Nitrification in freshwater sediments as studied with microsensors and fluorescence in situ hybridization

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Chapter 1

General introduction

The benthic nitrogen cycle and nitrification

Over the last decades the global nitrogen cycle (N-cycle) has received substantial attention since the amount of N that is cycled on a global scale has greatly increased due to human activities. Moreover, a variety of negative consequences for the living world have been noticed. From a human point of view the eutrophication of aquatic ecosystems by excess N is a major problem. The nutrients NO_3^- and NH_4^+ can reach ground and surface waters by leaching from fertilized soils and insufficient treatment of wastewater can additionally supply NH_4^+ to surface waters. As a consequence of the high external loading with NO_3^- and NH_4^+ growth of especially planktonic primary producers may be enhanced, which can have profound effects on the quality of receiving waters (Smith, 2003). The enrichment of NH_4^+ itself is critical due to the toxicity of its uncharged form NH_3 to aquatic life (Hargreaves, 1998; Pandey, 1999; Oliviero *et al.*, 2003). Moreover, NO_2^- which is an intermediate of several microbial N conversions in the sediment and which is toxic towards animal and human life (Bruning-Fann and J.B., 1993; Kelso *et al.*, 1999; Neumann *et al.*, 2001) can be enriched in ground and surface waters as consequence of a high external NO_3^- load (Kelso *et al.*, 1997; Stief *et al.*, 2002). Fossil fuel combustion and biomass burning are major contributors to the emission of gaseous NO + NO_2 (summarized as NO_x) into the atmosphere where this gas mix participates in the photochemically induced formation of tropospheric ozone (Kerr, 1990). Another gaseous N-species, N_2O, is an important greenhouse gas and is therefore associated with global climate changes (Dalal *et al.*, 2003). N_2O is a byproduct of nitrification and an intermediate of denitrification which is emitted e.g. from irrigated rice soils (Ghosh *et al.*, 2003) and also from estuarine and coastal sediments with high organic loading (Usui *et al.*, 2001).

In addition to the anthropogenic input of N to aquatic environments internal sources of N in benthic habitats are mostly originating from the degradation of organic matter within the sediment. Degradation of organic matter leads to the formation of NH_4^+, which is either lost to the overlying water or oxidized to NO_3^- via nitrification at the oxic sediment-water interface. At the oxic-anoxic interface within the sediment NO_3^- can be reduced to N_2 via the process of denitrification. Alternatively NO_3^- can again be converted to NH_4^+ via the dissimilatory pathway of NO_3^- ammonification (DNRA).

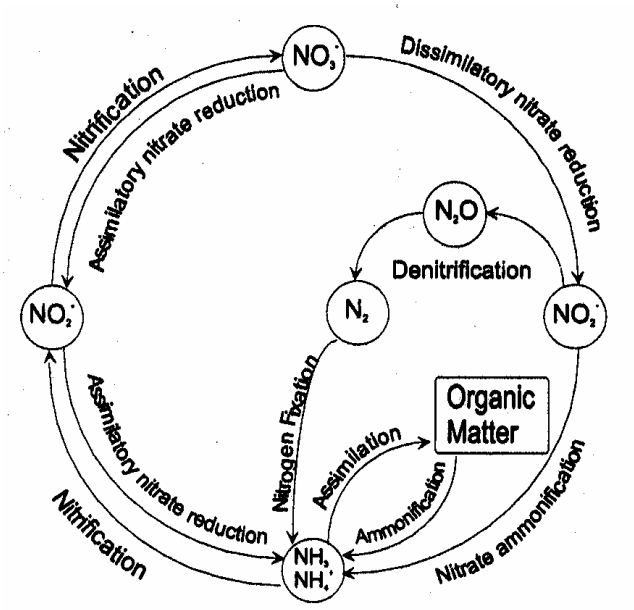

Fig. 1: The N cycle showing the chemical forms and key processes involved in the biogeochemical cycling of N (Herbert, 1999).

Denitrification and DNRA can be supplied with NO_3^- from either the nitrification layer or the overlying water. The quantitative dominance of either nitrification or denitrification strongly depends on the concentrations of O_2 and NO_3^- in the overlying water (Jørgensen, 2001): At low NO_3^- concentrations in the overlying water denitrification depends almost exclusively on the NO_3^- formed by nitrification and increases with O_2 concentrations and hence the thickness of the nitrification layer. The close coupling between nitrification and denitrification bears a great potential for the elimination of NH_4^+ and NO_3^- via gaseous N_2 from aquatic ecosystems (Jenkins and Kemp, 1984; Jensen *et al.*, 1993; Jensen *et al.*, 1994; Herbert, 1999).

Moreover, evidence increases for the performance of anaerobic NH_4^+-oxidation in O_2-limited or even completely anoxic environments (Jetten, 2001; Mortimer *et al.*, 2002; Schmidt *et al.*, 2002). Physiological studies demonstrate complete anoxic conversion of NH_4^+ by known aerobic NH_4^+-oxidizing bacteria (Bock *et al.*, 1995; Schmidt and Bock, 1997, 1998). Further potential of ammonia oxidation lies in the activity of newly identified anaerobic NH_4^+-oxidizers affiliated within the group of *Planctomycetales* (Strous *et al.*, 1999) oxidizing NH_4^+ with NO_2^- to N_2 (Vandegraaf *et al.*, 1995). This process has been called Anammox (Mulder *et al.*, 1995) and has so far

been described from engineered systems affiliated with wastewater treatment (Mulder *et al.*, 1995; Egli *et al.*, 2001). Evidence increases that Anammox is spread over a variety of natural environments (Jetten, 2001; Freitag and Prosser, 2003, Kuypers *et al.*, 2003).

Within the benthic N-cycle the process of nitrification is of particular importance since, on the one hand, it removes NH_4^+ and NO_2^- both being critical due to their toxicity to aquatic life (see above). On the other hand, nitrification produces NO_3^-, which can be readily removed from aquatic ecosystems by the process of denitrification. Moreover, processes of the benthic N cycle can be cross-linked via NO_3^- to the cycling of Fe (Hauck *et al.*, 2001; Ratering and Schnell, 2001), Mn (Hulth *et al.*, 1999) and S (Otte *et al.*, 1999; Zopfi *et al.*, 2001).

Autotrophic nitrification comprises two sequential steps, which are catalyzed by two groups of chemolithoautotrophic bacteria. Under oxic conditions NH_4^+-oxidizing bacteria (AOB) perform the oxidation of NH_4^+ to NO_2^-, while the NO_2^--oxidizing bacteria (NOB) oxidize NO_2^- to NO_3^-:

$$NH_4^+ + 1\,½\,O_2 + 2\,OH^- \rightarrow NO_2^- + 3\,H_2O \qquad \Delta G_0 = -287\ kJ\ mol^{-1} \quad [1]$$

$$NO_2^- + ½\,O_2 \rightarrow NO_3^- \qquad \Delta G_0 = -74.1\ kJ\ mol^{-1} \quad [2]$$

For the oxidation of 1 mol NH_4^+ 2 mol O_2 are consumed implying a relatively high O_2 demand for the performance of nitrification in aquatic sediments. Since nitrifiers are autotrophic organisms the small amount of energy obtained from these oxidation steps is then used for the production of biomass by CO_2 fixation. However, the biggest part of the energy is needed for their cell maintenance and even though nitrifiers optimize the amount of energy gained from the oxidation process (e.g. extension of the reactive surface, small cell size) their maximum growth rate is still low compared to e.g. heterotrophic bacteria (Prosser, 1989). Moreover, nitrifiers possess comparably high K_m values for O_2 and NH_4^+, i.e. 18 – 1400 µM NH_4^+ and 16 – 3600 µM O_2 for *Nitrosomonas* species and 1600 – 3600 µM NO_2^- and 62 – 260 µM O_2 for *Nitrobacter* spp. (Prosser, 1989; Schramm *et al.*, 1997). Heterotrophic bacteria can therefore easily outcompete nitrifying bacteria for their common substrates NH_4^+ and O_2 when the

availability is low and organic C is not limiting (Strauss and Lamberti, 2000, 2002) (Verhagen *et al.*, 1992).

Most commonly isolated and described AOB and NOB are the genera *Nitrosomonas* and *Nitrobacter*, respectively. Since methods are now available to detect and quantify nitrifiers in their natural environment (reviewed in (Schramm and Amann, 1999) evidence increases that other nitrifying organisms like the genera *Nitrosospira* and *Nitrospira* might be of greater importance in some environments, e.g. at low concentrations of NH_4^+, NO_2^- and O_2 (Schramm *et al.*, 1998; Okabe *et al.*, 1999; Schramm *et al.*, 2000).

In sediments O_2 is often depleted within the first few millimeters of the surface. Thus nitrification is limited to a very narrow zone. Nitrifiers therefore face aggravating conditions, i.e. they have to deal with a limited supply of O_2 and they have to compete with metabolically superior microorganisms for their sources O_2 and NH_4^+. Compared to other functional groups of bacteria like sulfate reducers, which can make up to around 9% of the total bacterial community of marine sediments (Ravenschlag *et al.*, 2000), cell numbers of nitrifying bacteria in sediments are relatively low. Cell numbers determined with the most probable number method range from $10^3 – 10^6$ ml^{-1} making up only around 1% of total bacterial cells (Smorczewski and Schmidt, 1991; Bianchi and Lefevre, 1999; Whitby *et al.*, 2001). It is therefore striking that the contribution of nitrifying bacteria to the microbial N conversions and to the overall O_2 consumption in sediments is so substantial. Nitrification can consume up to 90% of the total sedimentary O_2 consumption (see own data, Chapter 4) and nitrification rate is often comparable to the rate of denitrification (Lorenzen *et al.*, 1998; Meyer *et al.*, 2001).

For the fine scale investigation of nitrification measuring tools are needed that allow a sufficiently high spatial resolution for the exact determination of nitrification rates and for the identification and quantification of the nitrifying community. For this purpose, the combination of selective microsensors with fluorescence *in situ* hybridization provides a useful tool to target function and structure of nitrification also in sediments (Amann and Kühl, 1998). This approach has already been successfully applied in biofilms and aggregates of wastewater treatment plants (Schramm *et al.*, 1997; Okabe *et al.*, 1999; Gieseke *et al.*, 2001).

Investigating the ecology of nitrifying bacteria with microsensors and FISH

The activity of nitrifying bacteria as determined with microsensors

Because of their small size microsensors are useful tools to investigate processes occurring at a very small scale as it is the case in microbial mats, biofilms, and sediments (de Beer, 1999), where diffusion and reaction processes create steep physicochemical gradients within µm distances. The application of microsensors minimizes the disturbance of these gradients, of the three dimensional organization of microbial communities, and of other microenvironmental conditions such as boundary layers, diffusion and flow patterns on the microscale (Amann and Kühl, 1998). Moreover, the fine spatial resolution of the microsensors allows the functional separation of different layers and hence the spatially differentiated interpretation of processes that occur in close vicinity (e.g. nitrification and denitrification). Thus, the microsensor technique has great advantages compared to other methods of investigating the processes of the benthic N-cycle: Mass balances (Hansen *et al.*, 1981; Rysgaard *et al.*, 1995; Van Luijn *et al.*, 1999; Christensen *et al.*, 2000), potential nitrification rates (Hansen *et al.*, 1981) (Henriksen *et al.*, 1993) (Vouve *et al.*, 2000), and ^{15}N tracer techniques (Rysgaard *et al.*, 1994; Rysgaard *et al.*, 1995; Christensen *et al.*, 2000) (Usui *et al.*, 2001) (Vouve *et al.*, 2000) do either average across several microbial processes or they do not measure *in situ* activities. Microsensor measurements on the other hand only give net conversion rates and each of the alternative methods bears also some advantages, e.g. ^{15}N techniques are useful for the investigation of the coupled nitrification-denitrification since the source of NO_3^- can be differentiated properly (Nielsen, 1992).

In the present thesis microsensors for O_2 (Revsbech, 1989), NH_4^+, and NO_3^- (de Beer and Sweerts, 1989; de Beer *et al.*, 1991) have been applied to determine the activity of nitrifying bacteria in freshwater sediments (compare reaction equations [1] and [2]).

The microsensors for O_2, NH_4^+, and NO_3^- belong to two different types of electrochemical sensors that are commonly used for ecological studies: (1) potentiometric electrodes (e.g. for H^+, Ca^{2+}, NH_4^+, NO_2^-, and NO_3^-) measure an electrochemical potential difference across a liquid membrane and (2) amperometric electrodes (e.g. for O_2, N_2O, and H_2S) measure the current that is generated by the reduction of the respective substrate at the cathode of the sensor.

Potentiometric electrodes for NH_4^+ and NO_3^-. These electrodes contain an ion exchanging liquid membrane within the tip of the sensor (liquid ion exchanger – LIX). LIX sensors can be constructed with a capillary tip of 1 – 10 µm thus allowing an equivalent spatial resolution of the measurements. A typical LIX sensor is sketched in Fig. 2 A. Both, NH_4^+ and NO_3^- microsensors have been successfully used in freshwater sediments (de Beer and Sweerts, 1989; de Beer *et al.*, 1991). Their sensitivity towards interference from other ions, such as K^+ for NH_4^+ and HCO_3^- and Cl^- for NO_3^-, impedes their use in saline waters. Even in freshwater sediments the accuracy of vertical NO_3^- profiles can be affected by increasing concentrations of HCO_3^- with depth (Kühl and Revsbech, 2001). To overcome this problem a biosensor for NO_3^- has recently been developed that allows fine scale measurements in marine environments and in biologically reactive environments with high concentrations of HCO_3^- (Larsen *et al.*, 1997).

Amperometric Clark type O_2 electrodes. The Clark type O_2 sensor is the best known gas microsensor (Revsbech, 1989). This sensor consists of a gold-coated cathode, which is situated behind a silicone membrane. O_2 diffusing through the gas-permeable membrane is reduced at the cathode when a polarization voltage is impressed. O_2 microsensors can be constructed with a tip size of 1-100 µm, they are further characterized by the absence of interferences, linear calibration curves, fast response times (≥ 0.1 s) and low sensitivity to stirring of the external medium (Kühl and Revsbech). An O_2 microsensor with a guard cathode is sketched in Fig. 2 B.

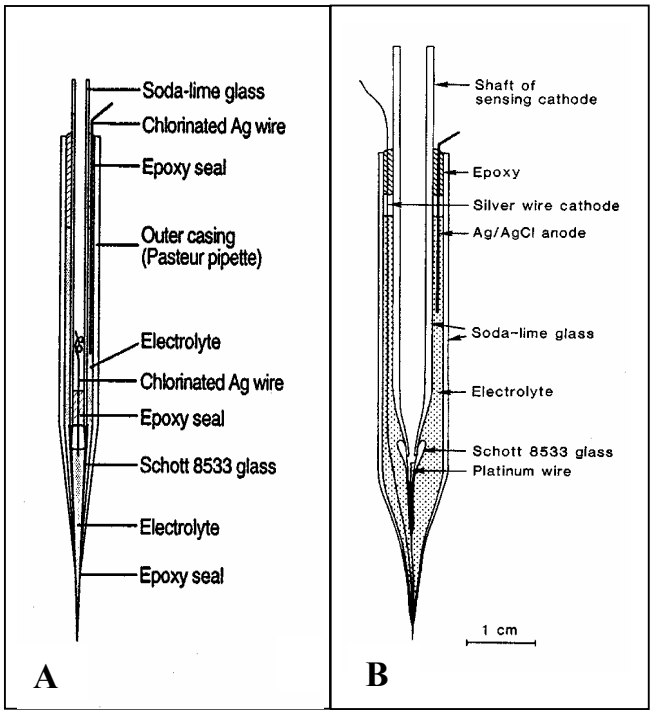

Fig. 2A, B: Schematic drawing of a Lix sensor (A) (from Jensen *et al.*, 1993) and a Clark type O_2 electrode (B) (from Revsbech, 1989)

Calculations. Vertical solute profiles are in steady state when conversion rates equal transport rates. Assuming diffusion as the only transport mechanism the activity of microorganisms, i.e. the conversion rate of a certain solute, can be determined by applying a one-dimensional diffusion-reaction model (Revsbech and Jørgensen, 1986) on steady state microprofiles. This model is based on Fick´s first law calculating the flux, i.e. the rate of mass transfer per unit area, for each data point of the concentration profile:

$$J = - D_s \cdot \delta c/\delta x \qquad [3]$$

Where J is the flux [mmol m^{-2} h^{-1}], D_s is the apparent diffusion coefficient in sediment as calculated from the diffusion coefficient in water D_0 and the porosity φ of the sediment ($D_s = D_o \cdot \varphi$) [m^2 h^{-1}], and $\delta c/\delta x$ is the concentration gradient between two depths x_1 and x_2.

The fluxes calculated for the single points in a concentration profile can be used for the calculation of local volumetric conversion rates [mmol m^{-3} h^{-1}] after (de Beer, 1999):

$$R = J_{ab} - J_{bc} / (0.5(x_a + x_b) - 0.5(x_b + x_c)) \quad [4]$$

The calculation procedure thus comprises the differentiation of the concentration profile using adjacent values. As a result a vertical profile of local conversion rates is obtained. The subsequent averaging of consecutive conversion values within a certain zone can partly reduce noise that may result from the differentiation procedure and which can sometimes strongly affect the calculated conversion rates.

The nitrifying activity within a given sediment can be expressed as: (i) the local volumetric conversion rates in single layers of the nitrification zone and (ii) the bulk areal conversion rate in mol m^{-2} h^{-1} within this zone as calculated by integration of volumetric conversion rates over the considered depth interval, i.e. the oxic layer. By integration over the NO_3^- consumption zone within the anoxic layer rates within in the anoxic layer the areal rate of denitrification activity can be determined. Thereby the quantitative importance of nitrification and denitrification for the budget of N conversions in the sediment can be estimated, i.e. net influx to or efflux of NO_3^- from the sediment. Alternatively, the total flux of a solute into or out of the sediment is represented by the linear concentration gradient within the diffusive boundary layer.

Identity and distribution of nitrifying bacteria: the application of FISH

Hybridization of intact cells with fluorescently labeled, 16S rRNA targeted oligonucleotides (fluorescence *in situ* hybridization, FISH) has been introduced to microbial ecology approximately a decade ago as an accurate method of characterizing microbial populations in their *in situ* environment (De Long *et al.*, 1989; Amann *et al.*, 1990). The ribosomal 16S rRNA is considered a powerful target because (i) ribosomes are a common feature of all organisms, (ii) ribosomes are of homologous origin and show functional constancy and hence the phylogeny of the organisms can be reconstructed based on the 16S rRNA molecules, (iii) the differentiation at any taxonomic level, i.e. from species to domains, is allowed because of a different degree of conservation within the rRNA molecules, and (iv) 16S rRNA sequences have been

described for many species and are available from public databases (e.g. http://www.ncbi.nlm.nih.gov/) (Schramm and Amann, 1999).

The method of FISH is part of the so called full cycle rRNA approach which comprises the extraction of DNA from environmental samples, the amplification of rDNA using PCR and specific primers, the cloning and sequencing of the amplified rDNA fragments and the subsequent design of specific probes to enumerate whole fixed cells in the original sample by in situ hybridization (Olsen *et al.*, 1986; Amann *et al.*, 1995). Thereby, quantitative information on the community structure can be obtained. Oligonucleotide probes that are commercially available are either provided with a ^{32}P label or with a fluorescent dye (Cy3, Cy5, Fluorescein). Radio labeled probes are commonly used for hybridization of extracted and immobilized rRNA (dot blot hybridization). The relative abundance of a certain 16S rRNA sequence is then expressed as the fraction of total 16S rRNA in the sample (Schramm *et al.*, 1997). The dot blot hybridization has been applied for the investigation of sulfate reducing bacteria in arctic sediments in which a low cellular RNA content was expected (Ravenschlag *et al.*, 2000). The hybridization of intact cells with fluorescently labeled oligonucleotides (fluorescence *in situ* hybridization (FISH)) enables the detection of a certain rRNA sequence within morphologically intact cells *in situ*, i.e. in their natural environment (Schramm *et al.*, 1997). Data about *in situ* distribution and abundance of the cells are thereby complemented by information on the morphology and on the spatial organization of the cells. Since a low cellular rRNA content limits the detectability of cells and thus the application of FISH in environmental samples the FISH method has been extended by the use of multilabeled oligonucleotides (DeLong *et al.*, 1999) or by the enzymatic amplification of the hybridization signal (Schönhuber *et al.*, 1997).

The FISH technique constitutes a major advantage compared to classical methods like the most probable number technique or viable plate counts since it enables the *in situ* detection and a reliable quantification of bacterial populations. In contrast, the use of isolation and cultivation techniques for quantitative purposes is limited by the “great plate count anomaly” (Staley and Konopka, 1985)): Species will be overlooked for which the cultivation conditions are not suitable or which have entered a non-culturable stage. Thus, by the application of cultivation-dependent techniques the

actual size and diversity of microbial communities can be underestimated dramatically (Amann *et al.*, 1995).

An important precondition for the *in situ* detection of a larger functional group of microorganisms like nitrifiers is their monophyletic origin allowing the detection of group specific signatures within the rRNA (Schramm, in press). The nitrifying bacteria are at present grouped into two monophyletic lineages of NH_4^+-oxidizing bacteria and four phylogenetically distinct groups of NO_2^--oxidizing bacteria. NH_4^+-oxidizing bacteria comprising the genera *Nitrosospira*, *Nitrosovibrio*, *Nitrosolobus*, *Nitrosomonas* and also the species *Nitrosococcus mobilis* are closely related organisms within the β subclass of the Proteobacteria. Two other species of this genus *Nitrosococcus oceani* and *Nitrosococcus halophilus* are located within the γ subclass of the Proteobacteria. NO_2^--oxidizing bacteria were assigned to the α, γ, and δ subclasses of Proteobacteria (genera *Nitrobacter*, *Nitrococcus*, and *Nitrospina*). A distinct phylum close to the δ subclass of the Proteobacteria comprises the genus *Nitrospira* (Koops and Pommerening-Roser, 2001). Based on 16S rRNA sequences of nitrifying bacteria a comprehensive set of probes targeting these nitrifiers has been developed and tested for FISH (Schramm, in press).

Alternatively to the 16S rRNA approach NH_4^+-oxidizing bacteria can be identified by targeting the functional gene encoding for the NH_4^+ monooxygenase (*AmoA*) which catalyzes the first step of NH_4^+ oxidation (McTavish *et al.*, 1993; Rotthauwe *et al.*, 1997; Phillips *et al.*, 2000). However, the enzyme catalyzing NO_2^--oxidation (NO_2^--oxidoreductase, NOR) has so far only been detected and quantified by immunological methods (Spieck *et al.*, 1996; Bartosch *et al.*, 1999; Maron *et al.*, 2003).

In the present thesis fluorescence *in situ* hybridization with monolabeled probes was used to address the identification and *in situ* distribution of nitrifying bacteria in several freshwater sediments.

Influence of sediment disturbances on nitrification

Benthic nitrification is controlled by a number of physico-chemical factors which include temperature, pH, salinity, O_2, NH_4^+ and dissolved CO_2 concentrations (Herbert, 1999) and the presence of inhibitory compounds like H_2S (Bagarinao, 1992). Moreover burrowing macrofauna can have profound effects on the performance of

nitrification (Pelegri and Blackburn, 1994, 1995a; Rysgaard *et al.*, 2000; Svensson *et al.*, 2001). Mixing the sediment artificially, as it is commonly used for factorial sediment experiments (van de Bund *et al.*, 1994; Svensson and Leonardson, 1996; Strauss and Lamberti, 2002) is also thought to influence nitrification activity (Svensson *et al.*, 2001). Sediments in sheltered areas and streams are influenced by movements of the overlying water body, which can have enormous effects on the sediment loading and hence the stratification of microbiota within the sediment (Wulff *et al.*, 1997). In such dynamic habitats motile organisms can have advantages compared to non-motile forms since they can easily recolonize newly formed surfaces (Wulff *et al.*, 1997). Moreover, nitrifiers may be severely handicapped under such conditions due to their low competitiveness with other organisms, i.e. their slow growth and low substrate affinities (Prosser, 1989; Schramm *et al.*, 1997).

Natural disturbance: sediment bioturbation

A variety of investigations have been carried out addressing the influence of bioturbation and bioirrigation on N mineralization in marine (Pelegri and Blackburn, 1994; Gilbert *et al.*, 1998; Bartoli *et al.*, 2000), estuarine (Pelegri *et al.*, 1994; Pelegri and Blackburn, 1995b; Rysgaard *et al.*, 1995), and freshwater sediments (Pelegri and Blackburn, 1996; Svensson *et al.*, 2001; Stief and de Beer, 2002). Macroinvertebrates living in or at the surface of sediments intensively act on their physical, chemical, and microbiological environment. While feeding on sediment particles and by their locomotive activity the animals actively rework the sediment thereby translocating sediment particles and associated microorganisms to more or less favorable microenvironments (Matisoff and Wang, 2000). The construction of burrows, which can reach deep into anoxic sediment layers, and the ventilation of these burrows lead to an extension of the oxidized sediment surface and to a 3-D microstratification as a consequence of steep gradients around the burrow walls (Kristensen *et al.*, 1985; Fenchel, 1996). Moreover, the animals may stimulate the generation of suitable organic substrates for bacteria by ingestion, digestion and the subsequent excretion of dissolved compounds which can be used for bacterial growth (Goedkoop *et al.*, 1997). By the use of common methods ^{15}N tracer techniques and mass balance studies bioirrigation of sediments was often shown to stimulate the activity of bacteria

involved in benthic N conversions (Kristensen *et al.*, 1985; Pelegri *et al.*, 1994; Svensson, 1997; Gilbert *et al.*, 1998; Bartoli *et al.*, 2000). The deposit feeding of several animals on the other hand, i.e. the ingestion and digestion of particles from the sediment surface causes significant grazing losses of particle-associated bacteria (Johnson *et al.*, 1989; Rouf and Rigney, 1993; Plante, 2000).

Using the high spatial resolution that is provided by microsensors the depth specific behavior of *Chironomus riparius* larvae has recently been shown to differently influence the bacterial community in freshwater sediments: deposit feeding by the larvae reduced the size of microbial populations at the surface of the sediment and ventilation of deeper sediment layers stimulated the growth and activity of subsurface microorganisms (Stief and de Beer, 2002). An individual larva and possible mechanisms of reworking the sediment are depicted in Fig. 3.

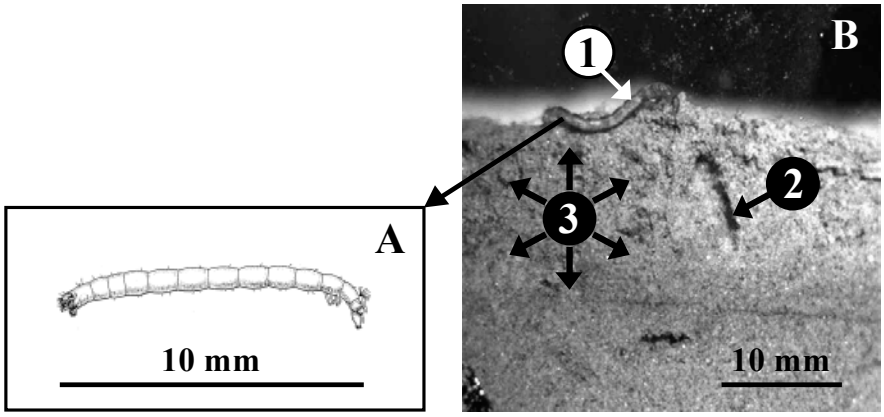

Fig. 3A, B: 4th instar larva of *Chironomus riparius* (Insecta, Diptera) (A) and a side view of a sediment bioturbated by *C. riparius* (B) showing a deposit feeding larva (1), a permanent burrow (2) and an intensely reworked zone (3).

Artificial disturbance of the sediment: sediment homogenization

Experimentally treated sediments are often used as a standardized basis for the study of certain ecological factors influencing nitrification (Caffrey *et al.*, 1993; Jensen *et al.*, 1993; Jensen *et al.*, 1994) because procedures such as sieving and homogenization reduce the heterogeneity within replicates (Svensson and Leonardson, 1996; Hansen and Jensen, 1998). Moreover, the sieving removes physical obstacles that may disturb

microsensor measurements. Nevertheless, the microbial stratification within the sediment is revoked during homogenization and sedimentary processes can be destabilized. During the settlement of homogenized sediment in experimental containers the particulate organic matter may accumulate at the sediment surface and thereby favor the thriving of heterotrophic bacteria (Stief *et al.*, accepted). Sieving and homogenization were shown to initially reduce microbial growth (Findlay *et al.*, 1990) and especially slowly growing organisms as nitrifying bacteria (Prosser, 1989) are assumed to recover more slowly from disturbance than other microorganisms (Svensson *et al.*, 2001). However, the sediments are allowed to restratify and stabilize prior to the start of the actual investigation (Rysgaard *et al.*, 1994; Pelegri and Blackburn, 1995a; Svensson *et al.*, 2001; Stief *et al.*, 2002). Steady state in the respective sediment is assumed to have reestablished when fluxes of the nutrients of interest have stabilized (Rysgaard *et al.*, 1994; Svensson *et al.*, 2001; Stief *et al.*, 2002). To clarify the potential problem of biased nitrification in such sediment incubations a precise investigation of the activity and distribution of nitrifiers is thus important.

Thesis outline

Sediment-dwelling macroinvertebrates influence benthic microbial processes by various mechanisms, e.g. by feeding, locomotive activities and by the construction of burrow-like structures. The process of nitrification plays a crucial role in freshwater sediments because it removes NH_4^+ and NO_2^- both being potentially toxic to aquatic live. Against the background of high loading of freshwater systems with NH_4^+ and NO_2^- bioturbated sediments bear ecological potentials as sinks for inorganic N compounds. The influence of bioturbation and bioirrigation on nitrification in freshwater sediments was therefore the main focus of this thesis. Special attention was given to the burrows of the investigated invertebrates where a high microbial activity (burrows as "microbial hot spots") and intense animal-microbe interactions are assumed.

Animal-related changes in nitrification might be based on changes in the structure and function of the nitrifying community, i.e. changes of the taxonomic composition, abundance, distribution and the activity of functional groups. The application of *in situ* techniques, i.e. fluorescence in situ hybridization (FISH) and microsensors allowed the determination of these parameters independent from cultivation. Moreover, the high spatial resolution of the two methods is indispensable, as nitrification is restricted to a very narrow zone of the sediment surface (around 2 mm). Depending on the concrete aim of single experiments FISH and microsensors have been supplemented by additional methods of microbial ecology (e.g. determination of nitrification potentials, total fluxes, and exoenzyme activities).

The objectives of this thesis were to i) evaluate the potential of the combined application of microsensors and FISH in freshwater sediments, ii) to obtain first insights into the *in situ* abundance, distribution and activity of nitrifying bacteria in model freshwater sediment, iii) to investigate burrows of macroinvertebrates as potential hot spots of nitrification and other mechanisms by which nitrifying bacteria are linked to bioturbating and bioirrigating macroinvertebrates.

In the following a short summary of the experiments that are included in this thesis is given (Experiment 1 – 3). Moreover, a short summary of additional experiments, which resulted in manuscripts with a co-authorship is given. It is complemented by a

more detailed description of experimental parts that were accompanied by some difficulties (Experiment 4 –6).

The first experiment (Chapter 2) was dedicated to evaluate the potentials and limitations of the combination of the two *in situ* methods (FISH and microsensors) for the investigation of nitrifying bacteria in freshwater sediments. The combined approach proved to be applicable to freshwater model sediment. The two functional groups of nitrifiers, i.e. NH_4^+- and NO_2^--oxidizing bacteria (AOB and NOB) were quantified independent from cultivation by FISH and their *in situ* metabolic activity was successfully measured with microsensors. For the first time, *Nitrospira* spp. was identified as the dominant NO_2^--oxidizing bacterium in a freshwater sediment. With the detection of *Nitrospira* spp. in various other environments this stresses the importance of *Nitrospira* spp. and not *Nitrobacter* spp. for *in situ* NO_2^--oxidation.

In the second experiment (Chapter 3) the experimental approach of the first experiment was applied to compare model sediment (´manipulated´) with untreated (´natural´) stream sediment. Since the pre-treatment of sediment is commonly accomplished prior to factorial experiments the impact of this artificial disturbance on the performance of nitrification was investigated. Using FISH and microsensors the activity and distribution of nitrifying bacteria was successfully determined: measurements in the natural sediment revealed comparably low activities and abundances of AOB and NOB and were considered as a snap-shot in a probably dynamic and heterogeneous environment. The manipulated sediment revealed higher activities and abundances of nitrifiers compared to the natural sediment, which was ascribed to a better substrate availability within the uppermost sediment layer. Moreover, we observed that in the manipulated sediment NOB persisted longer than AOB did under unsuitable conditions, i.e. at depths where the substrate and O_2 supply was low.

In the third experiment (Chapter 4) FISH and microsensors were used to address the impact of sediment-dwelling *Chironomus riparius* larvae (Insecta, Diptera) on nitrification in two freshwater sediments with different organic content. *C. riparius* larvae are known to affect microbial processes by their movements, their ventilation behavior and their feeding. With this they change the O_2 microdistribution within the

sediment and the distribution of particle-associated microorganisms. Nitrification was thus expected to be affected by *C. riparius*. The experiment revealed that the sediment type was decisive for the larval behavior and thus for the animal impact on benthic nitrification: in the organic poor sediment the extensive sediment reworking and the feeding of the larvae reduced the nitrification activity measured therein. In contrast, in the organic rich sediment net nitrification was neither influenced in the bulk sediment or due to the presence of burrows, which the larvae constructed in this sediment type. The abundances of AOB and NOB were not influenced by the larvae neither in the organic-poor nor in the organic-rich sediment.
The methodological design and the results of experiment 1 – 3 have been submitted for publication.

Why reduced nitrification activity was not reflected by reduced abundances of the nitrifiers (result of experiment 3) was investigated in experiment 4. It was assumed that the larval feeding (gut passage of particles and associated bacteria) reduced the metabolically active part of the nitrifying population but that the ribosome content of this inactive fraction was still high enough for detection with FISH. Thus, an experiment was conducted to quantify metabolically active vs. inactive nitrifiers on the fecal pellets of the larvae. Microsensor profiles were successfully recorded at the surface of the fecal pellets (300 µm in diameter) to determine the nitrification activity.
For the visualization of metabolically active cells FISH was combined with a live-dead-stain, which was supposed to allow a microscopic differentiation of metabolic active vs. inactive cells of a certain nitrifier population. The live-dead-stain which was chosen is based on the esterase substrate cFDA yielding the fluorescent carboxyfluorescein (cF) upon hydrolysis (Bunthof *et al*. 2001). When applied separately each stain (cF and FISH/DAPI) yielded signals, which could be quantified by epifluorescence microscopy. However, cells of the killed control and living cells showed the same cF signal intensity. Moreover, cF and DAPI signals were overlapping, meaning that cF containing objects could not be undoubtedly identified as bacterial cells. In future investigations metabolic activity of single cells could be targeted by showing DNA synthesis in general (Pernthaler *et al*. 2002a) or by monitoring the precursor rRNA of certain bacterial populations (Oerther *et al*. 2000, Schmid *et al*. 2001).

In Experiment 5 the contribution of single burrows of the mayfly *Ephoron virgo* (Insecta, Ephemeroptera) to microbial mineralization and to the N cycle was investigated. Investigations with *Ephoron virgo* larvae were made in a bypass flow channel of the river Rhine (Ecological lab of the University of Cologne). *E. virgo* burrows reach up to 10 cm deep into the sediment and are typically lined with fine particulate organic matter that is trapped inside the burrows due to permanent ventilation of the burrow lumen (*E. virgo* as filter-feeder). For experimental purposes larvae from lab cultures had been inserted into the sediment of the flow channel. The burrows were visible through the walls of the channel thus allowing microsensor measurements not only within the lumen but also within the walls of the burrows. Measurements showed that concentrations of O_2 and NO_3^- within the lumen are nearly as high as in the water column. Nitrification rates as well as denitrification rates were distinctly higher in the walls than in the surrounding sediment. Also exoenzyme activities were higher than in the burrow-free sediment. The results showed that *E. virgo* larvae changed the physico-chemical and microbial properties of the sediment. With this they could have a considerable impact on large scale biogeochemical processes in the sediments of the river Rhine. The results of this part of the experiment have been submitted for publication.

In a supplementary part, data on the microbial activity along the burrow walls were supposed to be complemented by the identification and quantification of total bacterial numbers (DAPI) and nitrifiers in particular (FISH/DAPI). In order to get such ´microbial imprints` of the burrow linings a variety of approaches was tested. Small sediment cores containing one branch of a burrow were taken and were sliced horizontally. The aim of this approach was to get thin slices or an imprint of a sediment cross-section on slides or filters which could be hybridized and then evaluated microscopically. Though clearly visible as distinct compartments, the burrow walls were very fragile and their sampling turned out to be difficult, i.e. sampling resulted in a collapse of the burrows when the cores were sliced. Freezing of the whole cores was supposed to overcome this problem. The perfusion of the samples with a fixative prior to the freezing (to maintain an intact cell structure) was likewise impeded by the fragility of the sediment structure. In a second approach several rows of microscopic slides had been exposed at the wall of the transparent flow channel at the time when the incubation had started. At the time of the activity measurements

(after 3 months) several burrows were visible behind the slides and it was hoped that after the recovering, fixation and embedding of the slides and of the attached particles and after FISH the burrow linings and bacteria therein would be visible by microscopy. This succeeded with one of the slides, which bore a biofilm with the shape of two opposite lines. Unfortunately, no cells were visible within this biofilm. This was either due to washout during the treatment of the slides or because the surface of the slides were not suitable for adhesion of sedimentary bacteria.
Despite the problem of getting the microbial imprint of the burrow walls also the quantification of bacteria in bulk sediment (treatment of samples as described in the method sections of the manuscripts (Chapters 2 – 4)) was difficult. This was due to low signal intensities and misleading DAPI signals (a lot of yellow fluorescent objects were interfering). Low signal intensities could not be overcome by the application of HRP probes (Pernthaler *et al*. 2002a). Finally, the samples could not be evaluated.

Experiment 6 was aimed to get microsensor profiles and microbial imprints of the burrow linings of *Chironomus riparius* and *Chironomus plumosus* larvae. Both can densely colonize (highly eutrophic) sediments of small streams and lakes. The incubations were made in slender cuvette-form aquaria. Microsensor measurements within the lumen allowed calculations of N conversions within this sediment compartment. In contrast, high concentration changes and the fragility of the burrow walls hampered the recording of steady state profiles within the burrow walls. Microscopic slides that had been exposed to the sediment did not bear biofilms of the burrow linings. Only the water-exposed part of the slides was densely colonized by bacteria. To correlate measured activities of the lumen with bacterial abundances it was necessary to compare cores with and without burrows (method see Chapter 4). The data were supplemented by measuring the nitrification potentials of sediment samples, which integrate over the (potential) activity and abundance of nitrifiers. In addition to the diffusive fluxes of NH_4^+ and NO_3^- measured with the microsensors, total fluxes were measured in order to determine the effective contribution of the burrows to the exchange of N between water and sediment.

A manuscript on the microsensor measurements within *Chironomus* burrows, nitrification potentials, as well as on diffusive and total fluxes in the sediments will be written.

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Chapter 2

In situ distribution and activity of nitrifying bacteria in freshwater sediment

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Summary

Nitrification was investigated in a model freshwater sediment by the combined use of microsensors and fluorescence in situ hybridization with rRNA-targeted oligonucleotide probes. In situ nitrification activity was restricted mainly to the upper 2 mm of the sediment and coincided with the maximum abundance of nitrifying bacteria, i.e. $1.5 \cdot 10^7$ cells cm^{-3} for ammonia-oxidizing Beta-proteobacteria (AOB) and $8.6 \cdot 10^7$ cells cm^{-3} for *Nitrospira*-like nitrite-oxidizing bacteria (NOB). Cell numbers of AOB decreased more rapidly with depth than numbers of NOB. For the first time, *Nitrospira*-like bacteria could be quantified and correlated with in situ nitrite oxidation rates in a sediment. Estimated cell-specific nitrite oxidation rates were 1.2-2.7 fmol NO_2^- $cell^{-1}$ h^{-1}.

Introduction

Nitrification in sediments has been shown to occur in narrow zones, i.e., within a few or sometimes even less than one mm (Jensen *et al*., 1993; Jensen *et al*., 1994; Lorenzen *et al*., 1998). This observation provides a challenge for the exact determination of rates and for the quantification and identification of the nitrifying community with sufficiently high spatial resolution. For the analysis of processes and populations in nitrifying biofilms and aggregates, the combined use of microsensors and fluorescence in situ hybridization (FISH) has been successfully applied (reviewed by Schramm, 2003).

This approach has so far not been used for the investigation of nitrification in freshwater sediments, partially because the low abundance of nitrifiers and the background fluorescence of sediment material were assumed to reduce the applicability of FISH (Prosser and Embley, 2002). Although numerous studies have addressed the diversity of nitrifying bacteria in sediments with molecular methods (e.g., Hastings *et al*., 1998; Kowalchuk *et al*., 1998; Whitby *et al*., 2001), information about abundance and fine-scale distribution of ammonia-oxidizing bacteria (AOB) in sediments is scarce, and nitrite-oxidizing bacteria (NOB) have been widely neglected.

The objectives of the present study were therefore (i) to evaluate the potentials and limitations of the combined use of microsensors and FISH in freshwater sediments, and (ii) to obtain first insights into the in situ abundance, vertical distribution, and activity of AOB and NOB in a model freshwater sediment.

Results and Discussion

Sediment pre-incubation

Sediment from a small lowland stream (fine sand, low organic content) that had been sieved to remove macrofauna was allowed to settle in cylindrical chambers (Jensen *et al*., 1994). Sediment chambers were incubated in the dark with stream water close to the in situ conditions, i.e., O_2 adjusted to air saturation, 15°C, NH_4^+ (50 µM) and NO_3^- (500 µM) added to concentrations only slightly higher than in situ (5-16 µM NH_4^+, 370 µM NO_3^-). After two weeks, the sediment NH_4^+ conversions had reached steady state which was proven by repeated bulk measurements of NH_4^+ in the overlying water (data not shown), demonstrating that NH_4^+ fluxes into the sediment remained stable over time. Macrofauna had been removed to allow for a stable stratification and to avoid disturbance of measurements. However, after this initial perturbation, care was taken to maintain near-in situ conditions during re-stratification of the sediment; it is therefore anticipated that the original nitrifying community re-established in the sediment columns, and that similar communities and distributions of nitrifiers are to be found under similar conditions in other freshwater sediments.

Activity of nitrifying bacteria

Nitrification activity as determined by microsensor measurements was restricted to the upper 2 mm of the sediment (Fig. 1A, B). Maximum conversion rates were found at the sediment surface, and nitrate production rates (0.2 µmol cm^{-3} h^{-1}) were comparable to rates obtained in other freshwater sediments by microsensor measurements (Jensen *et al*., 1993; Jensen *et al*., 1994; Lorenzen *et al*., 1998).

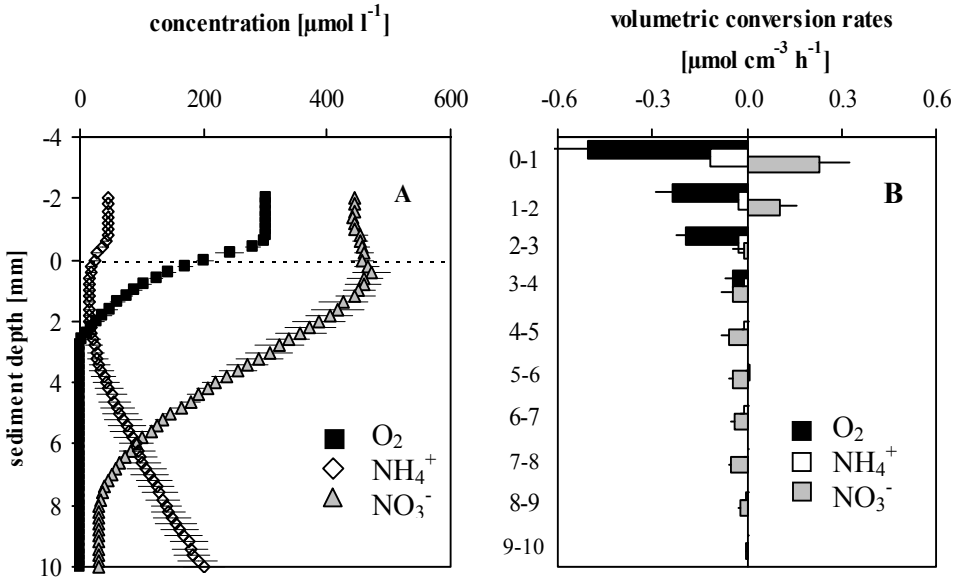

Fig. 1A, B: Mean values ± standard deviation for concentrations (A) and volumetric conversion rates (B) of O_2 (n = 5), NH_4^+ (n = 4), and NO_3^- (n = 4) along the depth of the freshwater model sediment. Dotted line corresponds to the sediment surface.

Microsensors for O_2 , NO_3^-, and NH_4^+ were prepared and calibrated according to published protocol (Revsbech, 1989; de Beer *et al*., 1997). Vertical concentration profiles in the sediment chambers were recorded using a measuring setup as previously described (Stief *et al*., 2002). Concentration profiles were used to calculate volumetric conversion rates of O_2, NH_4^+ and NO_3^- (de Beer and Stoodley, 1999; Stief and de Beer, 2002).

However, benthic macrofauna can alter conversion rates of processes within the nitrogen cycle, and stimulation (e.g., Pelegri and Blackburn, 1995, 1996) as well as inhibition (e.g., Stief and de Beer, 2002; Pelegri and Blackburn, 1996) of nitrification by macroinvertebrates have been reported. Therefore, the exclusion of macrofauna in our model sediment may have biased the obtained rates compared to undisturbed sediments. Depth-integrated reaction rates of spatially coinciding O_2 consumption, NH_4^+ consumption, and NO_3^- production were 1.00 ± 0.04 mmol m^{-2} h^{-1}, 0.18 ± 0.04 mmol m^{-2} h^{-1}, and 0.34 ± 0.14 mmol m^{-2} h^{-1}, respectively. The production of NO_3^- at a two-fold higher rate than consumption of NH_4^+ might be explained by two processes: (i) simultaneous NH_4^+ production by mineralization (de Beer *et al*., 1991) leads to an underestimation of NH_4^+ consumption by nitrification (Okabe *et al*., 1999); and (ii) NO_3^- reduction in anoxic sediment layers might provide additional NO_2^- for NO_3^- production in the oxic zone independent of NH_4^+ (Stief *et al*., 2002). Indeed, NO_3^-

consumption was detected between a sediment depth of 2.5 mm (i.e., after O_2 had disappeared) and 8.5 mm at rates of 0.02-0.06 µmol cm^{-3} h^{-1}, which is at the lower end of rates measured in other freshwater sediments (e.g., Jensen *et al*., 1994; Lorenzen *et al*., 1998).

Abundance and vertical distribution of nitrifying bacteria.

Total cell numbers were 2.4-3.4·10^9 cm^{-3}, with highest numbers at the sediment surface. Of these cells, 80% at the surface and about 50% in the deepest layers could be detected by FISH with a combination of probes EUB338 (Amann *et al*., 1990), EUB338-II, and EUB338-III (Daims *et al*., 1999). Unspecific signals (due to binding of the negative control probe NON338 (Manz *et al*., 1992) or autofluorescent cells) were detected for only ≤ 0.04% of all cells, which means that theoretically microbial populations with an abundance of less than 8·10^5 cells cm^{-3} were not detectable by FISH. Ammonia-oxidizing Beta-proteobacteria (β-AOB) as detected by probe Nso1225 (Mobarry *et al*., 1996) (Fig. 2A, B) accounted for only about 0.5% of all cells. Their cell numbers decreased even further with depth (Fig. 3). Due to these low numbers, further identification of β-AOB by FISH was not attempted.

NOB could be identified by hybridization with probe Ntspa662 (Daims *et al*., 2001a) as members of the genus *Nitrospira* (Fig. 2C, D) which accounted for maximal 2.7% of all cells. Identification was confirmed by hybridization with probe Ntspa712 specific for the phylum *Nitrospira* (Daims *et al*., 2001a) which yielded similar numbers (data not shown). *Nitrobacter*, the most commonly isolated NOB, was not detected by hybridization with probe Nit3 (Wagner *et al*., 1996). After identification of *Nitrospira* as key nitrite oxidizer in engineered systems like freshwater aquaria (Hovanec *et al*., 1998), activated sludge (Juretschko *et al*., 1998; Daims *et al*., 2001a) and bioreactors (e.g., Schramm *et al*., 1998; Okabe *et al*., 1999; Gieseke *et al*., 2001), this is yet another example of the importance of *Nitrospira* not *Nitrobacter* for nitrite oxidation in situ. *Nitrospira*-like 16S rRNA gene sequences have also been retrieved from several natural aquatic habitats like deltaic mud, caves or lakes (summarized in Daims *et al*., 2001a), and *Nitrospira* spp. can be enriched from a wide range of habitats, including soils and hot springs (Bartosch *et al*., 2002; Alawi *et al*., 2003).

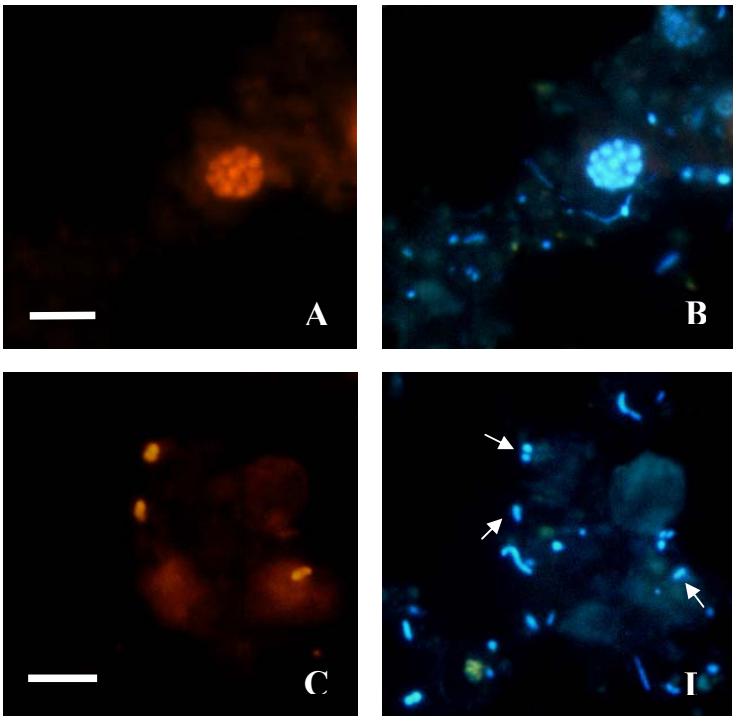

Fig. 2 A – D: Epifluorescence micrographs of a cluster of ammonia-oxidizing β-proteobacteria hybridized with probe Nso1225 (A) and *Nitrospira*-like bacteria hybridized with probe Ntspa662 (C), and the respective DAPI counterstain (B, D). Scale bars are 5 µm; arrows indicate *Nitrospira*-like cells.

Sediment cores (diameter 2.5 cm, taken from sediment columns after microsensor measurements) were sectioned horizontally and fixed with paraformaldehyde (Llobet-Brossa *et al*., 1998). Sediment sections were diluted 30-fold in a 1:1 mixture of 1× phosphate-buffered saline (10 mM sodium phosphate [pH 7.2], 130 mM NaCl) and 96% Ethanol, sonicated (3 x 60 s, 20% pulse, 109 µm amplitude) with a type UW70 probe (Sonopuls HD70; Bandelin, Berlin, Germany), and aliquots of 20-30 µl were immobilized on gelatine-coated microscopic slides. FISH with CY3-labeled oligonucleotide probes (Hybaid Interactiva, Ulm, Germany), counter staining of all cells with 4′,6-diamino-2-phenylindole (DAPI; 0.5 µg ml^{-1}), and microscopic analysis was according to published protocol (Pernthaler *et al*., 2001).

However, this is the first report of not just detection but also quantification and distribution of *Nitrospira*-like NOB in a quasi natural system, and to correlate these data with in situ activity measurements. With a size of 0.4 - 0.8 µm (Fig. 2C,D) the cells were somewhat larger than previously detected by FISH (e.g., Schramm *et al*., 1998), but still well in the range of cultured *Nitrospira* (Bock, 1992; Ehrich *et al*., 1995).

Although cell numbers of *Nitrospira*-like NOB also decreased with depth, their abundance remained relatively high (0.4-1.0 % of all cells), even in the deeper, anoxic layers (Fig. 3). These results are in agreement with previous studies showing that (i) the abundance of nitrifying bacteria decreases with depth in freshwater sediments (Whitby *et al*., 2001); (ii) the numbers of NOB might be 3-30 times higher than that of AOB in sediments (Smorczewski and Schmidt, 1991), nitrifying aggregates (Schramm *et al*., 1999), or biofilms (Gieseke *et al*., 2001); and (iii) NOB of the genus *Nitrospira* maintain a ribosome content high enough for detection by FISH even in the absence of O_2 (Schramm *et al*., 1998; Okabe *et al*., 1999; Schramm *et al*., 2000; Gieseke *et al*., 2001). The latter might indicate an adaptation of *Nitrospira* sp. to survive or even thrive under anoxic conditions, or be simply a result of low turnover/slow decay of ribosomes in *Nitrospira* sp. (Morgenroth *et al*., 2000). Compared to the enumeration of nitrifying bacteria in freshwater sediments by the most probable number method (Hall, 1986; Smorczewski and Schmidt, 1991; Hastings *et al*., 1998; Pauer and Auer, 2000; Whitby *et al*., 2001), cell numbers determined by FISH in the present study are 1-4 orders of magnitude higher. This finding is consistent with the general observation that cultivation-dependent methods severely underestimate the actual size of microbial populations (Amann *et al*., 1995), which is also true for nitrifying bacteria (Hall, 1986).

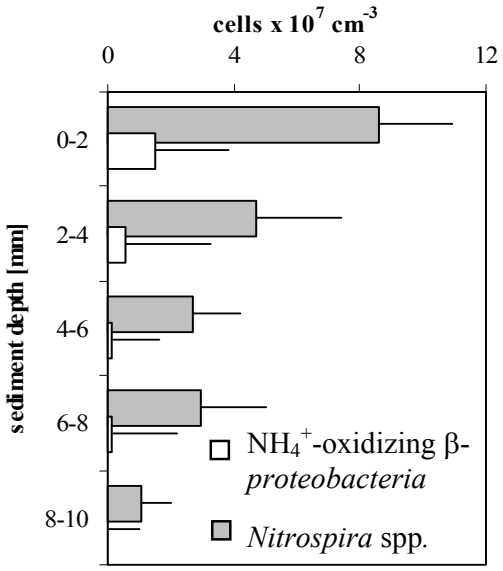

Fig. 3: Mean values ± standard deviation for cell numbers of ammonia-oxidizing β-proteobacteria hybridized with probe Nso1225 (Mobarry *et al*., 1996) (n = 3) and nitrite-oxidizing *Nitrospira* spp. hybridized with probe Ntspa662 (Daims *et al*., 2001a) (n = 3) along the depth of the sediment.
All FISH counts were corrected by subtracting cell numbers obtained with control probe NON338. For probes EUB338 and Ntspa662, 20 randomly chosen microscopic fields (corresponding to 1000 to 2000 DAPI-stained cells) were investigated. For probes Nso1225 and NON338, 200 to 400 microscopic fields were analyzed to account for the low cell numbers and the uneven distribution in the sample; total cell counts of 20 microscopic fields were then extrapolated to the area analyzed for FISH-signals. The absolute numbers of FISH-positive cells were calculated for each probe using the relative FISH-positive counts (as percentage of DAPI-stained cells) and the total cell counts from separately conducted DAPI stains of the sediment samples on black polycarbonate membrane filters (pore size, 0.2 µm; Osmonics Inc., Livermore, California, USA).

Cell-specific nitrification rates

Using volumetric conversion rates of NH_4^+ and NO_3^-, and cell numbers of β-AOB and NOB in the nitrification zone, the cell-specific NH_4^+ oxidation and NO_3^- production rates were estimated to be 1.3-8 fmol NH_4^+ $cell^{-1}$ h^{-1} and 1.2-2.7 fmol NO_3^- $cell^{-1}$ h^{-1}, respectively. The cell-specific NH_4^+ oxidation rates are at the lower end of rates found in the literature (1-30 fmol $cell^{-1}$ h^{-1}; Prosser, 1989), and are most likely underestimated due to the underestimation of NH_4^+ consumption rates (see above). In contrast, the cell-specific NO_3^- production rates are high compared to 0.01-0.07 fmol $cell^{-1}$ h^{-1} calculated for *Nitrospira* spp. in nitrifying aggregates (Schramm *et al*., 1999)

and biofilms (Gieseke *et al*., 2001). This observation might be explained in several ways: (i) different strains of *Nitrospira* might be present in the sediment and in the bioreactors, and, considering the broad phylogenetic diversity of the genus (Daims *et al*., 2001a), the specific NO_2^- oxidation rates might greatly differ between these different strains. To approach this hypothesis, *Nitrospira*-like 16S rRNA sequence data need to be retrieved from the sediment, a difficult task due to the relative low abundance of *Nitrospira*. Although in principle *Nitrospira*-specific PCR can be used, the published primers (Dionisi *et al*., 2002) do not target the whole genus *Nitrospira* and yield only sequence information of about 150 bp, too little for meaningful phylogenetic analysis. Therefore, further identification of *Nitrospira*-like NOB in the sediment has to await further method development. (ii) the cell-specific NO_2^- oxidation rates of *Nitrobacter* are much higher (5-40 fmol $cell^{-1}$ h^{-1} ; Prosser, 1989) than those reported for *Nitrospira*. Even though undetectable by FISH in the sediment, i.e. at cell numbers $\leq 8 \cdot 10^5$ cells cm^{-3}, *Nitrobacter* might significantly contribute to the production of NO_3^-; and (iii) the occurrence of other NOB, e.g., of the genus *Nitrococcus* or *Nitrospina*, which have so far only been detected in marine environments, cannot completely be ruled out.

Concluding remarks

The combined use of microsensors and FISH proved to be applicable to freshwater sediments. For the first time, the in situ abundance of nitrifiers in a sediment could be determined with sufficiently high spatial resolution to be combined with fine-scale activity measurements. *Nitrospira*-like bacteria were the dominant if not sole nitrite-oxidizers in the sediment. Limitations for the application of the combined approach to sediments are the potential occurrence of bioturbating macrofauna that may disturb microsensor measurements and interpretation of profiles in a real natural sediment, and the extremely time-consuming counting procedure during FISH analysis; despite an enormous counting effort, populations with lower abundance ($<10^6$ cells cm^{-3} of sediment) may remain undetectable by FISH due to the background of unspecific binding and autofluorescence. While the application of automated image analysis (e.g., Daims *et al*., 2001b) will in the future speed up FISH analysis even in difficult samples like sediments, it will most likely not improve its detection limit.

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Chapter 3

Distribution and activity of nitrifying bacteria in natural and manipulated stream sediment as determined with *in situ* techniques

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Abstract

Nitrification was studied with a combination of microsensors and fluorescence *in situ* hybridization (FISH) in sandy sediment of a small lowland stream. Comparative measurements were performed in both the intact field sediment ('natural sediment') and after processing sediment from the same site for a laboratory incubation experiment ('manipulated sediment'). In the natural sediment nitrification activity and abundances of nitrifiers were markedly low. In contrast, nitrification activity in the manipulated sediment (sieved, homogenized, and incubated for five weeks in the laboratory with NH_4^+-enriched stream water) was significantly higher than in the natural sediment. Abundances of NH_4^+-oxidizing bacteria (AOB) and *Nitrospira* sp. in the 0-2 mm layer were 12 and 3-5.5 times higher than in the natural sediment, respectively. AOB disappeared faster from anoxic sediment layers than *Nitrospira* sp., indicating that the latter was more persistent under anoxic conditions. Higher activities and abundances of nitrifiers near the sediment-water interface of the manipulated sediment were explained by (i) the additional NH_4^+ supply via the overlying water and (ii) by adverse conditions for nitrification in the field. In conclusion, the snapshot measurement in the natural sediment revealed the spatial heterogeneity created by stream dynamics, whereas the sediment manipulation provided semi-natural microcosms with reduced heterogeneity ready for factorial experiments.

Introduction

Metabolic activities of nitrification, the microbial oxidation of NH_4^+ to NO_3^-, have been measured with microsensors in intact field sediments (Sweerts and de Beer, 1989; Lorenzen *et al*., 1998; Meyer *et al*., 2001) as well as in model, i.e. sieved and homogenized sediments (Jensen *et al*., 1993; Jensen *et al*., 1994; Stief *et al*., 2002). Only fine scale measurements of this type allowed to precisely localize the narrow surface layer of active nitrification that may be overlooked by conventional pore water analysis (Henriksen *et al*., 1993; Rysgaard *et al*., 1995) or mass balance studies (Christensen *et al*., 2000; Van Luijn *et al*., 1999). Using microsensors the process of nitrification could be localized in the upper few mm of the sediment and the influence of other sedimentary processes like O_2 production by photosynthesis on nitrification and the coupling of nitrification and denitrification was demonstrated. However, the

pure process analysis of sedimentary nitrification leaves in the dark which microorganisms perform nitrification in this particular habitat, in which quantities these microorganisms occur, and how they are spatially organized in the sediment. So far the identification and quantification of nitrifying bacteria in natural sediments has been addressed, for instance, by the most probable number technique (Pauer and Auer, 2000; Smorczewski and Schmidt, 1991; Whitby *et al*., 2001; Bianchi and Lefevre, 1999). Cell numbers detected in these studies were 10^4 –10^5 g^{-1} for AOB and 10^3 g^{-1} for NOB and quantified nitrifiers could be identified as members of the genera *Nitrosospira*, *Nitrosomonas* and *Nitrobacter* using immunofluorescence analysis and by enrichment cultures. However, using this cultivation-dependent technique, the actual size and structure of microbial communities in general (Amann *et al*., 1995) and of nitrifying populations in particular (Hall *et al*., 1996) can be severely misjudged due to non-culturability of some microorganisms and selective enrichment of others. To date, this possible bias can be circumvented by using cultivation-independent techniques such as FISH (Amann and Kühl, 1998). The combined application of microsensors and FISH for nitrification studies has so far been used exclusively in wastewater biofilms (Schramm *et al*., 1997; Okabe *et al*., 1999; Gieseke *et al*., 2001) and in a model freshwater sediment (Altmann *et al.*, accepted), but not in intact field sediments as presented here.

While measurements in naturally heterogeneous field sediments help depict the spatial and temporal dynamics of a particular habitat, working with sieved and homogenized sediments is a commonly used approach for factorial experiments under defined conditions. Sieving removes physical obstacles that may disturb microsensor measurements and homogenization produces a high degree of similarity between replicate sediment cores (Svensson and Leonardson, 1996; Davidsson *et al*., 1997; Hansen and Jensen, 1998). On the other hand, the harsh pretreatment of sediments has been questioned because it destroys the vertical microbial stratification and has a negative effect on the persistence of slowly growing members of the microbial community, e.g. nitrifiers mixed into anoxic subsurface layers (Findlay *et al*., 1990; Svensson *et al*., 2001). Recovery of these microorganisms and the establishment of a close-to-natural microbial stratification may take as long as 2-3 weeks under laboratory conditions (Tuominen *et al*., 1999). For this reason a preincubation of the

manipulated sediments is usually scheduled prior to the actual experimental treatments (Svensson *et al*., 2001; Stief *et al*., accepted).

The major aim of our study was to investigate for the first time the activity and distribution of nitrifying bacteria *in situ*, i.e. in a natural stream sediment with microsensors and FISH. Secondly, we studied the performance of nitrification after the same natural sediment was sieved, homogenized and incubated in natural stream water and at ambient temperature. The same set of methods was used to follow the reorganization of nitrification in this manipulated sediment during a 5-week incubation in the laboratory.

Materials and Methods

Sampling site. Sandy sediment for i) measurements in the natural sediment and ii) for those in the manipulated sediment was sampled in May 2002 from a small lowland stream, Rittrumer Mühlenbach (Northern Germany). The stream crosses an area of glacial sand accumulation in the Wildeshausener Geest. Average flow velocity was 0.2 m s^{-1} causing ripple formation on the sediment surface, with detritus mainly accumulating in the troughs of this ripple system. Concentrations of NH_4^+ and NO_3^- in the overlying water at the day of sampling were 10 and 500 µmol l^{-1}, respectively. The organic content of the sediment ranged between 1.0 and 5.1 % (n = 15) and the sediment was only sparsely inhabited by macrofauna.

Experimental design. For the analysis of the natural sediment intact sediment cores of 15 cm length and 7.5 cm ∅ were taken in replicates with Plexiglas® cylinders from a water depth of 50 cm. To avoid disturbances of the sediment surface the enclosed water phase was hermetically sealed and the cores were carefully transported to the lab within 45 min. The microsensor measurements were performed immediately in natural stream water and at *in situ* temperature (9.6°C). During the measurements the overlying water was held turbulent to ensure the formation of a diffusive boundary layer. For the analysis of the manipulated sediment, in the same reach surface sediment (0-5 cm) was collected with a flat shovel and transferred into buckets. In the laboratory the sediment was immediately sieved to remove macrofauna, large detritus and pebbles, filled into beakers (∅ 9 cm) up to a height of 13 cm, and allowed to settle over night (Jensen *et al*., 1994). Six beakers were submersed in 3 basins containing 15

l of unfiltered stream water and were incubated in the dark at *in situ* temperature (9.6°C). O_2 concentration in the overlying water was adjusted to air saturation. To avoid NH_4^+ limitation of nitrification the concentration of NH_4^+ in the overlying water was kept constant by repeated additions of 50 µmol l^{-1} of a NH_4Cl stock solution. Concentrations of NH_4^+, NO_3^- and NO_2^- in the basins were checked regularly using photometric test kits by Merck (Germany). After 3 and 5 weeks of incubation microsensor measurements and sediment samples for FISH were taken.

Microsensor measurements. Microsensors for O_2 (Revsbech, 1989), NO_3^- (de Beer and Sweerts, 1989), and NH_4^+ (de Beer and van den Heuvel, 1988) were prepared, calibrated, and operated in a measuring setup as previously described (Stief *et al*., 2002). Replicate profiles were recorded at randomly chosen spots of the sediment surface down to a depth of 10 mm. Information on the nitrification activity was obtained by calculation of the fluxes of O_2, NO_3^-, and NH_4^+ at the sediment water interface (J_{swi}) and at the oxic/anoxic interface (J_{anx}) using Fick´s first law of diffusion. Diffusion coefficients for of O_2, NO_3^-, and NH_4^+ in the overlying water (D_w) were taken from the literature (Schramm *et al*., 1999) and corrected for the measuring temperature (9.6 °C) by applying the Stokes-Einstein-relation. The sediment diffusion coefficients of O_2, NO_3^-, and NH_4^+ (D_s) were calculated from an averaged profile of diffusivity as measured with a H_2 diffusivity sensor (Unisense A/S, Denmark) (Revsbech *et al*., 1998; Stief *et al*., accepted). Net nitrification activity, i.e. the areal conversion rate of NO_3^- and NH_4^+ within the oxic layer, was defined as the sum of J_{swi} and J_{anx} of each solute.

FISH and total cell counts. After the microsensor profiles had been recorded one sediment core (∅ 2.5 cm) was taken from each sediment cylinder or beaker, respectively, and was sectioned horizontally into 2 mm-thick slices down to a depth of 10 mm. Sediment slices were prepared for FISH as described in (Altmann *et al.*, accepted). For FISH a set of oligonucleotide probes specific for i) *Eubacteria* (probe mix EUB 338 (Amann *et al*., 1990), EUB II and EUB (Daims *et al*., 1999)), ii) NH_4^+-oxidizing β-*proteobacteria* (Nso1225, (Mobarry *et al*., 1996)), and iii) for the NO_2^--oxidizing genera *Nitrobacter* (Nit3, (Wagner *et al*., 1996)) and *Nitrospira* (Ntspa662, (Daims *et al*., 2000)) was used. Probes Nit3 and Ntspa662 were used with an equimolar amount of a competitor oligonucleotide as indicated in the references. To

check for unspecific binding samples were hybridized with control probe Non338 (Manz *et al*., 1992). All probes were purchased labeled with the fluorescent dye CY3 (Hybaid Interactiva, Ulm, Germany). FISH and counter staining of all cells with 4′,6-diamino-2-phenylindole (DAPI; 0.5 µg ml^{-1}) were performed according to published protocols (Pernthaler *et al*., 1998). Counting was adapted to the low numbers of FISH-positive cells and their uneven distribution in the aliquots as described in (Altmann *et al.*, accepted). To correct for cell losses during hybridization total bacterial cell numbers were determined separately by DAPI-staining of sonicated and diluted sediment samples on black membrane polycarbonate filters (pore size, 0.2 µm; Osmonics Inc., Livermore, California, USA). The absolute numbers of FISH-positive cells were calculated for each probe using the relative FISH-positive counts (as percentage of DAPI-stained cells) and the total cell counts.

Results

Natural sediment: Microsensor measurements. Concentration profiles of O_2, NH_4^+, and NO_3^- as measured in the natural sediment are presented in Fig. 1 A-C. To highlight the heterogeneity between replicate cores, profiles are shown for each individual core.

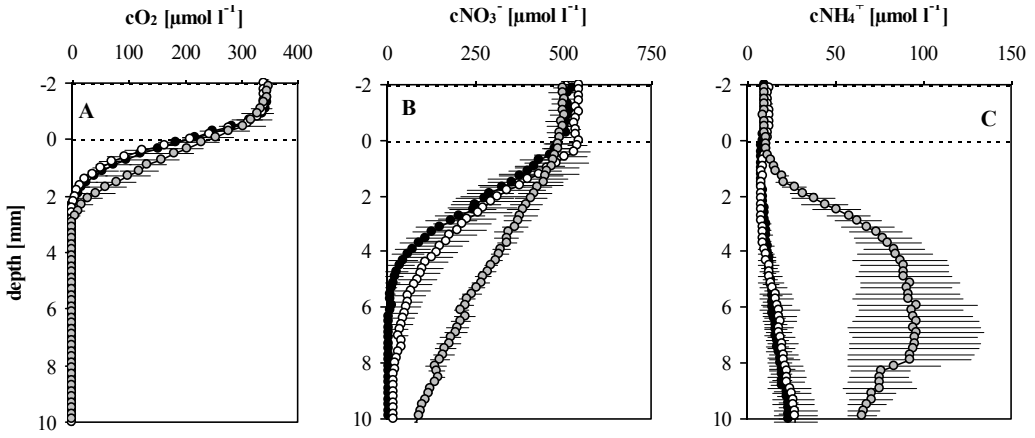

Fig. 1 Vertical concentration profiles of O_2 (A), NH_4^+ (B), and NO_3^- (C) as measured in the naturally stratified sediment of a lowland stream. Average profiles (+ 1 SD, n ≥ 3) of three replicate sediment cores (c1-3) are plotted separately.

In all three cores O_2 became depleted within the uppermost 2 mm of the sediment, but in core 3 this decrease in O_2 concentration was less steep indicating lower bacterial activity compared to the other cores. NO_3^- was continuously depleted over a depth of approximately 6 mm in cores 1 and 2, but did not reach 0 µmol l^{-1} within the sampled sediment depth of core 3. NH_4^+ concentration in the cores 2 and 3 decreased slightly within the upper 2 mm and increased again in the deeper layers. In contrast, core 1 was characterized by comparably high NH_4^+ concentration in deeper layers and a steep concentration gradient at the oxic/anoxic interface. The local fluxes of O_2, NH_4^+, and NO_3^- were directed into the oxic layer of the natural sediment (Tab. 1).

Table 1. Local fluxes of O_2, NH_4^+, and NO_3^- [µmol m^{-2} h^{-1}] within the presumable nitrification layer of the sediment from a lowland stream. Natural sediment: Means (± 1 SD, n ≥ 3) within each of three replicate cores. Manipulated sediment: Means of three replicate cores within one sampling date (± 1 SD, n ≥ 9). Positive values = production, negative values = consumption.

sediment type	core	time	J_{O2}	J_{NH4}	J_{NO3}
natural	1	week 0	-796 (62)	-89 (27)	-123 (86)
natural	2	week 0	-925 (121)	-10 (3)	+24 (179)
natural	3	week 0	-600 (60)	-12 (5)	-34 (15)
manipulated	1-3	week 3	-425 (57)	-101 (19)	+249 (152)
manipulated	1-3	week 5	-494 (75)	-77 (21)	+247 (53)

Natural sediment: Nitrifier abundances. Total cell numbers in the natural sediment were 8 – 13.9 · 10^9 cells cm^{-3} showing almost equal distribution throughout the sampled sediment depth. Overall detection rate with FISH, as determined with combined oligonucleotide probes EUB 338, EUB II, and EUB III, was 40-50% of total bacterial cells. Unspecific signals (i.e. binding of probe NON338 and autofluorescent cells) were not detected in this natural sediment. Absolute abundances and the vertical distribution of NH_4^+- oxidizing β-proteobacteria (AOB) and of the NO_2^--oxidizing bacteria (NOB) are shown in Fig. 2.

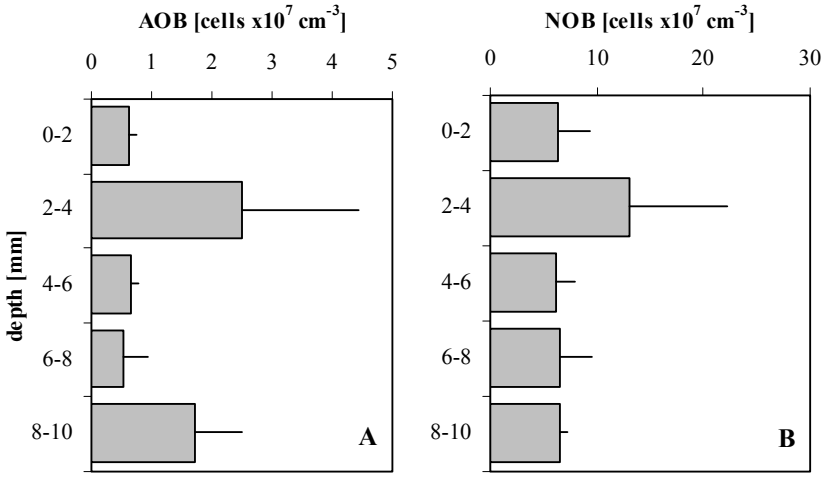

Fig. 2 Vertical distribution of NH_4^+-oxidizing β-proteobacteria (AOB, A) and NO_2^--oxidizing *Nitrospira* sp. (NOB, B) as revealed with FISH in the naturally stratified sediment. Average abundances (+ 1 SD) of three replicate sediment cores are given.

AOB abundance was highest in the 2-4 mm layer but peaked again in the deepest sampled layer, i.e. in 8-10 mm depth, accounting for 0.26% and 0.16% of total cells, respectively. FISH indicated that in the natural sediment NOB were represented by the genus *Nitrospira*, whereas the commonly isolated genus *Nitrobacter* spp. was not detected. Cells of *Nitrospira* ssp. were 5-9 times more abundant than the NH_4^+-oxidizing β-proteobacteria and here too the maximum numbers were found in the 2-4 mm layer where they made up 1.2% of all bacterial cells. Cells of *Nitrospira* ssp. were also detected in remarkably high abundances in the anoxic layers of the sediment.

Manipulated sediment: Microsensor measurements. In contrast to the natural sediment, the high similarity between replicate cores of the manipulated sediment allowed averaging of all measured profiles within one sampling date. Averaged concentration profiles of O_2, NH_4^+, and NO_3^- recorded after 3 and 5 weeks of incubation in the laboratory are shown in Fig. 3.

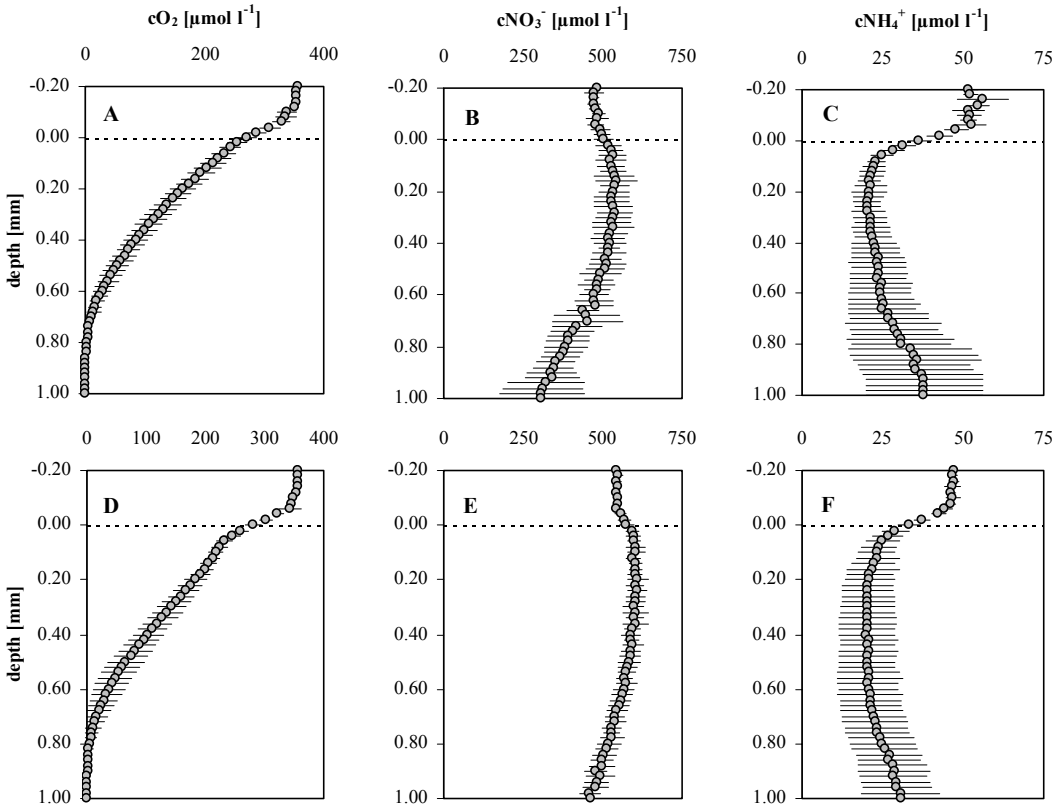

Fig. 3: Vertical concentration profiles of O_2, NH_4^+, and NO_3^- as measured in the manipulated sediment after a 3-week (A-C) and 5-week (D-F) incubation in the laboratory. Average profiles of three replicate sediment cores are plotted ($\pm$ 1 SD, n ≥ 9).

O_2 penetration depth after 3 weeks was 7 mm. NH_4^+ concentration that was held constant at 50 µmol l^{-1} in the overlying water, decreased within the upper 1 mm of the sediment and increased slightly in the deeper layers. The NO_3^- concentration peak in the upper 1 mm of sediment was followed by an extended zone of slightly decreasing NO_3^- concentration, but NO_3^- was not completely consumed within the sampled sediment depth. After 5 weeks of incubation the concentrations of O_2, NH_4^+, and NO_3^- showed the same vertical patterns than 2 weeks before, but O_2 penetration depth

increased from 7.0 to 7.6 mm. At both sampling dates fluxes of O_2 and NH_4^+ were directed into the nitrification zone, whereas NO_3^- was released from this zone (Tab. 1).

Manipulated sediment: Nitrifier abundances. Total cell numbers after the 3-week incubation were 9.5 – 11.1 · 10^9 cm^{-3} showing a homogeneous distribution throughout the sampled sediment depth. Numbers in the uppermost sediment layer were 9.9 · 10^9 cm^{-3} after another 2 weeks of sediment incubation, but were conspicuously lower in the layers below, i.e. 4.1 · 10^9 cm^{-3}. Overall detection rate with FISH as revealed by hybridization with the EUB probe mix was 70-80% in the oxic sediment and 50-60% in the deeper layers. Unspecific signals due to binding of control probe NON338 or autofluorescence were not detected. Absolute abundances of AOB and NOB varied spatially and temporally (Fig. 4): After the 3-week incubation the maximum abundance of AOB was found in the 0-2 mm layer of the sediment (Fig. 4A). In this layer AOB made up 0.7% of total bacterial cells.

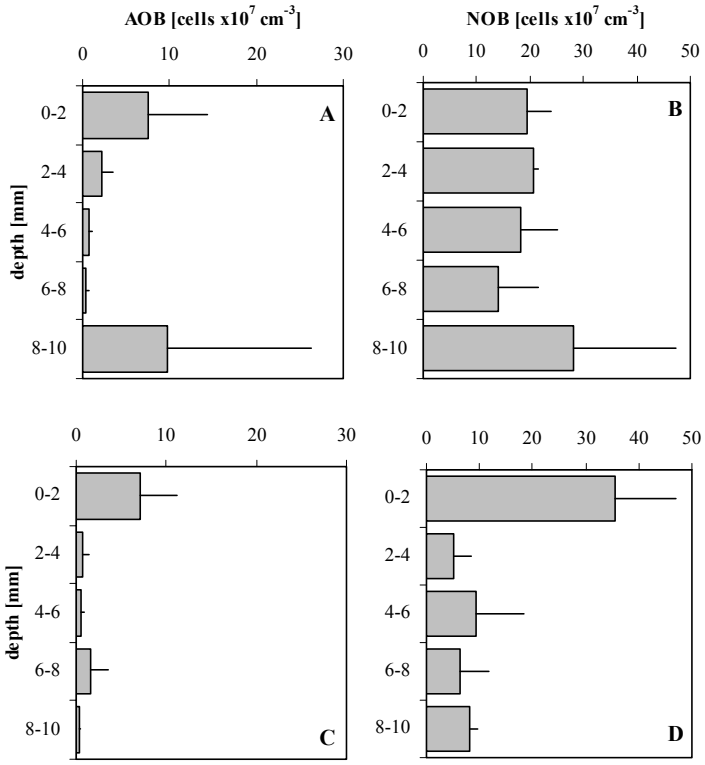

Fig. 4 Vertical distribution of AOB (A+B) and NOB (C+D) as revealed with FISH in the manipulated sediment after a 3- (A+C) and 5-week (B+D) incubation in the laboratory. Average abundances (+ 1 SD) of three replicate sediment cores each are given.

In deeper layers their proportion decreased to 0.04% of total cells. In the 8-10 mm layer their averaged abundance was exceptionally high, but this was due to the detection of two cell clusters (70 and 90 cells, respectively) in just one out of three replicate cores. After another 2 weeks of incubation the absolute abundances and the vertical distribution of the AOB were the same as before, except for the absence of a secondary abundance peak in the 8-10 mm layer (Fig. 4C). NOB in the manipulated sediment were also represented by the genus *Nitrospira*, whereas *Nitrobacter* spp. was not detected at any time of the incubation. Cells of *Nitrospira* spp. were evenly distributed over the whole sampled sediment depth after 3 weeks of incubation and made up approximately 1.9% of total bacterial cells (Fig. 4B). After 5 weeks, however, a distinct abundance maximum had developed at the sediment surface and cell numbers there had increased to 3.6% of total cells (Fig 4D).

Discussion

Natural sediment

The investigated stream sediment was characterized by negligible low nitrification activity as indicated by the absence of distinct oxic NO_3^- peaks in the majority of the measured profiles. On average NO_3^- was consumed throughout the whole sampled sediment depth. Dissimilatory NO_3^- reduction was probably the principal pathway of NO_3^- consumption in this sediment. Additionally performed measurements in illuminated sediments (light intensity 500 $\mu E\ m^{-2}\ s^{-1}$, data not shown) did not reveal measurable photosynthetic capacity and hence, in our sediments photosynthesis can be ruled out as a significant sink for NO_3^-. However, the areal rates of NO_3^- consumption were at the lower end of the rates reported in the literature (Sweerts and de Beer, 1989; Lorenzen *et al*., 1998; Meyer *et al*., 2001). These low rates of N cycling in our sediment (i.e., nitrification and denitrification) were probably due to its low content of readily usable organic matter, which limited the supply of NH_4^+ and CO_2 for nitrification and of electron donors for dissimilatory NO_3^- reduction. Secondly, the low rates may be indicative of the physical dynamics in natural stream sediment, as the water currents permanently reshape the sediment surface, e.g. cut off formerly oxic layers from O_2 (Wulff *et al*., 1997). Such a disturbance may inhibit nitrification in

particular, since O_2 is needed for the performance of this process (Caffrey *et al*., 1993; Sloth *et al*., 1995). Moreover, nitrifying bacteria are characterized by low growth kinetics (Prosser, 1989) meaning that recovery from disturbances may be particularly slow (Findlay *et al*., 1990; Svensson *et al*., 2001). Supportive of the dynamic character of the natural sediment is also the heterogeneity of solute conversions within and between the replicate sediment cores.

Applying oligonucleotide probes specific for NH_4^+-oxidizing β-proteobacteria (AOB) and the NO_2^--oxidizing genera *Nitrospira* and *Nitrobacter* (NOB) we localized and quantified members of the two physiological groups of nitrifying bacteria *in situ*, i.e. in a natural freshwater sediment that was sampled directly in the field. Most probable number counts (MPN) of culturable nitrifying bacteria in other natural freshwater sediments led to abundances in the order of 10^4 to 10^5 cells per g (Pauer and Auer, 2000; Smorczewski and Schmidt, 1991; Whitby *et al*., 2001) and were thus 1-2 orders of magnitude lower than those detected in our *in situ* study. This discrepancy is apparently due to the fact that methods like MPN or enrichment cultures are highly biased because they are cultivation-dependent and thus often underestimate the actual size of bacterial communities in general (Amann *et al*., 1995) and of nitrifying populations in particular (Hall *et al*., 1996). However, we are also aware of possible disadvantages of the FISH technique: With FISH the total population size of nitrifiers can be accurately estimated, but a discrimination of active vs. inactive cells is difficult because nitrifiers are able to maintain a relatively high ribosome content even if they are not active (Batchelor *et al*., 1997; Morgenroth *et al*., 2000). In our study this problem was overcome though by additionally using microsensors in order to give the precise site and the magnitude of nitrifying activity.

In the investigated natural sediment the genus *Nitrobacter*, which is the most commonly isolated NO_2^--oxidizer, was not detected with the *in situ* technique of FISH. Instead, we identified and quantified the genus *Nitrospira* as a major representative of NOB. This genus has recently been detected in several habitats by means of comparative 16S rRNA analysis (Hovanec *et al*., 1998; Juretschko *et al*., 1998; Cho and Kim, 2000; Li and Horikoshi, 1999; Todorov *et al*., 2000). Our finding thus further confirms the recently assumed dominance of *Nitrospira* rather than *Nitrobacter* not only in engineered systems and model sediments, but also in intact freshwater sediment.

Maximum abundances of both the AOB and NOB in the natural sediment occurred in the 2-4 mm horizon that was apparently anoxic during our microsensor measurements. In a previous study applying microsensors and FISH to model sediment (Altmann *et al.*, accepted) highest abundances of AOB and NOB were found in the uppermost sediment layer and coincided well with the conversion maxima of O_2, NH_4^+ and NO_3^-. However, in the present study, the abundance peak of AOB and NOB in the 2-4 mm layer clearly did not coincide with concentration minima or maxima of nitrification substrates or products. Two main reasons can be considered for the presence of the abundance peak in this apparently anoxic layer: i) In the field, i.e. just before the cores were taken and measured in the laboratory, O_2 may have also been present in the 2-4 mm layer. O_2 penetration depth may have decreased due to the lower current velocity during our measurements and hence advective transport of O_2 into the sediment (Huettel and Rusch, 2000) was probably less significant. If this scenario was true, then under field conditions the nitrifier population has met ideal growth conditions at the lower end of the oxic zone in which the supply of NH_4^+ from deeper layers was higher compared to the upper end of the oxic zone. ii) The layer of maximum abundances of AOB and NOB may represent the former surface of the sediment which has recently been slopped and cut off from O_2 supply by the horizontal shifting of ripple structures. The newly reshaped sediment surface may not have been recolonized by nitrifiers to form a new abundance maximum.

Manipulated sediment

In contrast to the natural sediment the sieved and homogenized sediment was characterized by a distinct nitrification zone in the uppermost layer of the sediment. Even though, nitrifiers obviously did not tap the full potential of nitrification, as was indicated by incomplete depletion of NH_4^+ within the oxic sediment layer. Since O_2 was present to a depth of 7 mm, nitrification was limited neither by NH_4^+ and O_2, but rather by other factors like a population size too small to consume the continuously supplied NH_4^+ to its limit or a shortage of CO_2 which was internally produced by mineralization. However, nitrification activity, i.e. the areal production of NO_3^- within the oxic zone was comparable to other freshwater studies applying microsensors to experimentally manipulated sediment (Jensen *et al*., 1993; Jensen *et al*., 1994; Stief *et al*., 2002). In contrast to the higher nitrification rates in the manipulated sediment,

dissimilatory NO_3^- consumption was lower than in the natural sediment as it was indicated by the incomplete consumption of NO_3^- within the sampled sediment depth. This was apparently due to a lack of electron donors for NO_3^- reduction as a consequence of the low sedimentary organic content. It seems likely that by sieving the sediment dissolved and fine particulate organic matter has partially been washed out from the sediment. Moreover, after the sediments had settled a sharp separation into the sandy sediment and a fluffy organic matter layer at the sediment surface of 2-3 mm thickness was observed suggesting that the sandy sediment below this fluffy layer became poor in at least particulate organics. Indeed organic content of the manipulated sediment was lower compared to the natural sediment and decreased even further with sediment depth (data not shown). Both nitrification and denitrification activity did not change during the 2 week incubation indicating the stability of the manipulated sediment during this period. Moreover, the sieving and homogenization of the sediment clearly reduced heterogeneity within and between the sediment cores as was indicated by the low variability between concentration profiles. From this point of view this pretreatment of sediment and a time course of 2 weeks after initial restratification hence presents a suitable basis for the performance of factorial experiments.

Sediment manipulation has most likely created also a homogeneous vertical distribution of both AOB and NOB at the start of the experiment. During the following laboratory incubation both populations showed a different pattern of vertical restratification: AOB were found at highest abundances in the 0-2 mm layer already after 3 weeks and this was still the case after 5 weeks of incubation. In contrast, NOB were still homogeneously distributed after 3 weeks of incubation, but finally showed signs of vertical stratification after 5 weeks of incubation. The AOB population quickly established an abundance maximum near the sediment-water interface at the expense of deeper layers, even though O_2 penetrated 7 mm deep into the sediment and was thus not limiting. This preferential growth within the uppermost sediment layer was probably due to the continuous supply of NH_4^+ via the overlying water, while in the deeper oxic layers AOB were probably limited by the low supply of NH_4^+ from the anoxic zone. A spatial “plasticity” of the microdistribution of nitrification activity, i.e. the shifting of nitrification activity in response to the NH_4^+ source was also demonstrated by (Jensen *et al*., 1993). Given that NO_2^- production by AOB was the

only significant source of NO_2^- in the sediment, NOB was expected to thrive better at the sediment water interface than in deeper layers, and so it was near the end of the experiment. Obviously though, NOB in this experiment persisted longer the unsuitable conditions in the deeper layers (i.e., lack of O_2 and NO_2^-) than did AOB. This superior persistence may have contributed to the delayed stratification of NOB compared to that of AOB. Similarly, a slower decay of *Nitrospira* spp. compared to that of ß-AOB was observed in anoxic layers by (Altmann *et al.*, accepted). This feature of *Nitrospira* sp. was assumed to be either due to its adaptation to survive or even thrive under anoxic conditions or to be a result of the slow decay of ribosomes in this NO_2^--oxidizer. Supportive of the superior persistence of NOB in deeper sediment layers were also the higher NO_2^-- compared to NH_4^+-oxidation rates which could be evoked in slurry incubations of various sediments (P. Stief, unpublished results).

Comparison of natural and manipulated sediment

Despite the destruction of the microbial stratification by sieving and homogenizing, the manipulated sediment was finally characterized by higher abundances of AOB and NOB as well as by conspicuously higher nitrification activity. This was probably due to the additional supply with NH_4^+, which can be a major factor limiting nitrification in sediments (de Beer *et al.*, 1991; Jensen *et al.*, 1993). On the other hand, sediment pretreatment and laboratory incubation have obviously produced a loss of electron acceptors and NH_4^+ within the sediments, which resulted in lower consumption of O_2 and lower dissimilatory consumption of NO_3^-. Possible reasons for this loss are the washout of dissolved substances during sieving and the "dilution" of otherwise patchily distributed particulate organic matter during homogenization and by the accumulation of fine particulate organic matter at the sediment surface. This may constitute non-favorable conditions for the performance of both nitrification and denitrification because NH_4^+, CO_2, and electron donors may become limiting. In our case though, at least NH_4^+ limitation was counteracted by supplying it via the overlying water.

As expected, the natural sediment was more heterogeneous than the manipulated sediment giving an idea of the spatial and temporal dynamics in the sampled stream habitat. Measurements conducted directly in the field are useful for investigations of the complexity of a particular habitat, for comparisons between different natural

habitats and for investigations of the seasonality within certain habitats. The pretreatment of the sediment, however, produced a high degree of similarity between replicate cores which is useful for the performance of factorial experiments under defined conditions. In our case the complementary use of both approaches also allowed to address the adaptive capacities of the sedimentary microorganisms to changed conditions.

Conclusions

The application of microsensors combined with FISH for the investigation of nitrification in a natural stream sediment was challenging because of the generally lower activities and cell abundances compared to e.g. waste water biofilms. Still, to date it is the only acceptable *in situ* approach to study nitrification activity with the appropriate spatial resolution and to determine the abundance of nitrifiers without the bias of cultivation-dependent techniques. With the use of FISH, the two physiological groups of nitrifiers, AOB and NOB, could be separately studied with respect to both their prevalence in a natural sediment and their fate upon sediment manipulation and incubation. Microsensor data, on the other hand, helped to reveal which of the identified nitrifiers were actually active and what effect their activity had on the nutrient exchange between the sediment and the overlying water. In this sense, our field measurements represent a snapshot of the situation in a small stream at a given time of the year, whereas the laboratory incubation revealed the potential of the same sediment for nitrification and the ability of the inhabiting nitrifiers to adapt to changing conditions.

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Chapter 4

Nitrification in freshwater sediments as influenced by insect larvae: Quantification by microsensors and fluorescence in situ hybridization

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Abstract

Sediment-reworking macroinvertebrates can stimulate nitrification by increasing the oxygen penetration into sediments or reduce it by grazing on nitrifying bacteria. We investigated the influence of *Chironomus riparius* larvae (Insecta: Diptera) on the activity, abundance and distribution of the two groups of nitrifiers, i.e. NH_4^+-oxidizing (AOB) and NO_2^--oxidizing bacteria (NOB), in two freshwater sediments with a combination of microsensor measurements and fluorescence in situ hybridization (FISH). In the organic-poor sediment nitrifying activity was reduced by the animals, whereas no effect was detected in the organic-rich sediment. We interpret this difference as a result of the variable larval grazing behavior in the two sediment types: In the organic-poor sediment larval feeding activity on the sediment surface was laterally extensive, while it was locally restricted to the immediate surroundings of permanent burrows in the organic-rich sediment. Surprisingly, the animals did not cause any significant changes of the abundances of AOB and NOB. This implies that the observed reduction of nitrification activity was not density-regulated, but rather a matter of lowered metabolic activity due to partial digestion and re-deposition of particle-associated bacteria into the sediment.

Introduction

Nitrification, the microbially mediated oxidation of NH_4^+ to NO_3^-, plays a central role in the N cycle of aquatic environments, since it links the mineralization of organic matter (formation of NH_4^+) to the recycling of N to the atmosphere (denitrification of NO_3^-). Especially with respect to the growing eutrophication of aquatic environments this link inheres a great significance for the elimination of N from polluted ecosystems (Herbert 1999). The impact of sediment-dwelling macroinvertebrates on benthic microorganisms has been studied intensively. This resulted in a rather complex picture of the interactions between the animals and the sedimentary microbial community. The burrowing and bioirrigation of sediment-dwellers increases the surface area and substrate availability for benthic nitrifiers (Kristensen *et al.* 1985; Pelegri and Blackburn 1994; Svensson 1997; Gilbert *et al.* 1998; Bartoli *et al.* 2000). On the other hand, grazing by deposit-feeding invertebrates can also significantly reduce the abundance or the metabolic activity of particle-associated bacteria (Johnson *et al.*

1989; Rouf and Rigney 1993; Plante 2000). Depth specific behavior of benthic macrofauna, i.e. larval grazing at the sediment surface, vertical particle relocation, and ventilation of deeper layers, can lead to simultaneous stimulation of bacterial activity and reduction of microbial biomass in different layers of the same sediment (Stief and de Beer 2002).

While measuring the activity of the nitrifying bacteria their identity and distribution in bioturbated freshwater sediments have been ignored so far. However, the qualitative and quantitative analysis of the nitrifying populations at a high spatial resolution can provide further information on the mechanisms of how benthic macroinvertebrates influence nitrification, since the nitrifiers may respond differently to the presence of animals than other bacterial groups. Fluorescence *in situ* hybridization (FISH) (DeLong *et al.* 1989) has been successfully applied in the past to identify and quantify bacterial populations in aquatic environments and to determine their spatial distribution (Llobet-Brossa *et al.* 1998; Pernthaler *et al.* 1998). The combination of FISH with microsensors has proved as a powerful tool to investigate *in situ* structure and function of nitrifying communities in biofilms (Schramm *et al.* 1997; Gieseke *et al.* 2001) and in a freshwater sediment (Altmann *et al.* accepted).

In our laboratory experiments, we combined the use of microsensors and FISH to evaluate the influence of *Chironomus riparius* larvae on the activity, composition and distribution of NH_4^+- and NO_2^--oxidizing populations. We hypothesized that larval grazing and ventilation would be accompanied by spatially coincident changes of AOB and NOB abundances and activities.

Materials and Methods

Sediment sampling and incubation. Surface sediment (0 to 5 cm) was collected from two field sites differing in grain size and organic content: (i) Sandy sediment (mean grain size 200 µm) was sampled from a small lowland stream with a sandy bed, Rittrumer Mühlenbach (near Wildeshausen, Northern Germany). The sediment had a low organic content (i.e., a mean weight loss on combustion of 2.5% (SD = ± 0.3%, n = 20)) and was only sparsely inhabited by chironomids of not identified species, (ii) silty sediment (mean grain size <100 µm) was collected from the Hetter, a man-made brook in the former floodplains of the River Rhine near Rees, Northwest Germany.

The organic content of this sediment averaged 12.7% (SD = ± 2.2%, n = 20) and contained moderate densities of *Chironomus* spp. The sediments were sieved (mesh size of 1 mm) to remove macrofauna and large detritus, filled into Perspex cylinders (∅ 9 cm, height 13 cm) and allowed to settle over night. Replicate cylinders were pre-incubated in the dark at 15 °C in basins containing 24 l of aerated stream water until the sediment had reached steady state, i.e. NH_4^+ fluxes into the sediment remained stable over time (Stief *et al.* 2002). NO_3^- and NH_4^+ concentrations in the water were kept constant at 500 and 50 µmol l^{-1}, respectively, by repeated addition of aliquots from $NaNO_3$ and NH_4Cl stock solutions. After pre-incubation *Chironomus riparius* larvae (4^{th} larval stage, mean body length 1 cm), taken from a laboratory-bred population, were introduced into the sediment cylinders at an abundance of 1 individual per cm^2 (Stief and de Beer 2002). Cylinders were checked regularly for dead and emerged larvae, which were then replaced.

Six sediment cylinders each were incubated with organic-poor, sandy sediment and organic-rich, silty sediment. Three sediment cylinders of each experimental run served as controls, i.e. they did not receive larvae, while the remaining cylinders served as animal treatments. After another 14 days of incubation at 15 °C and in the dark, microsensor measurements and fixation of sediment samples for FISH were done within three days.

Microsensor measurements. Microsensors for O_2 (Revsbech 1989), NO_3^-, and NH_4^+ (de Beer and v. d. Heuvel 1988; de Beer and Sweerts 1989) were prepared, calibrated, and operated in a measuring setup as previously described (Stief *et al.* 2002). In the organic-poor sediment measurements were performed at random spots on the sediment surface, whereas in organic-rich sediment spots in between animal burrows were chosen. Replicate profiles were recorded down to a depth of 10 mm. Local volumetric conversion rates at different depths were calculated based on the second derivative of the concentration profiles. Within each conversion rate profile five consecutive values were averaged to obtain a spatial resolution of 1 mm. This was done to more clearly arrange data, to reduce noise resulting from the calculation of the second derivative, and to approximate the spatial resolution of the FISH. Standard deviations for the mean of replicate profiles were then calculated by error propagation. To obtain the depth-integrated rate of nitrification the local conversion rates of O_2 and NO_3^- were

averaged across the nitrification zone, i.e. the overlap of the oxic and NO_3^- production zone (Stief and de Beer 2002).

Total flux measurements. In additional microcosms of organic-rich sediment the total solute exchange (affected by diffusion plus advective transport of solutes and reaction processes) was determined from the concentration changes in the overlying water over time. The water was aerated and adjusted to 100 µmol l^{-1} NH_4^+ and 500 µmol l^{-1} NO_3^-. The NH_4^+ and NO_3^- concentrations in the overlying water were analyzed at the start and after 4 h using photometric test kits by Merck (Germany). Linearity of the concentration changes during the first 4 h of the incubation was ascertained in one cylinder by sampling the overlying water every hour. Total fluxes (J) were calculated as:

$J_{total} = (\Delta C\ V) / (\Delta t\ A)$,

where ΔC is the concentration change over the time interval Δt, V is the volume of the overlying water, and A is the cross section area of the sediment surface.

Estimation of heterotrophic O_2 consumption. Based on the depth-integrated conversion rates of O_2 and NO_3^- in the nitrification zone and the stoichiometry of nitrification, i.e. $O_2 : NH_4^+ : NO_3^- = 2 : 1 : 1$, the heterotrophic O_2 consumption was estimated. This was done by subtraction of the O_2 consumption due to nitrification from the overall consumption of O_2.

FISH and total cell counts. One sediment core (∅ 2.5 cm) was taken from each sediment cylinder, and sectioned horizontally into 2 mm-thick slices down to a depth of 10 mm. Sediment slices were fixed with paraformaldehyde (Llobet-Brossa *et al.* 1998) and diluted 30-fold in a 1:1 mixture of phosphate-buffered saline (composed of 130 mmol l^{-1} NaCl, 3 mmol l^{-1} NaH_2PO_4 and 7 mmol l^{-1} Na_2HPO_4 [pH 7.2]) and 96 % ethanol. Suspensions were sonicated with a type UW70 probe (3 x 60 s, 20 % pulse, 109 µm amplitude, Sonopuls HD70; Bandelin, Berlin, Germany). Aliquots of 20-30 µl of a ten-fold dilution of the sonicated samples were immobilized on gelatine-coated microscopic slides. For FISH oligonucleotide probes specific for i) NH_4^+-oxidizing β-*proteobacteria* (Nso1225, (Mobarry *et al.* 1996)) and ii) for the NO_2^--oxidizing genera *Nitrobacter* (Nit3, (Wagner *et al.* 1996)) and *Nitrospira* (Ntspa662, (Daims *et al.* 2001)) were used. Probes Nit3 and Ntspa662 were used with equimolar amounts of

competitor oligonucleotides as indicated in the references. Correction for unspecific binding was achieved by subtracting cell numbers hybridized with control probe Non338 (Manz *et al.* 1992) from the FISH counts. All probes were purchased labeled with the fluorescent dye CY3 (Hybaid Interactiva, Ulm, Germany). FISH and counter staining of all cells with 4′, 6-diamino-2-phenylindole (DAPI; 0.5 µg ml^{-1}) were performed according to published protocols (Pernthaler *et al.* 1998). Counting was adapted to the low numbers of FISH-positive cells and their uneven distribution in the aliquots as described in (Altmann *et al.* accepted). Total cell counts were determined separately by DAPI-staining of sonicated and diluted sediment samples on black membrane polycarbonate filters (pore size, 0.2 µm; Osmonics Inc., Livermore, California, USA). The absolute numbers of FISH-positive cells were calculated for each probe using the relative FISH-positive counts (as percentage of DAPI-stained cells) and the total cell counts.

Results

Larval behavior in the two sediment types

In both sediments the larvae of *C. riparius* buried quickly into the sediment. However, the larvae behaved differently in the two investigated sediment types. In the organic-poor sandy sediment, they reworked the top 5 mm and made the surface look fluffier than in the larvae-free controls. The larval fecal pellets (diameter of 300 µm) extensively covered the surface of the sediment and were also found in the subsurface layer down to a depth of approximately 5 mm. In the organic rich sediment, however, the larvae constructed a high number of permanent burrows, which they aerated actively by undulating body movements. Because of this localized activity pattern, particle redistribution was not as intense as in the organic-poor sediment. The fecal pellets were mainly deposited around the burrow openings.

Larval effects on nitrification activity

After a two-week incubation with *C. riparius* larvae, vertical gradients of O_2, NH_4^+, and NO_3^-, representing steady state conditions, were recorded. O_2 penetrated 1.8 mm into the organic-poor control sediment, while the presence of the larvae enhanced O_2 penetration to 3.8 mm (Fig. 1A). Local O_2 consumption rates were significantly reduced in the presence of animals (Fig. 1D).

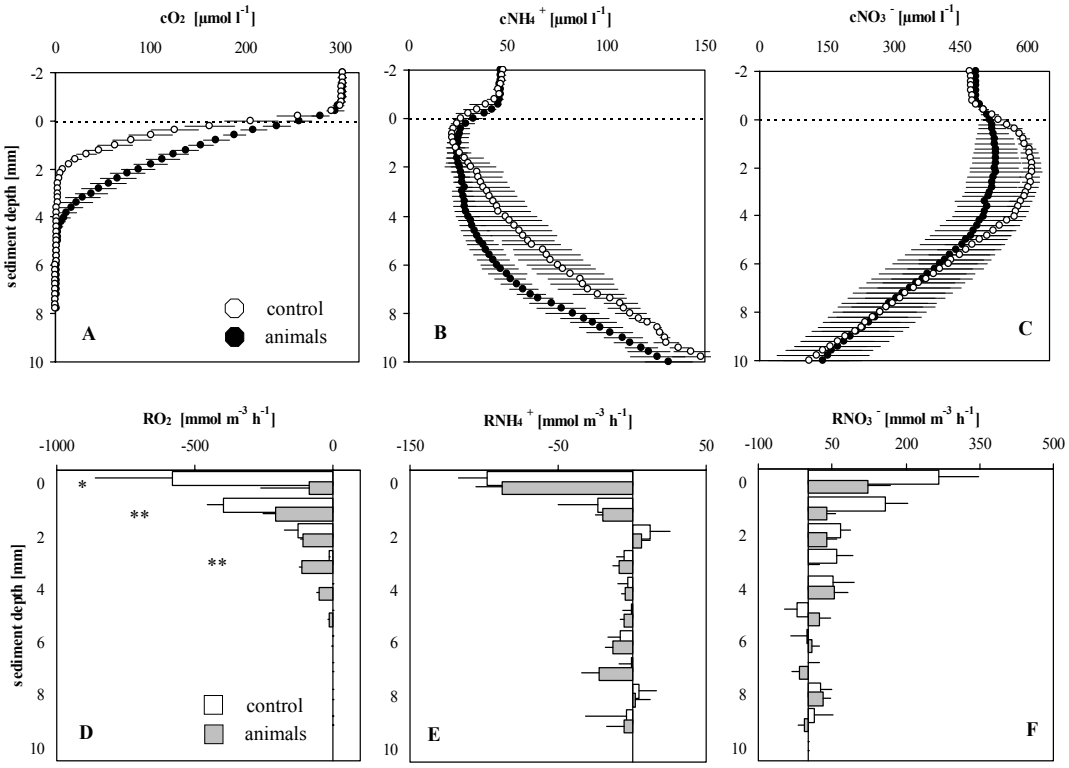

Fig. 1A-F: Vertical concentraton profiles (A-C) and local conversion rates (D-F) in the **organic-poor** sediment after a two-week incubation with and without *C. riparius* larvae. Means and standard deviation of 3 replicate sediment beakers are given. Dotted line corresponds to the sediment surface. Welch corrected t-test between animal and control treatments revealed significant differences at $p < 0.05$ (*) and $p < 0.01$ (**). Positive values = production, negative values = consumption.

NO_3^-, the end product of nitrification, was produced down to a sediment depth of 4-5 mm in the organic-poor sediment (Fig. 1F), thereby exceeding the O_2 penetration depth, both in the animal and in the control treatments. Superficial NO_3^- production was greater than the NO_3^- consumption below the oxic zone, rendering the organic-poor sediment a source for NO_3^- to the overlying water (data not shown). Although some animal-related decrease of superficial nitrification seems obvious the larvae did

not influence the NO_3^- efflux from the sediment. NH_4^+ consumption was observed only within the uppermost 2 mm of the sediment (Fig. 1E) and was not influenced by the larvae.

In the organic rich sediment the animal-related increase of O_2 penetration was less pronounced (from 1.4 to 1.8 mm, Fig. 2A) and no clear effect of the presence of larvae on overall O_2 consumption was observed (Fig. 2D). The highest NO_3^- production and NH_4^+ consumption rates were measured in the 0-1 mm horizon (Fig. 2E+F), but both rates were not influenced by the presence of larvae.

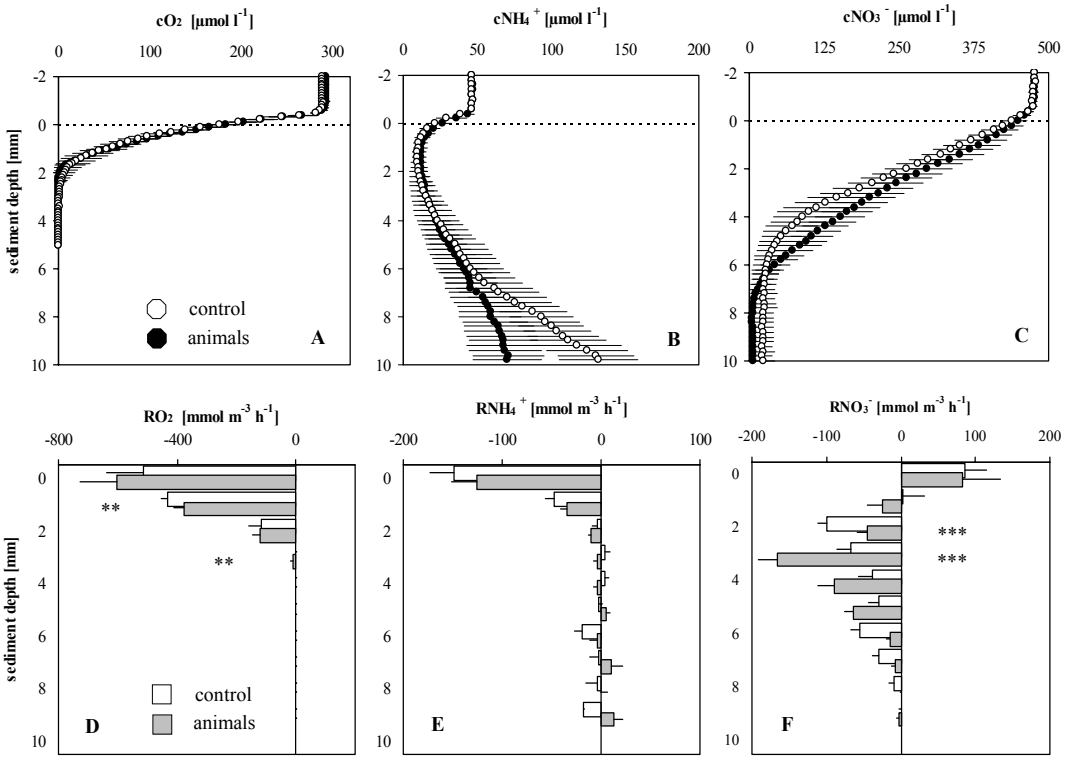

Fig. 2A-F: Vertical concentration profiles (A-C) and local conversion rates (D-F) in the **organic-rich** sediment after a two-week incubation with and without *C. riparius* larvae. Means and standard deviation of 3 replicate sediment beakers are given. Dotted line corresponds to the sediment surface. Welch corrected t-test between animal and control treatments revealed significant differences at $p < 0.01$ (**) and $p < 0.001$ (***). Positive values = production, negative values = consumption.

In contrast to the organic-poor sediment, consumption of NO_3^- below a sediment depth of 1 mm exceeded the superficial production of NO_3^-. Thus, the organic-rich sediment acted as a sink for NO_3^- from the overlying water (data not shown). The maxima of NO_3^- consumption were shifted downwards in the presence of animals and exceeded those of the control treatments significantly (Fig. 2F). Total flux measurements revealed an increased uptake of NO_3^- and NH_4^+ by the animal-inhabited sediment (Fig. 3).

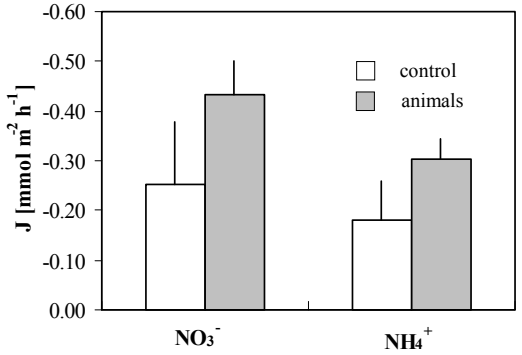

Fig. 3: Total fluxes of NO_3^- and NH_4^+ in the **organic-rich** sediment after a two-week incubation with and without *C. riparius* larvae. Negative values indicate uptake of NO_3^- and NH_4^+ by the sediment. Means and standard deviation of 3 replicate sediment beakers are given. Welch corrected t-test between animal and control treatments revealed no significant differences ($p > 0.05$).

Larval effects on heterotrophic activity

The depth-integrated conversion rates of O_2 and NO_3^- used for this calculation are listed in Table 1. The partitioning between the O_2 consumed by heterotrophic processes and by autotrophic nitrification was clearly different in the two sediment types (Fig. 4A+B). O_2 was mainly consumed by nitrification in the organic-poor sediment, whereas this process was of minor importance for the O_2 consumption in the organic-rich sediment, where heterotrophic processes consumed a major portion of O_2.

Table 1. Depth-integrated conversion rates of O_2 and NO_3^- (J) within the nitrification zone of organic-poor and organic-rich sediment either devoid or inhabited by *C. riparius* larvae. Organic-poor: Means of three replicate cores within one sampling date (± 1 SD, n ≥ 3), organic-rich: Means of three replicate cores within one sampling date (± 1 SD, n ≥ 5). Positive values = production, negative values = consumption.

J [mmol m^{-2} h^{-1}]	organic-poor		organic-rich	
	control	*animals*	*control*	*animals*
O_2	-0.915 ± 0.236	-0.489 ± 0.183	-0.699 ± 0.117	-0.957 ± 0.206
NO_3^-	0.404 ± 0.027	0.199 ± 0.121	0.105 ± 0.098	0.016 ± 0.110

Moreover, in the organic-poor sediment the presence of the animals caused a decrease in O_2 consumption by nitrification. The heterotrophic O_2 consumption remained unchanged. In the organic-rich sediment, we found no animal-related change in the O_2 consumption due to nitrification but the heterotrophic O_2 uptake was enhanced significantly in the presence of the larvae.

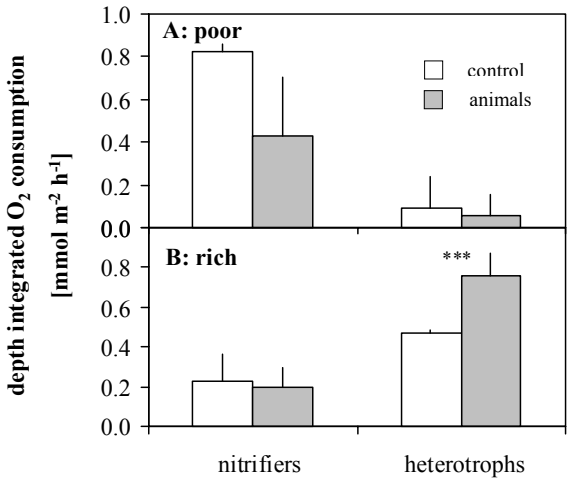

Fig. 4A, B: O_2 consumption by nitrification and by heterotrophic processes in the organic-poor (A) and organic-rich sediment (B) after a two-week incubation with and without *C. riparius* larvae. Means and standard deviation of 3 replicate sediment beakers are given. Welch corrected t-test between animal and control treatments revealed significant differences at $p < 0.001$ (***).

Identity and distribution of nitrifiers

Total bacterial cells in the organic-poor sediment were equally distributed over depth accounting for 3.2 to 3.6 · 10^9 cm^{-3} in both the control and the animal inhabited sediment. In the organic-rich sediment total cells varied between 4.8 and 12.2 · 10^9 cm^{-3}, but this was rather due to in-core variability than to the activity of the larvae.

The maximum abundances of both the AOB and the NOB coincided spatially with the highest conversion rates of O_2, NH_4^+, and NO_3^-, i.e. they were found in the 0-2 mm horizon of both sediment types (Fig. 5A – D).

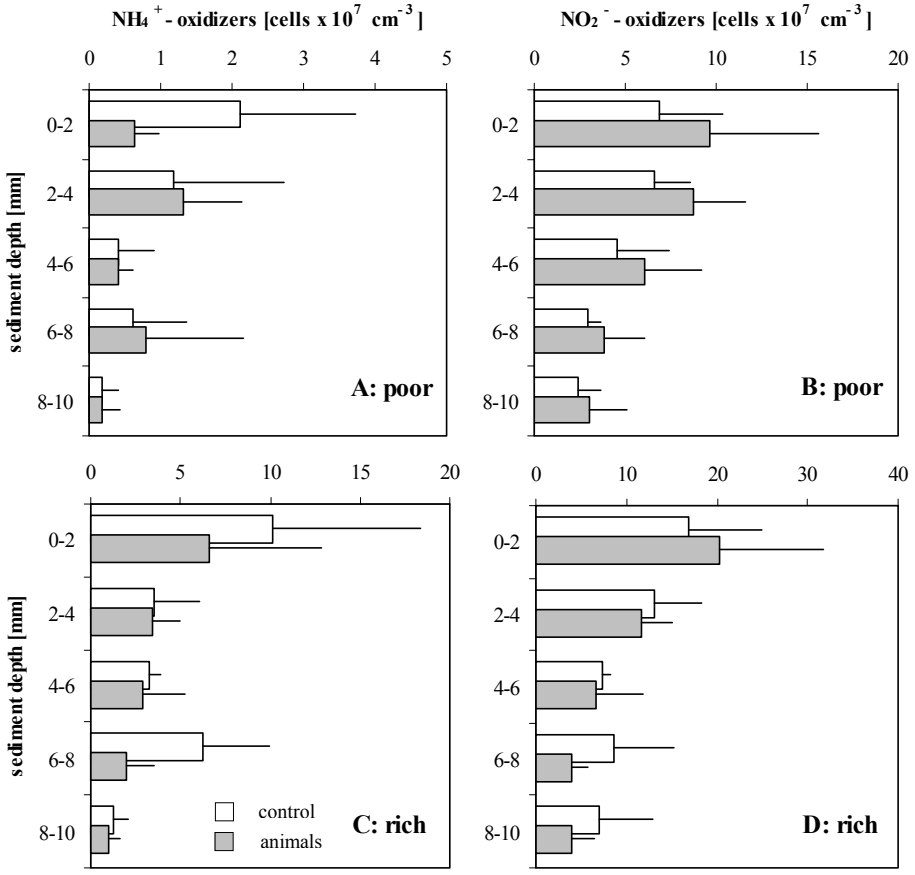

Fig. 5A-D: Absolute abundances of the NH_4^+-oxidizing β-*proteobacteria* (AOB) and the NO_2^- -oxidizing genus *Nitrospira* (NOB) in the organic-poor sediment (A, B) and the organic-rich sediment (C, D) after a two-week incubation with and without *C. riparius* larvae. Means and standard deviation of 3 replicate sediment beakers are given. Welch corrected t-test between animal and control treatments revealed no significant differences ($p > 0.05$).

In this layer the NH_4^+-oxidizing ß-proteobacteria, as determined with probe Nso1225, accounted for 0.6 and 1.2% of all cells in the organic-poor and the organic-rich sediment, respectively. NO_2^--oxidizing bacteria of the genus *Nitrospira* as detected with probe Ntspa662 accounted for about 2% of all cells in both sediment types. The abundances of *Nitrospira* sp. remained relatively high even with increasing depth, making up at least 1.0% of all cells even in the lowest sampled sediment layer. *Nitrobacter* sp., the most commonly isolated NOB, was not detected with probe Nit3 in any of the sediment treatments. In both sediments the presence of the chironomids had no significant influence on the abundances and the distribution patterns of AOB and NOB.

Discussion

In our experiment we combined the use of microsensors with FISH to evaluate the influence of *C. riparius* larvae on both the activity and the distribution of the NH_4^+- and NO_2^--oxidizing bacteria (AOB and NOB). Based on the results of a previous study we hypothesized that the grazing of *C. riparius* would decrease the nitrifying activity in the top layer of the sediment and that ventilation of deeper layers would increase the nitrifying activity (Stief and de Beer 2002). These differences in nitrifying activity were assumed to be regulated by the abundance of nitrifiers. The presence of larvae reduced the nitrification activity in the top layer of organic-poor, but not in the organic-rich sediment. The abundance of neither AOB nor NOB was affected by the presence of animals in any of the two sediments.

Larval behavior in the two sediment types

The differential behavior of the chironomids, i.e. the extensive lateral sediment reworking in the organic-poor sediment and the locally restricted activity in the organic-rich sediment has major implications for interpretation of the microsensor measurements. In the organic-poor sediment the vertical profiles recorded at random spots of the sediment surface can be taken as representative of the average distribution of solutes (see also Stief and de Beer 2002) because of the larval-induced homogenization of the surface. In contrast, in the organic-rich sediment the microsensor technique revealed only the animal impact on the bulk sediment in

between animal burrows, while the additional N conversions inside the burrows were excluded. In this case, the overall effect of the chironomid larvae on total benthic N conversions was more accurately represented by the total fluxes as determined with whole core incubations (see also Matisoff *et al.* 1985; Archer and Devol 1992).

Larval effects on nitrification and heterotrophic O_2 demand

The stimulation of benthic nitrification by chironomid larvae has been demonstrated in several previous studies and was discussed to be a consequence of increased O_2 penetration due to ventilation and extended surface area and the release of NH_4^+ by the animals (Mayer *et al.* 1995; Pelegri and Blackburn 1996; Svensson 1998). However, in our experiment, the animals lowered the overall nitrification rates in the organic-poor sediment despite an improved O_2 supply. Because the abundance of nitrifiers remained almost unchanged in the presence of animals this decrease can be interpreted as a reduction of the metabolic activity of single cells or, what is more likely, a decrease of the active proportion of the nitrifying population. We assume that the ingestion and digestion of organic particles by the larvae and the subsequent deposition and distribution of fecal pellets within the surface layer of the sediment reduced the overall metabolic activity of the particle-associated bacteria. The reduction of activity and abundances of particle-associated bacteria by deposit-feeding invertebrates has also been demonstrated in several other studies (Johnson *et al.* 1989; Rouf and Rigney 1993; Plante 2000). The metabolic recovery of different groups of bacteria, which have survived gut passage, occurs likely at different rates. Nitrifiers, for example, are poor competitors with heterotrophic bacteria because of their slow growth (Prosser 1989) and comparably high K_m values for O_2 (Schramm *et al.* 1997) and may thus recover less fast from the gut passage than heterotrophs. Indeed, the O_2 consumption due to nitrification was reduced in the presence of the larvae, whereas heterotrophic O_2 consumption remained unchanged. Microsensor measurements on single fecal pellets would here be helpful to further illuminate the role of the feces in overall sedimentary nitrification activity.

In the bulk sediment between the burrows pervading the organic-rich sediment the microsensor approach did not reveal an animal-induced effect on nitrification. In contrast, the animal-inhabited sediment was characterized by a higher total uptake of both NO_3^- and NH_4^+ as revealed by the total fluxes. This was probably due to the high

number of permanent burrows displaying a high microbial activity (Kristensen *et al.* 1985; van de Bund *et al.* 1994; Svensson 1997). Although the presence of burrows enhanced the uptake of NH_4^+ their contribution to nitrification in particular remains unclear because at the same time no net production of NO_3^- occurred. The burrows even acted as an additional sink for NO_3^- probably due to dissimilative NO_3^- consumption in the burrow walls and the surrounding sediment. Though the microsensor approach did not reveal an animal effect on nitrification the heterotrophic O_2 consumption in the sediment between the burrows was increased by the animals. This could be a consequence of the fertilization of heterotrophic microbes, but not nitrifiers, with dissolved compounds released into the overlying water during the feeding activities of the chironomids (Goedkoop *et al.*, 1997). Similar observations were made during an experiment with chironomids: Control sediment cores that were exposed to the same overlying water as the animal-inhabited cores showed increasing O_2 consumption rates (M. Koschorreck, pers. comm.).

Identity and distribution of nitrifiers

In our investigation the presence of *C. riparius* larvae influenced the activity of nitrifiers but not the abundance of AOB and NOB as it was revealed by FISH. Thus, *C. riparius* larvae did neither efficiently decimate the nitrifying population, nor did they significantly promote the growth of nitrifiers in the ventilation layer of the sediments during the course of the experiment (2 weeks). The vertical patterns of activity and abundance of nitrifiers do not necessarily match within the sediment column because metabolically inactive cells may survive in both the oxic and even in the anoxic sediment layers for extended periods (Gieseke *et al.* 2001; Morgenroth *et al.* 2000; Schramm *et al.* 2000). Due to their ability to maintain a relatively high ribosome content even at a stage of low metabolic activity, as given under starvation and other stress conditions, nitrifiers are detectable with FISH even if no nitrifying activity can be recorded with the microsensors. Applying methods for the detection of the single cell activity may solve this dilemma in the future. For example, precursor rRNA has been monitored and shown to be a promising tool for the determination of not only size but also *in situ* activity of certain bacterial populations (Oerther *et al.* 2000; Schmid *et al.* 2001). DNA-synthesizing cells can be detected by the uptake of the

halogenated thymidine analogue bromodeoxyuridine and consecutive FISH (Pernthaler *et al.* 2002).

Summary and conclusions

Depending on the sediment type, the larvae showed a different behavior in terms of structuring the sediment by their feeding and burrowing activities. As a consequence nitrification was influenced differently: (i) in the organic-poor sediment its activity was reduced by the extensive lateral larval grazing while (ii) its activity remained rather unaffected in the space between animal burrows constructed in the organic-rich sediment. In the latter case however, total flux measurements revealed the larval burrows as additional sinks of NO_3^- rather than a site of net nitrification activity. Extrapolated to a real world benthic ecosystem the sediment type may be decisive in regulating the impact of *C. riparius* on the N cycle and hence the recycling of N to the atmosphere.

This work stresses the need of further work applying a range of techniques to better understand the complex interactions between sediment-dwelling animals and the microbial community involved in benthic N cycle. Microsensor measurements i) directly within the burrows and ii) at the surface of the larval feces would greatly enhance our knowledge of local effects of bioturbation activities on nitrification. This knowledge would even be further improved by the quantification of the actually active portion of nitrifying bacteria.

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Chapter 5

Summary

Zusammenfassung

Summary

Nitrification is a central process of the benthic N cycle, which sets the preconditions for the removal of excess N from anthropogenically impaired aquatic environments. The fact that nitrification in freshwater sediments usually occurs within a thin oxic surface layer necessitates techniques with sufficiently high spatial resolution for the *in situ* investigation of this important microbial process. In previous studies of nitrifying biofilms in wastewater treatment systems microsensors and fluorescence in situ hybridization (FISH) served this purpose within even thinner oxic surface layers than in sediments. Stimulated by this great achievement, the combination of microsensors and FISH was also used in the present thesis to study some of the major ecological features of nitrifying bacteria in freshwater sediments *in situ*.

Microsensors alone have been applied in some investigations of freshwater sediments before, but FISH applicability was assumed to be hampered by low cell numbers of nitrifiers and high background fluorescence in the sediment. Thus, the first experiment (Chapter 2) was dedicated to evaluate the potential of this combined approach for the investigation of *in situ* distribution and activity of nitrifying bacteria in model freshwater sediment. Nitrification activity in this sediment as measured with microsensors was highest in the uppermost 2 mm of the sediment, i.e. 0.2 µmol cm^{-3} h^{-1}, and comparable to rates measured in other freshwater sediments. Maximum abundances of both NH_4^+- and NO_2^--oxidizing bacteria (AOB and NOB) as determined with FISH were 1.5 x 10^7 cells cm^{-3} and 8.6 x 10^7 cells cm^{-3}, respectively, and coincided spatially with maximum nitrification activity. Cell numbers of AOB and NOB were 1-4 orders of magnitude higher compared to previous studies using the most probable number technique. The present study also demonstrated the relevance of the genus *Nitrospira* (and not *Nitrobacter* spp.) for *in situ* NO_2^--oxidation not only in wastewater treatment systems, but also in freshwater sediments. For the first time, cell-specific nitrification rates of the genus *Nitrospira* could be calculated for freshwater sediment and added up to 1.2-2.7 fmol NO_2^- $cell^{-1}$ h^{-1}.

In a second experiment (Chapter 3) the combined approach of microsensors and FISH was applied to investigate the activity and distribution of nitrifying bacteria in intact field sediment ('natural sediment'). The results were compared to sediment from the

same sampling site, which had been sieved and homogenized in preparation for a laboratory incubation experiment ('manipulated sediment'). The genus *Nitrospira* was the prevalent NO_2^--oxidizer not only in the model sediment used in the first study, but also in the natural stream sediment used here. Nitrification activity in the natural sediment was negligible and abundances of nitrifiers in the apparently oxic layer were low. However, maximum abundances of both AOB and NOB were found in the 2-4 mm layer, which was anoxic at the time of the microsensor measurements. This may have been a consequence of the downward shift of the abundance maximum towards better availability of NH_4^+ in the field and the reduction of the O_2 penetration depth due to a lower current velocity during the laboratory measurements. Alternatively, it may be indicative of the physical dynamics in the stream sediment, e.g. the horizontal shifting of sediment ripples that slop formerly oxic layers. In the manipulated sediment nitrification activity and abundances of both AOB and NOB were conspicuously higher than in the natural sediment which was ascribed to the better availability of NH_4^+ throughout the incubation. *Nitrospira* spp. could be detected with FISH for an extended period of time in anoxic layers, into which they had been transported due to homogenization. In contrast, the AOB population was quickly restricted to the oxic surface layer in which they were supplied with O_2 and NH_4^+ from the overlying water. The pretreated sediment was characterized by a lower spatial heterogeneity compared to the natural sediment, which is beneficial for the conduction of factorial experiments. In the third experiment microsensors and FISH were applied to investigate the impact of *Chironomus riparius* larvae (Insecta: Diptera) on the activity and distribution of AOB and NOB in two sediments with different organic contents. *C. riparius* burrowed within the upper 10 mm of the sediments, changed the O_2 microdistribution therein and grazed on detritus particles inclusive of the attached microorganisms. However, in dependence on the sediment type the larvae behaved differently and thereby influenced the nitrification activity in a different way: In the organic poor sediment the larvae extensively reworked the sediment and overall nitrification activity was significantly lowered. Since the larvae did not cause any significant changes in the abundances of AOB or NOB, the reduction of nitrifying activity probably resulted from a general loss of metabolic activity of the nitrifiers. This may have been a consequence of larval deposit feeding on attached bacteria and the defecation of metabolically inactivated cells back into the sediment. In the organic rich sediment larval feeding was locally

restricted to the immediate surroundings of the sediment burrows. Hence, the microsensor measurements did not reveal an animal-related impact on nitrification activity in the sediment between the burrows. Abundances of AOB and NOB were again not influenced by the presence of *C. riparius*. However, total fluxes of NH_4^+ and NO_3^- into the sediment were higher in the presence of larvae. Thus, microbial activities were indeed apparent in the burrows, but they did not contribute to net nitrification in the sediment as a whole. This study adds the plasticity of larval behavior to the complexity of factors influencing benthic nitrification in bioturbated sediments.

Within the scope of this thesis microsensors and fluorescence in situ hybridization (FISH) were successfully applied to investigate *in situ* activities and distribution of NH_4^+-oxidizing bacteria (AOB) and NO_2^--oxidizing bacteria (NOB) at a fine scale in freshwater sediments. While with FISH these two physiological groups of nitrifiers were identified and quantified, the microsensor measurements revealed the sediment layers in which the nitrifiers were actually active and what effect their activity had on the N exchange between the sediment and the overlying water. Findings of special interest were (i) the quantitative prevalence of the NO_2^--oxidizing genus *Nitrospira*, so far only known for its occurrence mainly in wastewater treatment systems, in both model and natural stream sediments, (ii) the detectability of AOB and NOB with FISH in anoxic sediment layers as a consequence of their high ribosome content even when not actively nitrifying, and (iii) the plasticity of larval behavior in dependence on the sediment type which adds even more complexity to the array of factors influencing benthic nitrification in bioturbated sediments.

Zusammenfassung

Nitrifikation ist ein zentraler Prozess innerhalb des benthischen N-Kreislaufes, welcher die Voraussetzungen für die Entfernung von überschüssigem N aus anthropogen beeinträchtigten aquatischen Lebensräumen schafft. Die Tatsache, dass Nitrifikation in limnischen Sedimenten normalerweise in einer sehr dünnen oxischen Oberflächenschicht vorkommt, erfordert Messtechniken mit einer ausreichend hohen räumlichen Auflösung, um *in situ* Untersuchungen dieses wichtigen mikrobiellen Prozesses vornehmen zu können. In früheren Studien, die sich mit der Untersuchung nitrifizierender Biofilme aus Kläranlagen befassen, konnten Mikrosensoren und die Fluoreszenz in situ Hybridisierung (FISH) in sogar noch dünneren oxischen Oberflächenschichten als im Sediment erfolgreich angewendet werden. Angeregt durch diese Ergebnisse wurde die Kombination der Mikrosensoren mit der FISH in der vorliegenden Arbeit genutzt, um einige der hauptsächlichen ökologischen Aspekte nitrifizierender Bakterien in Süßwassersedimenten zu untersuchen.

Mikrosensoren wurden bereits in früheren Untersuchungen von Süßwassersedimenten eingesetzt, die Anwendbarkeit von FISH wurde bisher jedoch aufgrund sehr geringer Zellzahlen der Nitrifizierer und hoher Hintergrundfluoreszenz im Sediment in Frage gestellt. Aus diesem Grund bestand das Ziel der ersten Studie (Kapitel 2) darin, das Potential dieses kombinierten Methodenansatzes für die Untersuchung der *in situ* Verteilung und Aktivität nitrifizierender Bakterien in einem Modell-Süßwasser-Sediment zu erkunden. Die Nitrifikationsaktivität in diesem Sediment, gemessen mit Mikrosensoren, war am höchsten innerhalb der obersten 2 mm des Sedimentes, d. h. 0.2 µmol cm^{-3} h^{-1}, und war vergleichbar mit Raten, die in anderen Süßwassersedimenten gemessen wurden. Maximale Abundanzen von NH_4^+- und NO_2^--oxidierenden Bakterien (AOB and NOB), bestimmt mit FISH, betrugen 1.5 x 10^7 Zellen cm^{-3} and 8.6 x 10^7 Zellen cm^{-3}, und stimmten räumlich mit der maximalen Nitrifikationsaktivität überein. Die Zellzahlen von AOB und NOB waren 1-4 Größenordnungen höher als die in früheren Studien mit Hilfe der „Most Probable Number"- Methode ermittelten Abundanzen. In der vorliegenden Untersuchung wurde eine quantitative Bedeutung der Gattung *Nitrospira* (und nicht *Nitrobacter* spp.) für die *in situ* NO_2^--Oxidation festgestellt. Dies galt bisher vor allem für Biofilme aus

Kläranlagen. Zum ersten Mal konnten auch zellspezifische Nitrfikationsraten für die Gattung *Nitrospira* im Süßwassersediment berechnet werden, sie betrugen bis zu 1.2-2.7 fmol NO_2^- $Zelle^{-1}$ h^{-1}.

Innerhalb der zweiten Studie (Kapitel 3) wurde diese Methodenkombination angewendet, um die Aktivität und Verteilung nitrifizierender Bakterien in ungestörtem Sediment im Freiland ('natural sediment') zu untersuchen. Die Ergebnisse wurden mit einem Sediment vom gleichen Standort verglichen, welches in Vorbereitung auf ein Laborinkubations-Experiment jedoch gesiebt und homogenisiert wurde ('manipulated sediment'). Die Gattung *Nitrospira* war nicht nur im Modell-Sediment aus der ersten Studie, sondern auch in dem natürlichen Fließgewässersediment der vorherrschende NO_2^--Oxidierer. Die Nitrifikationsaktivität im natürlichen Sediment war unbedeutend und die Abundanzen der Nitrifikanten in der oxischen Oberflächenschicht waren gering. Die maximalen Abundanzen von AOB und NOB waren dagegen in der darunterliegenden 2-4 mm Schicht zu finden, welche zur Zeit der Mikrosensor-Messungen anoxisch war. Dies könnte durch eine Verlagerung des Abundanzmaximums an den unteren Rand der oxischen Schicht (mit höherer NH_4^+-Verfügbarkeit) und eine Verringerung der Eindringtiefe von O_2 im Labor verursacht worden sein. Eine andere Erklärungsmöglichkeit bieten die dynamischen Umlagerungsprozesse im Fließgewässersediment, z.B. horizontal wandernde Sedimentrippel, die ehemals oxische Zonen überlagern. Im behandelten Sediment waren sowohl die Nitrifikationsaktivitäten als auch die Abundanzen der AOB und NOB auffallend höher als im natürlichen Sediment, was auf die bessere NH_4^+-Verfügbarkeit während der Laborinkubation zurückgeführt werden kann. Bakterien der Gattung *Nitrospira* waren mit der FISH über einen längeren Zeitraum in anoxischen Sedimentschichten detektierbar, in die sie durch die Sedimenthomogenisierung verlagert worden waren. Im Gegensatz dazu war die AOB Population bereits nach kurzer Inkubationszeit auf die oxische Schicht beschränkt, in der sie mit O_2 und NH_4^+ aus der Wassersäule versorgt wurden. Das vorbehandelte Sediment war durch eine, verglichen mit dem natürlichen Sediment, niedrigere räumliche Heterogenität gekennzeichnet (Abundanzen und Aktivitäten der Nitrifikanten), was es für Experimente geeignet macht, bei denen die Untersuchung ausgewählter Faktoren im Vordergrund steht.

In der dritten Studie (Kapitel 4) wurden Mikrosensoren und FISH genutzt, um die Auswirkung von *Chironomus riparius* Larven (Insecta: Diptera) auf die Aktivität und Verteilung von AOB und NOB in zwei Sedimenten mit unterschiedlichem organischen Gehalt zu untersuchen. Die Fress- und Grabaktivität von *C. riparius* beschränkte sich auf die oberen 10 mm des Sediments und führte dort zu Veränderungen der kleinräumigen O_2-Verteilung. Die Larven ernährten sich von Detrituspartikeln und den daran angehefteten Mikroorganismen. In Abhängigkeit vom Sedimenttyp verhielten sich die Larven unterschiedlich und beeinflußten so die Nitrifikationsaktivität auf verschiedene Weise: Im organisch-armen Sediment wurde das Sediment durch die Fress- und Grabtätigkeit intensiv und weiträumig umgelagert; dies führte zu einer signifikanten Verringerung der Nitrifikationsaktivität. Da es gleichzeitig zu keiner signifikanten Änderung der Abundanzen von AOB und NOB kam, ist der Rückgang der Nitrifikationsaktivität wahrscheinlich nur mit einer allgemeinen Reduktion der metabolischen Aktivität der Nitrifikanten zu erklären. Dies könnte eine Folge der Aufnahme und Verdauung partikelassoziierter Bakterien sein, die anschließend - metabolisch inaktiviert - wieder in das Sediment ausgeschieden wurden. Im organisch-reichen Sediment beschränkte sich die Nahrungsaufnahme der Larven auf die unmittelbare Umgebung ihrer permanenten Wohnröhren. Aus diesem Grund konnten mit Mikrosensormessungen zwischen den Wohnröhren keine durch Larven verursachten Veränderungen der Nitrifikationsaktivität festgestellt werden. Auch in diesem Sedimenttyp waren die Abundanzen von AOB und NOB nicht durch die Larven beeinflußt. In Gegenwart der Larven waren allerdings die Gesamtfluxe von NH_4^+ und NO_3^- vom Wasser ins Sediment erhöht. Das heißt, dass zwar mikrobielle Umsetzungen in den Wohnröhren stattfanden, dass diese aber keine Auswirkungen auf die Nettonitrifikation des Gesamtsediments hatten. Diese Studie zeigt, dass die Plastizität des Larvenverhaltens ein weiterer wichtiger Faktor ist, der die Nitrifikation im Sediment beeinflusst.

Im Rahmen dieser Doktorarbeit wurden Mikrosensoren und Fluoreszenz in situ Hybridisierung (FISH) erfolgreich für die Untersuchung der *in situ* Aktivitäten und der Verteilung von NH_4^+-oxidierenden (AOB) und NO_2^--oxidierenden Bakterien (NOB) mit hoher räumlicher Auflösung in Süßwassersedimenten verwendet. Während mit FISH die beiden physiologischen Gruppen der Nitrifikanten identifiziert und

quantifiziert werden konnten, lieferten die Mikrosensormessungen Informationen darüber, in welchen Sedimentschichten die Nitrifikanten tatsächlich aktiv waren und welche Auswirkungen diese Aktivität auf den Austausch von N-Verbindungen zwischen Sediment und Wassersäule hatte. Von besonderem Interesse waren folgende Untersuchungsergebnisse: (i) die quantitative Bedeutung der NO_2^--oxidierenden Gattung *Nitrospira*, deren Vorkommen bisher vor allem in Kläranlagen bekannt war, (ii) die Detektion von inaktiven AOB und NOB in anoxischen Sedimentschichten mit FISH aufgrund ihres hohen Ribosomengehaltes, und (iii) das komplexe Larvenverhalten von *Chironomus riparius* als regulierender Faktor für die benthische Nitrifikation.

List of Publications

Contributions to the manuscripts presented in this thesis

1) Altmann, D., Stief, P., Amann, R., de Beer, D. and A. Schramm.

In situ distribution and activity of nitrifying bacteria in freshwater sediment.

This manuscript has been accepted for publication in Environmental Microbiology

Outline and concept of the project by P.S. and A.S., experimental design by P.S. and D.A., microsensor construction and measurements by P.S., fluorescence in situ hybridisation by D.A., writing by D.A. with help of coauthors

2) Altmann, D., Stief, P., Amann, R. and D. de Beer

Distribution and activity of nitrifying bacteria in natural and manipulated stream sediment

as determined with *in situ* techniques.

This manuscript will be submitted to Aquatic Microbial Ecology

Experimental design by D.A. and P.S., microsensor construction by D.A., microsensor measurements by D.A., fluorescence in situ hybridisation by D.A., writing by D.A. with help of coauthors

3) Altmann, D., Stief, P., Amann, R., and D. de Beer

Nitrification in freshwater sediments as influenced by insect larvae: Quantification by

microsensors and fluorescence in situ hybridization.

This manuscript has been submitted to Microbial Ecology

Outline and concept of the project by P.S. and D.A., experimental design by P.S. and D.A., microsensor construction by D.A. and P.S., microsensor measurements by D.A. and P.S., fluorescence in situ hybridisation by D.A., writing by D.A. with help of coauthors

Further publications

1) Stief, P., Schramm, A., Altmann, D., and D. de Beer
Decreasing nitrification rates, despite substrate-enrichment, in freshwater sediment microcosms: A microsensor study.
This manuscript has been accepted for publication in FEMS Microbiology Ecology.

2) Stief, P., Altmann, D., Kureck, A., and D. de Beer
Microbial activities in the burrow environment of the potamal fly *Ephoron virgo*.
This manuscript has been submitted for publication to Freshwater Biology.