected.

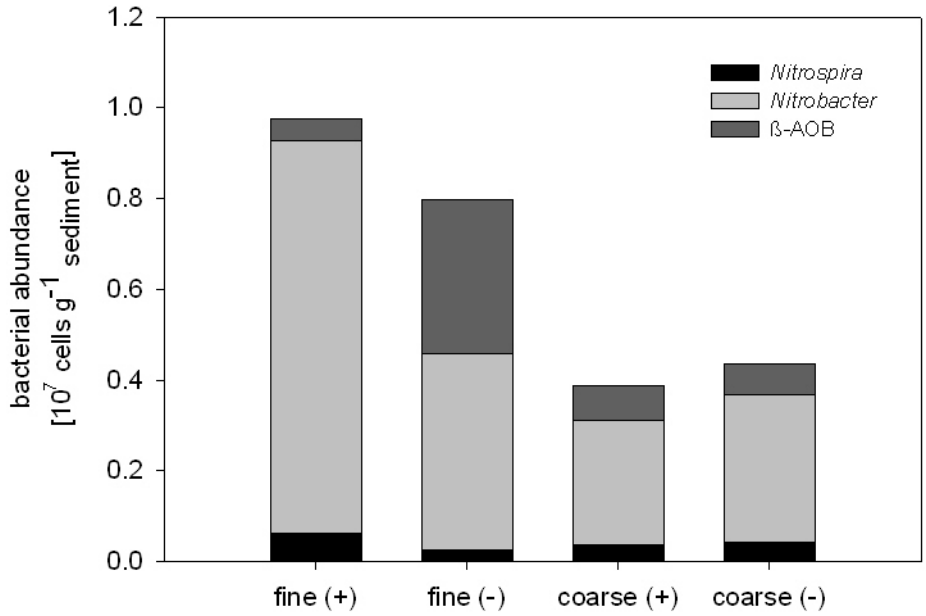

Fig. 5.2: Nitrifying bacteria detected by FISH. For *Nitrospira* Ntspa 712 and cNtspa 712 probes were used. The probes NIT3 and Nso1225 were used for *Nitrobacter* and β-AOB, respectively. Fine and coarse represent the type of sediment used during the experiment and (+) and (-) refer to the presence or absence of *N. diversicolor*.

Tab. 5.4: One-way ANOVA results from the nitrifying bacteria.

	df	MS	F	Sig.
Nitrospira				
Between groups	3	0.067	4.854	**0.033**
Within groups	8	0.014		
Total	11			
Nitrobacter				
Between groups	3	21.794	2.163	0.170
Within groups	8	10.076		
Total	11			
ß-AOB				
Between groups	3	5.784	8.219	**0.008**
Within groups	8	0.704		
Total	11			

Nitrification potential

For both sediments nitrification potentials were measured at different time intervals and the rates $t_0 - t_3$ as results of linear regression are presented.

Fine sediment:

Nitrification potential rates for the fine sediment ranged from 0 to 0.63 µg N g^{-1} h^{-1} (Fig. 5.3).

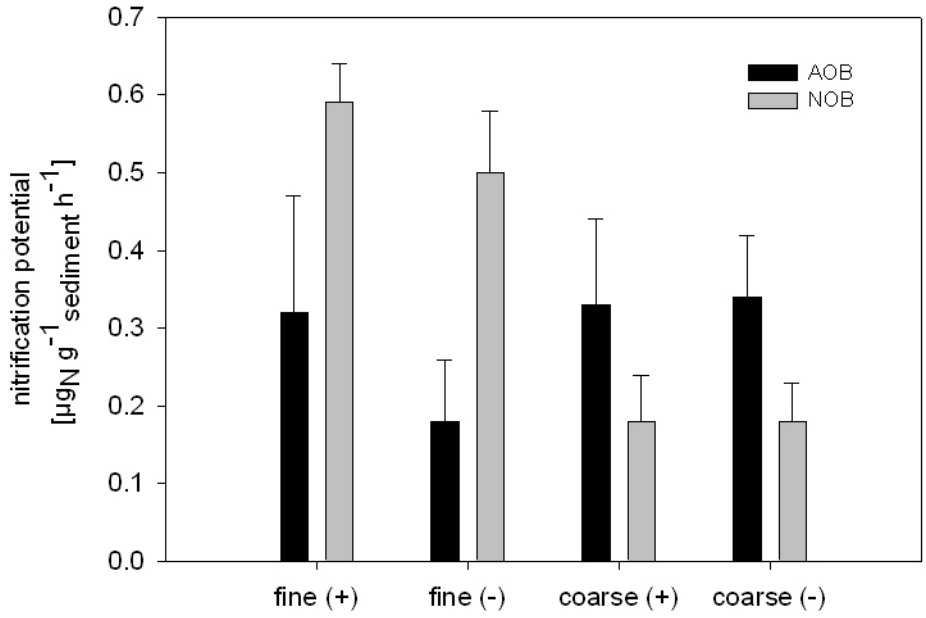

Fig. 5.3: Nitrification potentials (AOB = ammonia oxidizing bacteria; NOB = nitrite oxidizing bacteria) Fine and coarse represent the type of sediment used during the experiment and (+) and (-) refer to the presence or absence of *N. diversicolor*. Bars present means + standard deviation (n = 3).

In the treatment without worms the rates were on average lower than in the treatment with worms. Rates for ammonia oxidation were 0.32 ± 0.15 µg N g^{-1} h^{-1} for +worm treatments and 0.18 ± 0.08 µg N g^{-1} h^{-1} for –worm treatments, respectively. Nitrite oxidation rates were 0.59 ± 0.05 µg N g^{-1} h^{-1} for +worm and 0.50 ± 0.08 µg N g^{-1} h^{-1} for –worm treatments, respectively. No statistical differences for ammonia and nitrite oxidation between the treatments with and without worms were detected.

Coarse sediment:

For the analyzed coarse sediment the nitrification potentials ranged from 0.14 to 0.45 µg N $g^{-1}h^{-1}$ (Fig. 5.3). Ammonia oxidation rates were higher compared to nitrite

oxidation rates. Rates for ammonia oxidation ranged from 0.22 to 0.45 µg N g^{-1} h^{-1} and were 0.33 ± 0.11 µg N g^{-1} h^{-1} for +worm treatments and 0.34 ± 0.08 µg N g^{-1} h^{-1} for –worm treatments, respectively. Nitrite oxidation rates ranged from 0.14 to 0.25 µg N g^{-1} h^{-1}. Rates were 0.18 ± 0.06 µg N g^{-1} h^{-1} for +worm and 0.18 ± 0.05 µg N g^{-1} h^{-1} for –worm treatments, respectively. No significant differences for ammonia and nitrite oxidation between the treatments with and without worms were detected.

5.5 Discussion

Bacterial abundance

Total bacterial abundances were well within the range reported for other marine and freshwater sediments (Llobet-Brossa et al. 1998; Kuwae & Hosokawa 1999, Altmann et al. 2003). The presence of *N. diversicolor* did obviously enhance the bacterial abundance in both types of sediments, though the differences on flumes without worms were statistically significant for the fine sediment only. This was due to high variation of bacterial numbers in the coarse sediment. Including all data into the analyses for total bacterial abundances of the coarse sediment led to no statistically significant differences between treatments with and without worms. Considering the low count of total bacterial abundances as an outlier would change the situation severely.

It could be expected that due to dense sediment layers, caused by fine sediments, dissolved nutrients are only available at the sediment surface. Caused by an enhancement of surface area (Fenchel 1996) and the active water movement caused by the worms (Riisgard et al. 1996) nutrient as well as oxygen availability for bacteria should be increased especially in fine sediments. Kristensen (1984) suggested nutrient recycling by bacteria for ammonia excreted by *Nereis virens*. Such a nutrient recycling was also reported by Reichardt (1988) for psychrophilic bacteria. Predation pressure of *N. diversicolor* on bacteria might further keep bacteria in the exponential growth phase and consequently enhance bacterial abundance. Nevertheless, no statistically significant effect of the sediments was revealed.

Taxonomic composition of nitrifying bacteria

In some studies bacteria of the genus *Nitrospira* have been identified as the dominating nitrite oxidizing bacteria (NOB) in marine and freshwater systems (Hovanec et al. 1998, Altmann et al. 2003). In contrast, in our study bacteria of the genus *Nitrobacter* were the most important NOB, differing significantly from *Nitrospira* by factors of more than 14 and 16 in the fine sediment and more than 7 in the coarse sediment. In total they accounted for 50 – 89% of all 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). The β-AOB were found in significantly higher abundances in the absence of worms for the fine sediment. This is unexpected considering the ammonia excretion of the worms implying increased nutrient availability in the presence of the worms. The β-AOB are gram-negative as most of the sediment surface bacteria (Moriarty & Hayward 1982). Lucas and Bertru (1997) described lytic activity for gram-negative bacteria in extracts of the digestive tract of *N. diversicolor*. Plante & Mayer (1994) stated that deposit-feeders may grow on a diet of bacteria. Grossman & Reichardt (1991) showed that the digestive removal of bacterial carbon could attain 95% for *Arenicola marina*. Harvey & Luoma (1984) supposed that organic matter gained from bacteria such as exopolymeres and cellular envelopes may be of trophic importance. Therefore, it could be assumed that the feeding and digestion of β-AOB by *N. diversicolor* could be the explanation for the reduced abundance of ammonia oxidizing bacteria and therefore the alteration of the nitrifying bacterial community. In contrast to this assumption is that *Nitrospira* sp. and *Nitrobacter* sp. are also gram-negative bacteria and therefore the proportions of bacteria should have been similar. The location of the β-AOB could be a possible explanation, so that the bacteria could be more accessible for the worms.

Nitrification potential

The increased numbers of nitrifying bacteria in the presence of *N. diversicolor* for the fine sediment should also have led to increased nitrification potentials. Significantly higher ammonia and nitrite oxidation rates could not be detected. This is in contrast to findings of Kristensen (2000) and Heilskov & Holmer (2001). Yingst & Rhoads (1980) suggested that feeding of bioturbating invertebrates on the

microbial community in their close vicinity keep these bacteria in an active physiological state, explaining the elevated nitrification potentials in the presence of *N. diversicolor* for the fine sediment. For the coarse sediment ammonia oxidation potential exceeded nitrite oxidation potential. These findings are in contrast to natural sediments where higher nitrite oxidation potentials compared to ammonia oxidation potentials are commonly found (Schwoerbel 1999). Interactions of denitrification with other processes of the nitrogen cycle such as anammox could be an explanation. According to Dalsgaard et al. (2003) the requirements for anammox, the anaerobic bacterial oxidation of ammonia with nitrite, are nitrate rich waters and anoxic conditions. These conditions might occur within the flumes and may have reduced nutrients available for nitrite oxidizing bacteria. The reduced nutrient availability could have led to nitrite oxidizing bacteria at a low active physiological state. Alteration of this physiological state can only be achieved by increased nutrient concentrations over a certain time period and the time available for the slurry assays was too short to complete this change. Syakti et al. (2006) reported lag phases of two days for two strains of bacteria and Dean (1957) showed that bacteria which remained longer in liquid media had a decreased lag phase on plates.

Conclusions

N. diversicolor causes a bioturbation effect by its burrowing behaviour which increases the total bacterial abundance. It may alter the nitrifying bacterial community and hence the nitrification potential of sediments inhabited by the rag worms is increased. This effect is influenced by the type of sediment. Bioturbation caused by worms increases nitrification potential until a certain grain size is exceeded, afterwards this effect diminish. The presented study concentrated on the effects caused by macrobenthic organisms on bacterial abundances, community structures and nitrification potentials, further research is required to reveal the complex influence of *N. diversicolor* on benthic microbial food webs such as ingestion and digestion of different bacterial strains.

6. CONCENTRATION OF FIXED PLANKTON SAMPLES VIA SETTLING: HOW LONG IS LONG ENOUGH?

6.1 Abstract

Enumerating plankton cells, especially ciliates, usually requires settling samples in order to concentrate the cells. The time sufficient to settle all ciliates has, however, never been established. Therefore an improvement of this method, which mostly relied on experience regarding the settling times until now, was an important part of this work. Ciliate density was used to calculate the theoretical settling time of fixed ciliates with the Stokes equation. To determine ciliate density (g ml^{-1}) we modified and established a density gradient centrifugation method. We found that ciliate density was in the range of 1.02-1.08 g ml^{-1}. Additionally, empirical sinking rates were gathered semi-automatically with a digital camera system. The theoretical and experimental settling times were within the same range, though there were clear differences for some species. Therefore we recommend working with the empirical data that were more reliable: 1.7 mm min^{-1} for fixed marine (salinity 41‰) ciliate samples and 2.4 mm min^{-1} for fixed freshwater samples. Using these rates means saving up to 95% of time for settling compared to old, experience derived times. We found that marine samples had significantly higher settling times than freshwater samples. Though ciliate density was significantly correlated with settling rates, there was no correlation with particle size and shape.

6.2 Introduction

The sinking velocities of unfixed cells or particles have been addressed several times, mostly regarding phytoplankton, marine snow or other particles (e.g. Hamm 2002; Kiorboe et al. 2002; Kiorboe et al. 2003; Ptacnik et al. 2003; Peterson et al. 2005). Especially for fixed phytoplankton the Utermöhl method (Utermöhl 1958) is long known and used but data regarding the duration of settling of the fixed samples rely mostly on experience or on estimation. For phytoplankton at least few studies were conducted to achieve reliable sinking velocities (Padisak et al. 2003 and literature cited therein). The Utermöhl method is regularly used for ciliates but as far as we know, there is no data available for the exact settling time, though this is the critical point for obtaining reliable abundances.

In our study, experimentally collected data about sinking velocities were compared with theoretical data, following the Stokes equation. We determined ciliate densities, cell size, and cell shape from freshwater and marine ciliate cultures with strongly different salinity to calculate the theoretical sinking velocities. Empirical sinking velocities were gathered semi- automatic with a digital camera system. We expected to find (1) an increase in sinking velocity with an increase in cell size / diameter, (2) that differences in cell shape compared to spherical forms affect the sinking velocity and finally (3) that marine samples will settle more slowly than freshwater samples due to the increased density in sea water.

6.3 Material and Procedures

Ciliate cultures

We used seven ciliate cultures, five freshwater cultures and two from marine systems. *Tetrahymena pyriformis* and *Colpidium colpoda* were isolated from the River Salzach (Austria), *Cyclidium glaucoma* was provided by the University Bielefeld (M. Bergtold; Germany), *Paramecium aurelia* was obtained from CCAP (England), *Euplotes octocarinatus* was provided from the University Stuttgart (H.-D. Görtz; Germany). The marine cultures were assemblages of several species, of different size and cell shape, which were grown from natural, prefiltered (100 µm) seawater. Marine ciliates are extremely sensible to changes in the environment and therefore we could not grow single species cultures but only the assemblages. The Baltic Sea cultures were isolated from the Bay of Kiel (Germany) and the Red Sea ciliates were isolates from the Gulf of Aqaba. Freshwater ciliate stocks were cultured in a 1:1 mixture of Volvic mineral water and SMB-medium (1.5 mM NaCl, 0.05 mM KCl, 0.4 mM $CaCl_2 * 2\ H_2O$, 0.05 mM $MgCl_2 * 6\ H_2O$, 0.05 mM $MgSO_4 * 7\ H_2O$, 2.0 mM sodium phosphate buffer, pH 6.8). Ciliates from the Baltic Sea and the Red Sea were cultured in artificial seawater with 16 and 40 ‰ salinity, respectively.

Determination of ciliate density in terms of g ml^{-1} (Fig. 6.1)

Ciliate density was determined with a density gradient centrifugation. Ciliates were fed 30 minutes with black Indian ink to increase visibility of the ciliate band after the

density gradient centrifugation. The ingested ink particles were not expected to change cell density, as the Ink was just a carbon source having the same effect as other ingested particles. Thereafter the ciliates were fixed with glutardialdehyde (2 % f.c.). Glutardialdehyde was chosen as in other fixatives acids are used, e.g. in Bouin´s (formaldehyde saturated with picric acid with 20 % glacial acetic acid), which lead to precipitation of the Percoll that was used for the centrifugations. Cell shrinkage is a well-known phenomenon after fixation, with different degrees of shrinkage depending on the fixative used (Leackey et al. 1994; Stoecker et al. 1994) what might increase cell density and so affect the settling velocity. The cell shrinkage is lower with Glutardialdehyde than with Bouin´s solution, the latter is widely used for fixation of ciliates. Thus the settling velocity in Bouin`s samples should be even higher than the values we observed, due to the expected higher density. After fixation the cultures were concentrated via centrifugation (Heraeus Multifuge 4 KR, 500 rpm, 10 min). The density gradient was built up for 30 minutes with 14500 rpm (Sigma Percoll, autoclaved; Sorvall centrifuge RC-5B+, SLA-1500 rotor, Nunc 10 ml Oak Ridge centrifuge tubes). After that, one tube was loaded with differentially coloured density marker beads (Amersham Biosciences, 1.018, 1.033, 1.049, 1.062, 1.075 g ml^{-1}, 15 µl of each bead + 1925 µl sterile SMB-Medium) and the remaining gradients were loaded with 2 ml of the concentrated and fixed ciliate cultures (4-9 replicates per species). Gradients were then centrifuged for 25 minutes with 2000 rpm (Sorvall RC-5B+, SLA-1500 rotor). The distance from water / Percoll surface to the bands (beads or ciliates) was measured with a caliper gage (Fig. 6.1). Ciliate densities were calculated by the proportion of the bead bands and the ciliate bands. For each species densities were calculated with the help of standard curves, derived from the marker bead data (Fig. 6.2; Table 6.1).

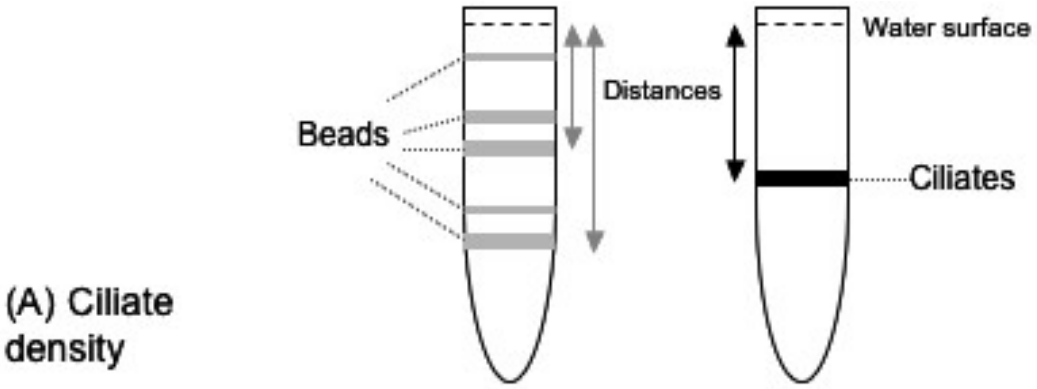

Fig. 6.1: explains the procedure following the density gradient centrifugation; the distances between the water surface and the bead bands were measured and used to create the standard curves. With the standard curves and the distance from surface to the ciliate bands, the ciliate densities were calculated.

Table 6.1: Equations and R^2 of the calibration curves, which were used to calculate ciliate densities. Calibration curves were made with the marker bead data.

Species	Equation of calibration curve	R^2
Cyclidium glaucoma	$f(x) = 3{,}442\ 10^{-2} * x + 1{,}018$	0.95
Tetrahymena pyriformis	$f(x) = 3{,}60\ 10^{-2} * x + 9{,}953E\text{-}1$	0.79
Paramecium aurelia	$f(x) = 9{,}508\ 10^{-2} * x + 9{,}296E\text{-}1$	0.95
Euplotes octocarinatus	$f(x) = 2{,}44\ 10^{-2} * x + 1{,}017$	0.94
Colpidium colpoda	$f(x) = 4{,}395\ 10^{-2} * x + 9{,}997E\text{-}1$	0.99
Red Sea	$f(x) = 9{,}015\ 10^{-2} * x + 9{,}998E\text{-}1$	0.89
Baltic Sea	$f(x) = 1{,}144\ 10^{-1} * x + 1{,}004$	0.97

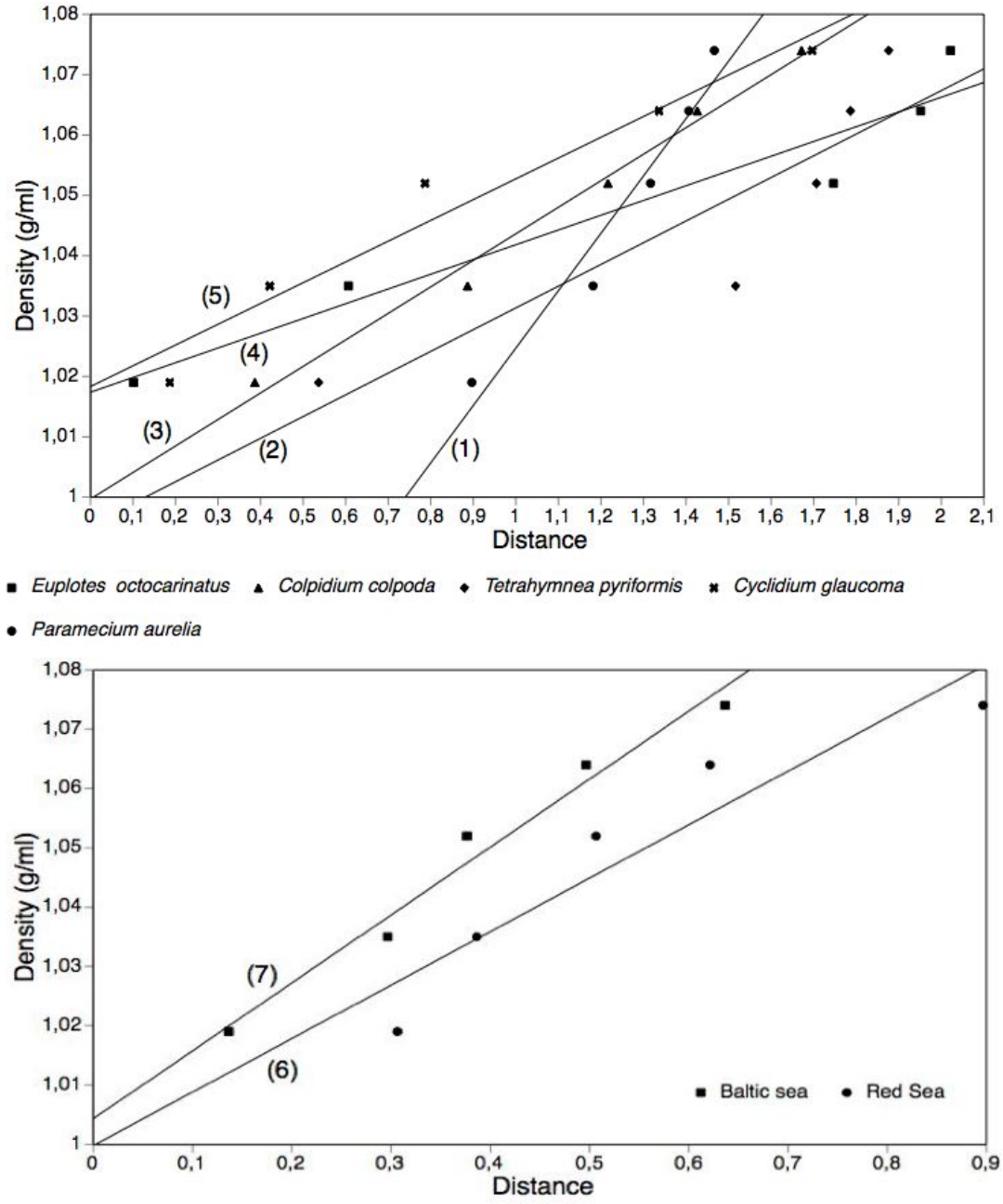

Fig. 6.2: Calibration curves for ciliate density of the freshwater (top) and marine cultures used in the study (bottom). The x-axis shows the distances, measured from water surface to the different bead bands; the y-axis shows he density, g ml^{-1}. The calibration curves were made with the density marker beads data for each culture and replicate separately. (1): *P. aurelia*, (2): *T. pyriformis*, (3): *C. colpoda*, (4): *E. octocarinatus*, (5): *C. glaucoma*, (6): Red Sea, (7): Baltic Sea.

Empirical sinking velocities of fixed ciliates (Fig. 6.3)

Ciliates from working stocks were fixed with glutardialdehyde (2 % f.c.) and transferred into a sedimentation chamber (Hydrobios, ∅ = 2.56 mm, h = 18.5 mm, V = 9.8 ml). The chamber was immediately placed on an inverted microscope (Nikon Eclipse TE2000-U) and picture sequences were taken automatically using a Nikon Digital Sight DS-SM camera and DS-L1 imaging computer. The intervals between two pictures were 15-30 seconds (3-4 replicates). Ciliates on the bottom of the chamber were counted for each picture until the number did not increase any more (Fig. 6.3). The sinking velocity was calculated from the time until constancy and height of the chamber. We never observed effects of convection.

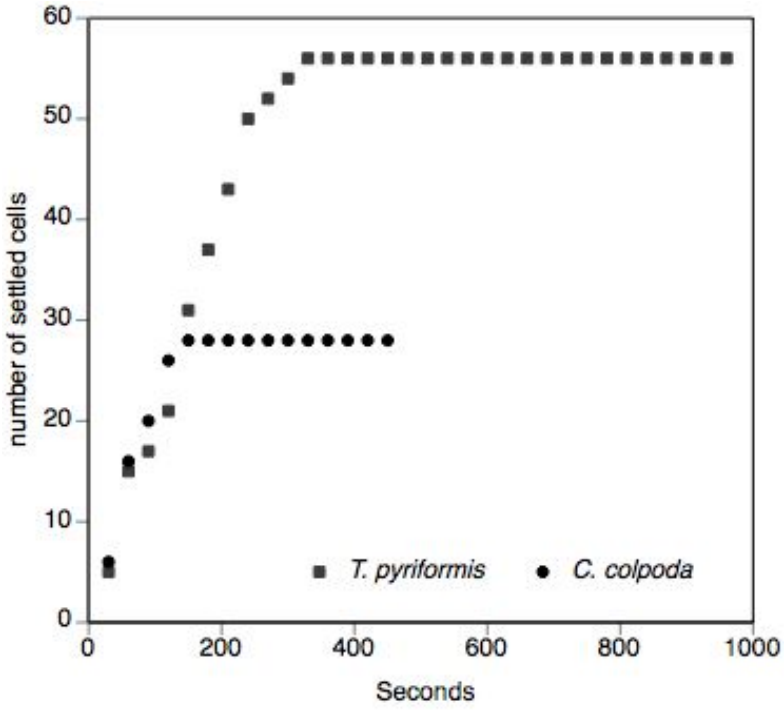

Fig. 6.3: Exemplary data for the empirical determination of settling velocity. The x-axis shows the time period (seconds) in which pictures were automatically taken. The y-axis shows the number of already settled cells.

Calculation of sinking velocity – Stokes equation

Sinking velocities were calculated following a modification of the Stokes Law (1) (Vogel 1983) to allow a comparison between empirical and theoretical settling velocities.

$$V = 2/9\ g\ r^2\ \frac{\delta - \delta_1}{\mu\ F} \tag{1}$$

The sinking velocity (V) was calculated with the following parameters: g = acceleration of gravity; r = particle radius; δ = density of the sinking particle; δ_1 = density of the medium; F = form resistance; μ = viscosity of the medium.

To calculate form resistance we had to develop a formula, which did not include velocity as parameter, otherwise we would have had to use our empirical settling velocities. For that we solved formula (2) and (1) for V and equalled them to get formula (3). Formula (2) and those for α and β ((4) and (5)) were derived from McNown and Malaika (1950). α and β were needed to calculate K (a proportionality factor) and with that form resistance correctly, considering the cell shapes of the species used in our study (same for (6) and (7)).

$$F = \left(3\pi\mu VD\right)K \tag{2}$$

$$D = 2\left(abc\right)^{1/3}\ ;\ K = \frac{16}{3}D\left(\alpha + \beta\right)$$

a, b, c = semi axis of the cell (half-axis of length, width and height)

$$F = \sqrt{\frac{2}{9}g\ r^2\ \frac{\delta - \delta_1}{\mu}\ K\left(3\pi\mu D\right)} \tag{3}$$

$$\alpha = \frac{2}{\sqrt{a^2 - c^2}}\tanh^{-1}\frac{\sqrt{a^2 - c^2}}{a} \qquad \beta = \frac{2a^2}{\left(a^2 - c^2\right)^{3/2}}\left(\tanh^{-1}\frac{\sqrt{a^2 - c^2}}{a} - \frac{\sqrt{a^2 - c^2}}{a}\right) \tag{4}$$

a>b=c; a is the axis in sinking direction; calculates shortest possible settling time due to the orientation of the cell.

$$\alpha = \frac{2}{\sqrt{b^2 - a^2}}\tanh^{-1}\frac{\sqrt{b^2 - a^2}}{b} \qquad \beta = \frac{a^2}{\left(b^2 - a^2\right)^{3/2}}\left(\frac{b\sqrt{b^2 - a^2}}{a^2} - \tanh^{-1}\frac{\sqrt{b^2 - a^2}}{b}\right) \tag{5}$$

a=c<b; a is the axis in sinking direction; calculates longest possible settling time.

Formula (4) and (5) were used for *T. pyriformis*, *C. colpoda*, *P. aurelia* and *C. glaucoma*. Theoretically there are two possibilities for the orientation of the cell regarding the moving direction. First, when the length axis is orientated parallel to the moving direction, meaning the shortest possible settling time (4) and (6). Second, for the case that the axis, which is orientated parallel to moving direction is not the length axis, meaning the longest possible settling time, (5) and (7). Due to the special shape of *Euplotes* for this species the following formulas (6) and (7) were used (for details see McNown & Malaika 1950).

$$\alpha = \frac{2F(\delta_1,K_1)}{\sqrt{a^2-c^2}} \qquad \beta = \frac{2a^2\left[F(\delta_1,K_1)-E(\delta_1,K_1)\right]}{\left(a^2-b^2\right)\sqrt{a^2-c^2}} \tag{6}$$

$$\delta_1 = \sin^{-1}\frac{\sqrt{a^2-c^2}}{a} \qquad k_1 = \sqrt{\frac{a^2-b^2}{a^2-c^2}}$$

a>b>c; a is the axis in sinking direction; calculates shortest possible settling time

$$\alpha = \frac{2F(\delta_2,K_2)}{\sqrt{b^2-a^2}} \qquad \beta = \frac{2a^2}{c^2-a^2}\left[\frac{c}{ab} - \frac{E(\delta_2,K_2)}{\sqrt{b^2-a^2}}\right] \tag{7}$$

$$\delta_2 = \sin^{-1}\frac{\sqrt{b^2-a^2}}{b} \qquad k_2 = \sqrt{\frac{b^2-c^2}{b^2-a^2}}$$

b>c>a; a is the axis in sinking direction; calculates longest possible settling time

For formulas (6) and (7), E and F are elliptic integrals, which were solved with tabulated values from Bronstein & Semendjajew (1974).

6.4 Results

The methods used in this paper were established and protocols were elaborated to easily adopt the procedures. The comparison of the empirical sinking rates with the theoretical data showed that there might be some effects on the sinking velocities,

which could not be completely covered by the modified Stokes equation. Therefore we attach great importance to the comparison of two different methods to get reliable data.

Table 6.2: Characteristics of the ciliate cultures, used for the studies. Calculated sinking velocity is based on Stokes equation (1) and is compared to the experimentally determined velocity. The salinities for the marine cultures are given, while FW indicates freshwater cultures, n.d. = not determined.

Ciliates	Size (µm)	Cell shape	Salinity (‰)	calculated sinking velocity (mm min^{-1})	experimental sinking velocity (mm min^{-1})	Ciliate density (g ml^{-1})
Cyclidium glaucoma	23	spherical	FW	2.1	9.4	1.04
Tetrahymena pyriformis	60	ovoid	FW	2.4	2.4	1.02
Paramecium aurelia	140	ovoid-flattened	FW	5.3	4.5	1.08
Euplotes octocarinatus	100	flattened	FW	4.4	10.3	1.05
Colpidium colpoda	110	ovoid with nose	FW	2.8	7.7	1.02
Baltic Sea Large	38	mostly ovoid	16	n.d.	0.6	1.08
Baltic Sea Small	19	mostly ovoid	16	n.d.	0.5	1.08
Red Sea Large	20	ovoid and flattened	40	n.d.	1.7	1.05
Red Sea Small	52	ovoid and flattened	40	n.d.	2.7	1.05

Ciliate density

Ciliate densities were specified for both, the freshwater and the marine cultures and they were all within the same range. The freshwater species densities were between 1.02 and 1.08 g ml^{-1}, the Red Sea and Baltic Sea ciliates had densities of 1.05 and 1.08 g ml^{-1}, respectively (Fig. 6.4; Table 6.2). The lowest densities were found for *T. pyriformis* and *C. colpoda* (1.02 g ml^{-1} for both), which were significantly lower then the highest density, found for *P. aurelia* and the Baltic Sea ciliates (1.08 g ml^{-1} for both; ANOVA $p<0.001$). Therefore we did not use a mean ciliate density for the calculation of the settling time, but calculated the velocity for each species on its own.

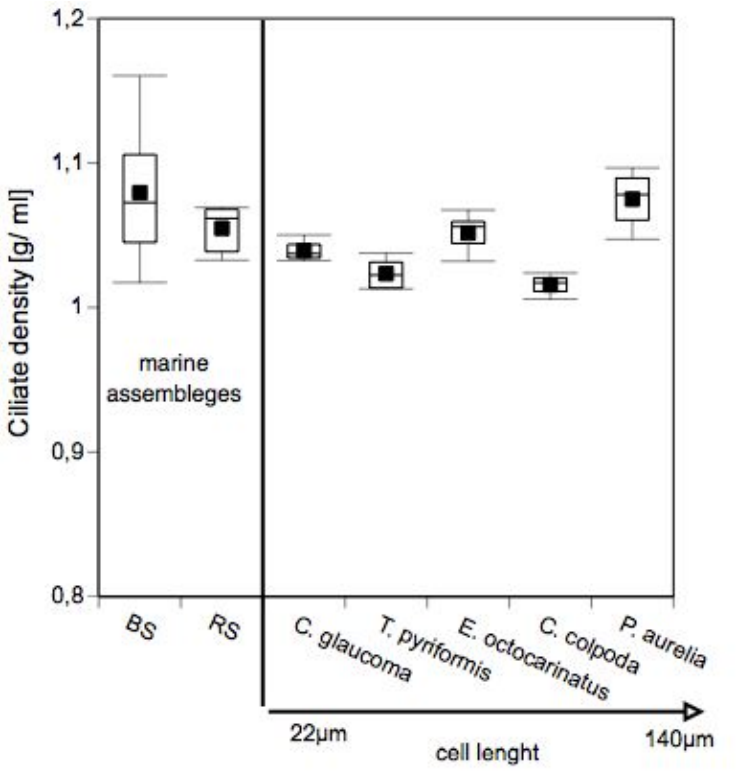

Fig 6.4: Box-plot of the ciliate densities (g ml^{-1}). The two marine assemblages are on the left side, while the freshwater species are arranged with increasing size on the right. BS: Baltic Sea; RS: Red Sea. The top, bottom, and line through the middle of the box correspond to the 75th percentile, 25th percentile, and 50th percentile (median), respectively. The whiskers on the bottom extend the 10th percentile (bottom decile) and top 90th percentile (top decile).

Sinking velocity of fixed ciliates

Sinking velocities were experimentally determined for the five different freshwater species and the two marine assemblages (Table 6.2). Ciliates differed in size, from the small marine species with 19 µm length to *P. aurelia* with 140 µm, and in cell shape (Table 6.2). The empirical sinking velocities varied between 2.4-10.3 mm min^{-1} for the freshwater species and between 1.7–2.7 mm min^{-1} for the ciliate assemblages from the Red Sea. The lowest values were found for the Baltic Sea ciliates (0.5-0.6 mm min^{-1}; Fig. 6.5). The settling rates of the marine ciliates were significantly lower then sinking velocities of the freshwater species, even if the unusual low values of the Baltic Sea culture were not considered (t-test, $p = 0.039$). Though there was a significant correlation between the empirical velocity and ciliate density ($p = 0.034$; Pearsons $r = 0.96$), there were no correlations between ciliate size and sinking velocity (Pearsons $r = 0.24$) or between form resistance and sinking velocity (Pearsons $r = -0.32$).

Sinking velocities were calculated for the freshwater species only, as the cell size in the marine assemblages was too diverse. The calculated velocities were between 2.1 and 5.3 mm min^{-1}, in the same range as the empirical values for the same species (Table 6.2; Fig. 6.5). For *C. glaucoma* and *C. colpoda* the calculated sinking velocities were clearly lower than the experimental velocity.

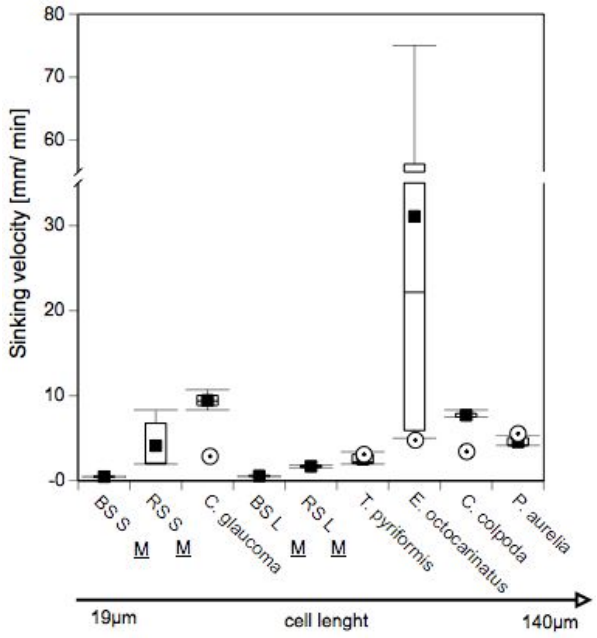

Fig. 6.5: Empirical (box-plots) and calculated (circles) sinking velocities arranged with increasing ciliate size from left to right. The calculated settling rates are given for the freshwater ciliates, but not for the marine cultures due to the diversity in the size structure. BS: Baltic Sea, RS: Red Sea, S: small, L: large. The top, bottom, and the line through the middle of the box correspond to the 75th percentile, 25th percentile, and 50th percentile (median), respectively. The whiskers on the bottom extend the 10th percentile (bottom decile) and top 90th percentile (top decile).

6.5 Discussion

We had three hypotheses regarding the settling of fixed ciliate samples, particularly ciliate samples: (1) settling velocities increase with increasing cell size / diameter. (2) Variations from the spherical body shape causes increased sinking times (3) marine samples will have lower settling velocities due to the higher density of seawater compared to freshwater. Sinking velocities were not correlated with cell size or form resistance, but there was a significant correlation with ciliate density. Cell shape was less important for the sinking velocities as the two species having the highest sinking velocities (*E. octocarinatus* 7.7 mm min^{-1}, *C. glaucoma* 9.4 mm min^{-1}) differed strongly in cell size and particularly in cell shape (Table 6.2). *Euplotes* is dorsoventrally flattened with a band of cilia, the membranell and several clusters of tightly arranged cilia, the cirri. This was expected to increase form resistance, while

Cyclidium is more like a spheroid, which should have the lowest form resistance. The prominent membranelles and cirri at the edge of the cell might cause the high variance within the replicates of *E. octocarinatus*. These membranelles may strongly affect the orientation of the cell and therefore increase the settling time compared to spheroid shaped cells. This became clear comparing minimal and maximal settling times of *Cyclidium* and *Euplotes*, 8.3-10.7 and 5-75 mm min^{-1}, respectively. Additionally, the calculated settling time of *Euplotes* was even lower than the lowest empirical velocity. This indicates that there were factors affecting the settling velocity, which cannot be covered by the mathematical formulas. Not body shape but the appearance of appendages affected the settling velocity, which was not considered by Stokes law. Therefore we had to falsify the first hypothesis and the second hypothesis as not body shape but the appearance of appendages, such as cirri and membranelles, was affecting the settling velocity. Therefore we conclude that the experimental determination of sinking velocities is more reliable than the theoretical approach.

The factors affecting the sinking velocities of fixed ciliates were cell density and salinity of the habitat, in terms of freshwater or marine systems. The marine ciliates had significantly lower settling velocities as the density of seawater is clearly higher than the density of freshwater while the density of the marine ciliates was in the range of the freshwater species, confirming the third hypothesis. The salinity of the medium needs to be considered when settling plankton samples, especially ciliates for enrichment.

Comments and recommendations

The methods for determining ciliate density and settling times both were established and can easily be repeated for other species. As there were significant differences in ciliate density between groups this parameter should be measured for each species on its own. The use of a mean ciliate density cannot be recommended. For the settling times, we recommend using the lowest empirical sinking velocities, meaning 1.7 mm min^{-1} for fixed marine (40‰) ciliate samples and 2.4 mm min^{-1} for fixed freshwater samples. As 40‰ was rather high, this value should be reliable for most marine systems.

Often 1 l samples are settled for planktonic systems, meaning for 1 l graduated cylinder with height of 33-37 cm, a settling time of 3.3-3.6 h for marine samples or

2.3-2.6 h for freshwater samples. These values are surprisingly short as until now often settling times up to one week were used for 1 l-graduated cylinders. For Utermöhl settling chambers the settling time can be drastically reduced now, from at least 24 h for 10 ml and 50 ml samples to 1 h and 2.8 h for marine samples and to the range of minutes for freshwater samples (8.3 and 42 minutes). That means that 95% less time is needed for settling ciliate samples, compared to the old settling times.

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8. APPENDIX

8.1 List of Publications

8.1.1 Do ciliates have an effect on the nitrogen cycle through grazing on nitrifying bacteria?

ELKE NEUBACHER, MARIO PRAST, ERNST-JOSEF CLEVEN & ULRIKE-G. BERNINGER

Outline, concept and experimental design for the project by M.P., counting and FISH by E.N., writing by E.N. and M.P. with help of coauthors.

Submitted to Hydrobiologia

8.1.2 The impact of ciliates on nitrifying bacteria in the River Salzach (Austria)

MARIO PRAST & ULRIKE-G. BERNINGER

Outline and experimental design by M.P., oxygen microsensors constructed by Gaby Eickert, LIX microsensors constructed by M.P., counting and FISH by M.P., writing by M.P. with help of coauthor.

In preparation

8.1.3 Impact of ciliates on nitrification and nitrifying bacteria in Baltic Sea sediments

MARIO PRAST, ADRIAN BISCHOFF, UWE WALLER, RUDOLF AMANN & ULRIKE-G. BERNINGER

Outline and experimental design by M.P., setup of flumes by A.B., microsensor construction, FISH and counting by M.P., writing by M.P. and A.B. with help of coauthors

Submitted to Marine Ecology Progress Series

8.1.4 Impact of *Nereis diversicolor* (O.F. Müller, 1776) on nitrification and nitrifying bacteria in two types of sediments

ADRIAN BISCHOFF & MARIO PRAST

Outline and experimental design by A.B., setup of flumes by A.B., counting and FISH by M.P., writing by A.B. and M.P.

Submitted to Estuarine, Coastal and Shelf Science

8.1.5 Concentration of fixed plankton samples via settling: how long is long enough?

MONIKA CLAESSENS & MARIO PRAST

Idea, concept and experimental design by M.C. and M.P., measurements by Alexander Breitner and Mislav Vulelija, analysis and writing by M.C. and M.P.

Submitted to Journal of Plankton Research

8.2 Erklärung gemäß §6 Abs. 5 der Promotionsordnung der Universität Bremen für die mathematischen, natur- und ingenieurwissenschaftlichen Fachbereiche

Hiermit versichere ich, dass ich die vorliegende Dissertation ohne unerlaubte fremde Hilfe angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Alle den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen sind als solche kenntlich gemacht.

Salzburg, den 25.04.2007

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8.4 Abbrevations

Anammox	anaerobic ammonium oxidation
ANOVA	analysis of variance
ATU	allylthiourea
β-AOB	ammonium-oxidizing β-proteobacteria
BS	beamsplitter
Cy3	cyanine 3
d	day
DAPI	4´,6-diamidino-2-phenylindol
EX	exication filter
f.c.	final concentration
FLB	fluorescently labeled bacteria
FLNB	fluorescently labeled nitrifying bacteria
FISH	fluorescence-*in-situ*-hybridization
h	hour
ind.	individuals
l	liter
LP	long-pass filter
m	meter
min	minute
n.d.	not determined
NOB	nitrite oxidizing bacteria
QPS	quantitative protargol staining
s	second
SD	standard deviation
SMB	synthetic medium for *Blepharisma*