ELSEVIER

FEMS Microbiology Ecology 42 (2002) 99–107

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Nitrification in the Schelde estuary: methodological aspects and factors influencing its activity

Monique J.M. de Bie [a], Mathieu Starink [a], Henricus T.S. Boschker [a,*], Jan J. Peene [a], Hendrikus J. Laanbroek [b]

[a] *Netherlands Institute of Ecology, Centre for Estuarine and Coastal Ecology, P.O. Box 140, NL-4400 AC Yerseke, The Netherlands*
[b] *Netherlands Institute of Ecology, Centre for Limnology, Nieuwersluis, The Netherlands*

Received 9 April 2002; received in revised form 20 June 2002; accepted 20 June 2002

First published online 28 August 2002

Abstract

We present a 15-month dataset on nitrification measurements in the Schelde estuary (Belgium and The Netherlands). Nitrification was estimated using the N-serve sensitive dark ^{14}C-bicarbonate incorporation technique. A peak of nitrification activity was observed in the freshwater part of the estuary. Downstream from this peak, nitrification declined, probably because of ammonium limitation. A range of nitrification inhibitors was tested on both a *Nitrosomonas europaea* culture and estuarine samples. It was found that methyl fluoride and acetylene stimulated dark ^{14}C-bicarbonate incorporation and those inhibitors were therefore considered inappropriate nitrification inhibitors in combination with this technique. The effect of the inhibitor N-serve was studied on the dark incorporation of ^{13}C-bicarbonate into polar lipid derived fatty acids to further identify the dominant chemoautotrophic processes. Inhibition of polar lipid derived fatty acid labelling in the presence of N-serve was complete, suggesting that nitrifying bacteria dominated the chemoautotrophic community.

Keywords: Nitrification; Estuary; Polar lipid derived fatty acid; Nitrification inhibitor; Ammonium concentration

1. Introduction

Eutrophication of estuaries is a common phenomenon. The high nutrient concentration in the output of these estuaries leads to algal blooms in predominantly N-limited coastal waters, which in turn might lead to anoxia. At the same time, estuaries are described to contain a well-developed self-cleaning system. Vast organic and inorganic nitrogen reductions occur due to intense microbial activity. This nitrogen removing function of estuaries is therefore of crucial importance to the water quality of coastal seas. Denitrification, which is the main process removing nitrogen from the estuary permanently, reduces nitrate to gaseous dinitrogen, which disappears to the atmosphere. Nitrate is thus an essential substrate for the removal of nitrogen from the estuary. This makes bacterial nitrification a key process because it is the only biological process that converts ammonium to nitrate. The nitrifying consortium consists of two different groups of chemoautotrophic bacteria. The first one oxidises ammonia to nitrite, while the second one oxidises nitrite to nitrate.

Estuarine nitrification activity typically shows a peak at intermediate salinity [1–6]. This increase in activity with increasing salinity is reported to be coupled to the oxygen condition in the estuary [2], indicating that nitrifying bacteria are oxygen limited upstream from the peak. The cause of the collapse in activity downstream from the peak is less clear, but might be explained by ammonia limitation or intolerance against the increasing salt concentration by the freshwater adapted community [1,2]. Another explanation for the appearance of the peak of activity is that it coincides with the maximum turbidity zone and that ammonia oxidisers prefer to be attached to particles instead of being free-living [3,7]. Nitrifying bacteria tend to form clusters or attach to particulate material and form flocks. In the Elbe estuary, between 50 and 100% of the nitrifying bacteria were attached to flocks [8]. In the Urdaibai estuary nitrification activity was associated with the fraction >3 μm [5]. Particulate material has a longer

* Corresponding author.
E-mail address: boschker@cemo.nioo.knaw.nl (H.T.S. Boschker).

0168-6496/02/$22.00
PII: S0168-6496(02)00325-2

residence time than water [9] and attachment of bacteria to flocks will increase their residence time, providing them a longer stay at one section in the estuary.

There are a number of studies that report nitrification rates in estuarine waters [2–6,10–14]. Many of these use a specific inhibitor to measure the nitrification activity. Nitrification inhibitors have been developed as agrochemicals to prevent loss of nutrient salts through nitrification after fertilisation. Nitrapyrine (N-serve, 2-chloro-6-(trichloromethyl)pyridine) (see [15] and references therein) is a commonly used inhibitor, which works by blocking the first step in nitrification, i.e. the oxidation of ammonia to hydroxylamine [16]. Disadvantage of N-serve is that it is sparingly soluble in water so that it has to be dissolved in an organic solvent. A range of other inhibitors has been used [17] (Table 1), but comprehensive comparative studies including several inhibitors are scarce.

This study presents an intercomparison of different nitrification inhibitors for a pure culture and a natural community of the Schelde estuary. Nitrification rates were measured once a month during a period of 15 months, using the N-serve-sensitive ^{14}C-bicarbonate incorporation technique. In addition, we studied the effect of N-serve on the incorporation of ^{13}C-bicarbonate into polar lipid derived fatty acids (PLFA) to further identify the dominant chemoautotrophic processes and populations [18]. Factors governing nitrification will be identified by comparing in situ nitrification rates with potential nitrification rates based on experimental manipulation of ammonium and oxygen concentration.

2. Method

2.1. Research area

The Schelde estuary (see Fig. 1) drains an estimated 21 000 km^2 of Northern France, Belgium and The Netherlands, an area with approximately 10 million inhabitants, and receives a nitrogen load of 56 000 ton N year^{-1} [19]. Salinity ranges from about 0.5 at Temse, the upper limit of saltwater intrusion, to about 30 at Vlissingen near the mouth of the estuary (Fig. 1). The estuary is vertically well mixed, although slight stratification can occur near Antwerp [20]. The average freshwater outflow of 105 m^3 s^{-1} [21] is small relative to the tidal exchange of about 45 000 m^3 s^{-1} [22]. Turbidity is high in the entire upper estuary with suspended matter concentrations ranging from 15 to 350 mg l^{-1}.

Table 1
List of inhibitors used in the inhibitor experiment

Inhibitor	Concentration used	Ref.	Comments
DMSO		[42]	Solvent
N-serve	50 μM	[16,42]	Dissolved in 80% ethanol
Allylsulfide	200 μM	[42,43]	Dissolved in DMSO
DCD	2 mM	[42]	Dissolved in DMSO
Acetylene (C_2H_2)	0.01 and 1%	[16,42,44]	
Methyl fluoride	10% (v/v)	[38,45]	
ATU	50 μM	[42]	Dissolved in DMSO

2.2. Sample collection and chemical analysis

Once a month from April 1997 till June 1998, surface water samples in the estuary were collected aboard the RV *Luctor* in the navigation channel at the indicated sample sites (Fig. 1). Concomitant with the samples for nitrification measurements, samples for nutrient analysis were taken. Ammonium, nitrite and nitrate were analysed with a SKALAR segmented flow autoanalyser system with a precision of approximately 1%. Salinity, temperature, and oxygen were measured with a CTD system, equipped with a polarographic oxygen sensor (THISHYDRO H2O).

2.3. Dark ^{14}C-bicarbonate incorporation measurements

Nitrification activity was measured by the dark ^{14}C-bicarbonate incorporation method [23]. With this method the carbon fixation by autotrophic ammonia oxidising bacteria was estimated by measuring the difference of inorganic carbon incorporation during dark incubation with and without a specific inhibitor of nitrification activity. At each station, 50-ml glass screw cap bottles were filled so that no headspace was left. To these bottles, 100 μl of 0.925 MBq ml^{-1} ^{14}C-$NaHCO_3$ were added. One set of bottles was treated with a combination of N-serve (5 mg l^{-1} final concentration) and chlorate (10 mg l^{-1} final concentration). A stock solution of N-serve was prepared in 80% ethanol and added to the empty incubation bottles about 4 h before sampling, to allow the ethanol to evaporate [24]. To prevent nitrite oxidising bacteria from taking up bicarbonate, chlorate was added to inhibit nitrite oxidation [25]. The water-soluble chlorate was directly added to the samples. Samples were incubated in triplicate for 20 h [24] in a rotating incubator in the dark at in situ temperature. Filtration and counting were carried out according to the protocol of Kromkamp and Peene [26].

During October 1997–June 1998, each month a triplicate set of bottles were incubated to distinguish between in situ and potential nitrification rates. Potential nitrification rates can be considered as a measure for the nitrifying biomass present at the time of sampling [27]. The bottles for in situ measurements were incubated with a minimal air headspace to prevent additional oxygen to dissolve during incubation. These bottles were incubated at in situ temperature. For determination of potential nitrification activity (PNA) bottles were incubated at room temperature (20–22°C) with 50% (v/v) air headspace and addition of 2 mM NH_4^+ (final concentration). In May 1998, an additional series of bottles were incubated with only

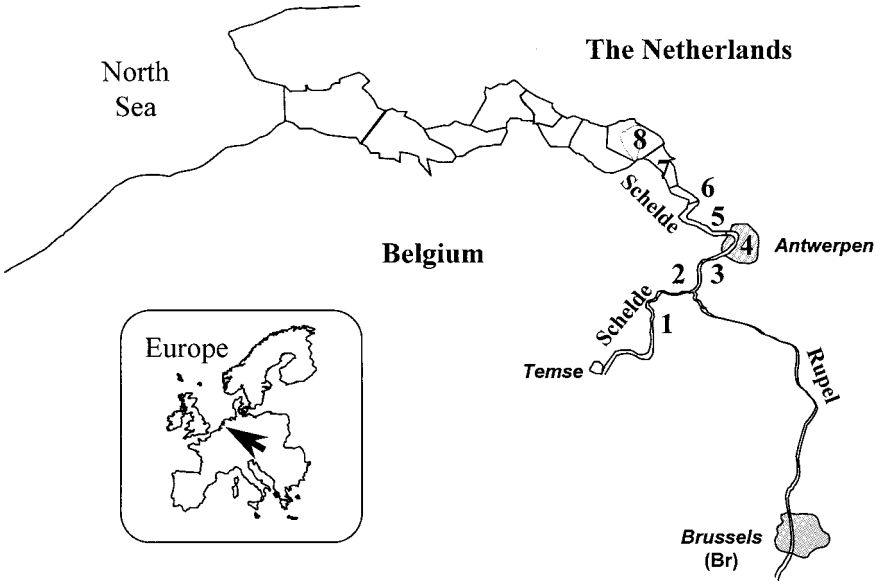

Fig. 1. Map of the Schelde estuary. Numbers indicate sample locations. The distance from the city of Antwerp to the mouth of the estuary is approximately 100 km.

2 mM NH_4^+ (final concentration) added, with a minimal headspace, to study the effect of ammonium on nitrification activity along the estuary.

In order to investigate the relative importance of flock attached and free-living bacteria, experiments were carried out where flocks in the water sample were allowed to settle for 1 h. Filtration was not feasible as a method to separate particulate from free activity, due to the high turbidity, which immediately caused clogging of filters. After 1 h of settling, potential nitrification activity was determined in the upper, clear water layer as described above. Obtained nitrification rates were compared with total sample measurements (with flocks) and particulate matter content was determined in both samples.

Standard errors on nitrification rates were calculated assuming a t-distribution of the results according to:

$$\Delta_{\overline{N}} = \frac{t_{\alpha,n-1}}{\sqrt{n}}\sqrt{\sigma_x^2 + \sigma_y^2}$$

where $\Delta_{\overline{N}}$ = standard error of the nitrification rate, $t_{\alpha,n-1}$ = critical value of t at probability α and $n-1$ degrees of freedom, where n = number of replicate measurements, σ_x = standard deviation of control measurements and σ_y = standard deviation of measurement with inhibitor.

2.4. Inhibitor comparison experiment

A range of known nitrification inhibitors was tested with the dark ^{14}C-incorporation method. Concentrations used and references are listed in Table 1. The background effect of the solvent dimethyl sulfoxide (DMSO), which is used to dissolve allylsulfide, dicyandiamide (DCD) and allylthiourea (ATU), was also tested. The acetylene used in the experiment was purified by passing it through a trap containing sulfuric acid followed by a trap containing 5 M NaOH according to Hyman and Arp [28]. The inhibition of ammonia oxidation was determined on a pure culture of *Nitrosomonas europaea* and on an estuarine water sample from Station 7, May 1998. Incubation was performed in triplicate as described above in the dark at room temperature with 2 mM ammonium (final concentration) and an air headspace of 50% of the total bottle volume.

2.5. ^{13}C-Bicarbonate incorporation into PLFA

In April 1997 and June 1998, we studied the ^{13}C-bicarbonate label incorporation into PLFA to determine the dominant autotrophic processes and populations at Station 3. This station in general showed near maximum nitrification activities. Duplicate 500-ml serum bottles were incubated on deck at in situ temperature for 3–4 h and received the following treatments: non-labelled control incubations, ^{13}C-bicarbonate-labelled in the light, and ^{13}C-labelled dark incubations with and without N-serve and chlorate as specific inhibitors of nitrification. The final ^{13}C-bicarbonate concentration was 0.2 mM (Isotec, $>99\%$ ^{13}C), which led to a 3.0% ^{13}C labelling of the total inorganic carbon pool, and inhibitors were used at concentrations as in the ^{14}C assay. At the end of the incubations, suspended material was collected on pre-combusted GF/F glass-fibre filters (Whatman), which were directly added to the PLFA extraction solvents to stop all activities.

PLFA on the filters were extracted and analysed as in Boschker et al. [29]. In short, lipids were extracted in chloroform/methanol/water using a modified Bligh and Dyer method and fractionated on silicic acid into different polarity classes. The most polar fraction containing the

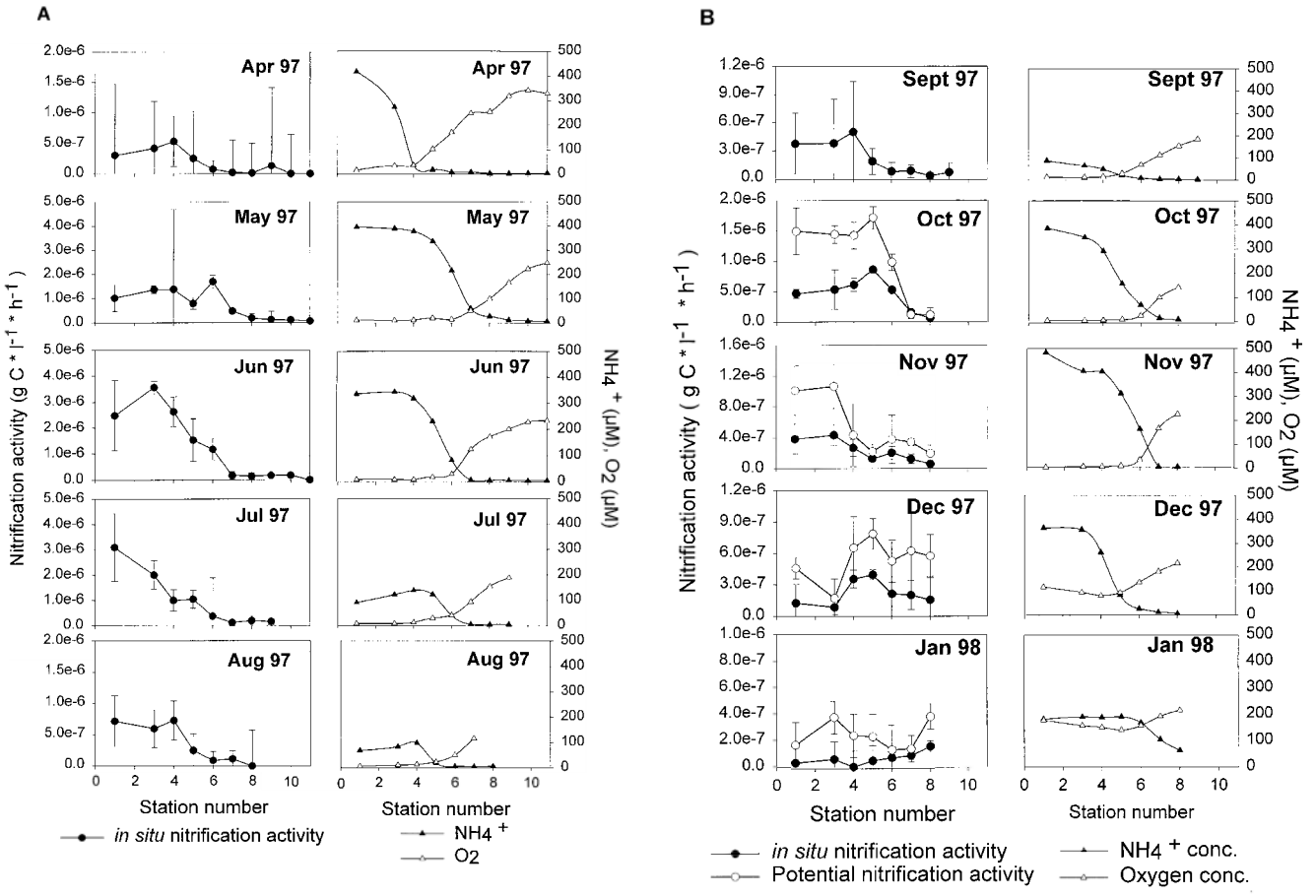

Fig. 2. In situ nitrification activity (closed circles, $n=3$) and potential nitrification activity (open circles, $n=3$). In potential nitrification activity incubations, both ammonium and oxygen were added. Error bars are calculated with a 95% confidence limit as explained in the text. Ammonia (closed triangles) and oxygen (open triangles) concentration along the estuary. Mind scale differences.

PLFA was derived to yield fatty acid methyl esters (FAME). Concentrations and isotopic composition of individual FAME were determined with a gas-chromatograph combustion-interface isotope-ratio mass spectrometer (GC-c-IRMS). Stable carbon isotope ratios for individual FAME were corrected for the one carbon atom in the methyl group that was added during derivation.

The rate of ^{13}C label incorporated into a specific PLFA was calculated as: incorporation rate $=((F_{tx}-F_{t0})\times[\mathrm{PLFA}]_{tx})$/incubation time, with the PLFA concentration in pmol C l^{-1} and the fraction ^{13}C at the start (F_{t0}) and the end (F_{tx}) of the incubations as: $F=^{13}C/(^{13}C+^{12}C)=R/(R+1)$. The carbon isotope ratio (R) was derived from the measured $\delta^{13}C$ values as: $R=(\delta^{13}C/1000+1)\times R_{\mathrm{VPDB}}$, with $R_{\mathrm{VPDB}}=0.0112372$.

3. Results

3.1. Estuary characteristics

Ammonium concentrations (Fig. 2, closed triangles) decreased from 200–500 µM in the upper reaches of the estuary to about 10 µM at Station 8. At the mouth of the estuary, the ammonia concentration was around 5 µM. Ammonium concentrations were typically higher in autumn months. Oxygen concentrations reached a maximum of about 300 µM (Fig. 2, open triangles) and never exceeded 100% air saturation in the entire sampling area. Oxygen concentrations were much lower during summer than during winter.

Salinity varied from below 1 at Station 1 to about 16 at Station 8, our most downstream sample station. At the mouth of the estuary the salinity was 30.

Temperature was 21°C in June 1998 but was never lower than 6°C even in winter months when ambient temperatures were close to zero. Temperature differences along the estuarine gradient were usually not more than 2°C (data not shown).

Suspended particulate matter varied from more than 300 mg l^{-1} in the upper estuary to about 15 mg l^{-1} at lower stations. A clear turbidity maximum could not always be detected but suspended matter concentrations were typically highest between salinity 1 and 5. Suspended particulate matter averaged 82.5 mg l^{-1} with maximal val-

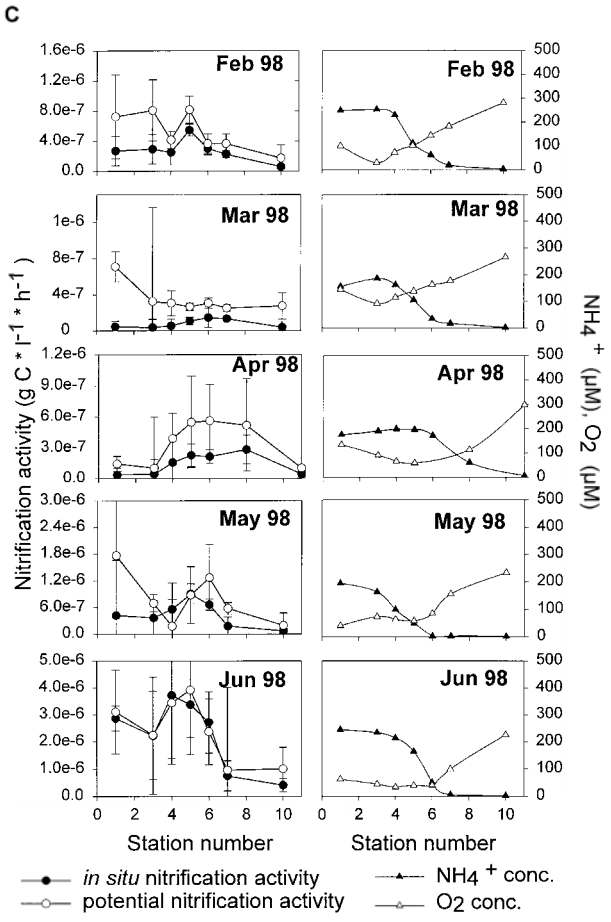

Fig. 2 (*Continued*).

ues of 363 mg l^{-1} in October 1997 (Station 1), 193.3 mg l^{-1} in February 1998 (Station 5) and 132 in May 1998 (Station 6).

3.2. Nitrification rates

Nitrification activity generally reached a maximum at one of the most upstream stations (Fig. 2, closed circles). Besides biomass, oxygen and ammonium concentrations, tidal currents further influence the exact location of this peak. Nitrification was low during winter and reached a maximum of 3.7 μg C l^{-1} h^{-1} in June (both 1997 and 1998, Fig. 2, closed circles). This maximal value was reached in a part of the estuary where oxygen concentrations were less than 100 μM, i.e. in the upper estuary in summer. This indicates that no severe oxygen limitation in this part of the estuary exists. Potential nitrification rates (Fig. 2, open circles) exceeded the in situ activity in the months October 1997–April 1998, which is probably due to lower in situ temperatures. In a more detailed study on the effect of ammonium, it was shown that in the lower estuary nitrification activity increased significantly ($P < 0.05$) upon addition of ammonium, indicating ammonium limitation at Stations 6, 8 and 10 (Fig. 3). Particle associated nitrification activities were 70, 86, 59, 80 and 57% for the months January, February, March, April and May, respectively.

3.3. Inhibitor comparison

In the *N. europaea* culture, bicarbonate incorporation was inhibited by all inhibitors tested (Fig. 4), with efficiencies varying between 90 and 96%. Highest inhibition was found with DCD and ATU (96%), but acetylene and methyl fluoride also inhibited 95% of C incorporation by the pure culture.

In the natural estuarine sample dark bicarbonate incorporation was inhibited by only 31–48% by the inhibitors allylsulfide, N-serve, DCD and ATU. This lower inhibition rate compared to the pure culture was expected since more C-fixing organisms are present in the natural sample than nitrifiers alone. Acetylene and methyl fluoride caused a marked increase of bicarbonate incorporation in natural samples by 45 and 60%, respectively.

The solvent DMSO inhibited ^{14}C incorporation by 46 and 40% in the pure culture and the natural community, respectively.

3.4. ^{13}C-Bicarbonate incorporation into PLFA

Results of the PLFA study were similar in April 1997 and June 1998 and only the latter data are shown here (Fig. 5). Concentrations of PLFA were high and both specific bacterial markers (e.g. i15:0 and a15:0) and polyunsaturated algal compounds (e.g. 20:5ω3) were detected in substantial amounts (Fig. 5A). PLFA labelled in the dark were mainly 16:0 and 16:1ω7c with some label in 16:1ω5c and 18:1ω7c (Fig. 5B). The two dominantly labelled PLFA are very general and occur in both bacteria and eukaryotes. However, the simple labelling pattern with two dominant compounds and the absence of label in polyunsaturated PLFA show that dark incorporation was by bacteria and not by phytoplankton. N-serve in combination with chlorate effectively inhibited the ^{13}C incorporation into PLFA (92% inhibition, Fig. 5B), indicating that most of the dark incorporation was by nitrifying bacteria. Labelling patterns in the light were very different with most of the label in polyunsaturated PLFA such as 16:2, 16:3, 18:2, 18:3, 18:4, 20:5ω3 and 22:6ω3 (data not shown, total incorporation rate into PLFA 7700 ± 330 pmol ^{13}C l^{-1} h^{-1}), which are typical fatty acids in green algae and diatoms that dominate the phytoplankton at the studied salinity [30,31]. Total dark incorporation rate into PLFA was 96 ± 4 pmol ^{13}C l^{-1} h^{-1}, which translates to a carbon based nitrification rate of about 1 μg C l^{-1} h^{-1} (calculated using the percentage ^{13}C labelling of bicarbonate pool and a general bacterial PLFA to biomass conversion factor of 0.04 g PLFA C g^{-1} biomass C [32]). This rate is within the range as found with the ^{14}C-method (Fig. 2) given the uncertainties in the PLFA to carbon conversion factor.

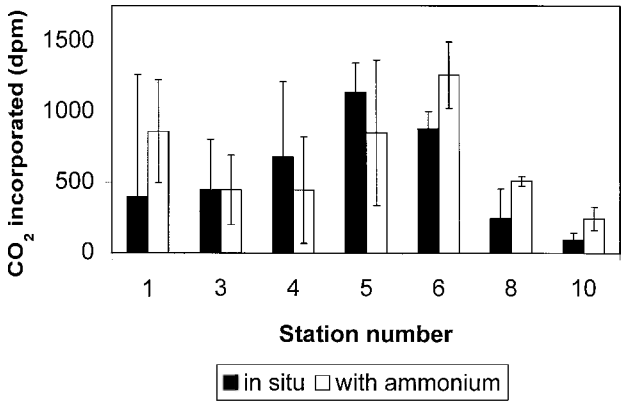

Fig. 3. Effect of ammonium addition on nitrification activity as measured in May 1998. Incubations were carried out at in situ oxygen concentration. Error bars represent 95% confidence limit, $n = 3$.

4. Discussion

4.1. Nitrification rates

We measured a maximum in situ nitrification rate of 3.7 μg C l^{-1} h^{-1} in June. Earlier measurements in the Schelde estuary in September 1977 showed maximum nitrification rates of 4.9 μg C l^{-1} h^{-1} (80 μM N l^{-1} day^{-1} ([2], Table 2). The peak activity in 1977 was observed around Station 7 [2], which is more downstream in the estuary than where the present maximal nitrification activity is observed (Stations 3–5). This observation is explained by the water quality history of the Schelde estuary. Waste loadings in the estuary used to be high, which induced intense microbial degradation leading to very high heterotrophic bacterial production rates [33] and accompanying oxygen depletion [20]. In recent years the water quality in the Schelde estuary has improved [6,34], which has caused a lower nitrification activity, with the peak activity located more upstream in the estuary. Secondary wastewater treatment in the catchment area has increased, which has resulted in decreased carbon and nitrogen loadings. Wastewater treatment might also have resulted in a decreased nitrifying biomass in the estuary, since untreated wastewater may be an important source of nitrifying bacteria [2,24].

Comparison with other estuaries (Table 2) shows that nitrification rates in the Schelde are still among the highest reported, despite the improved water quality of the estuary. In order to be able to compare the different nitrification rates with one another, reported nitrification rates have been recalculated (Table 2) using a conversion factor of 8.3 mol N/mol C [2]. Several authors have measured conversion factors for nitrification empirically: 14.3 (river) to 12.3 (sea) [13], 5.95 obtained with estuarine isolates of ammonium oxidisers [3,11], and 8.3 measured in the Schelde estuary [23]. The amount of ammonium necessary for growth can vary substantially among different growth phases or species of nitrifying bacteria. It has been shown that the composition of the nitrifying community is changing along the estuarine gradient [35], and it is therefore plausible that N/C ratios for nitrification differ along this gradient as well.

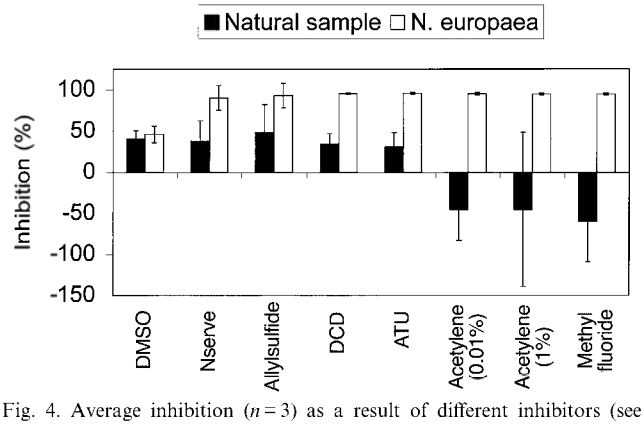

Fig. 4. Average inhibition ($n = 3$) as a result of different inhibitors (see Table 1 for used concentrations) in a pure culture of *N. europaea* and an estuarine sample. Standard errors are calculated by summation of the relative errors in the control and inhibited samples assuming *t*-distribution ($P = 0.05$).

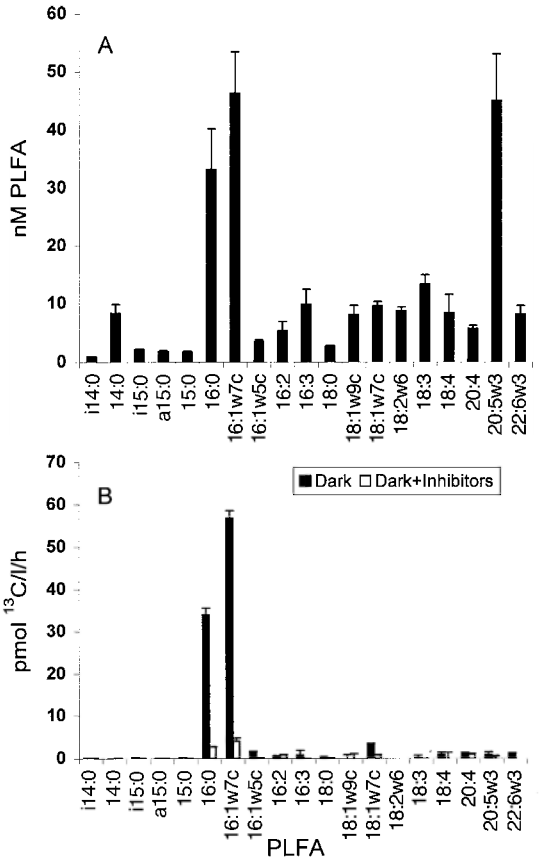

Fig. 5. Results of ^{13}C-bicarbonate incorporation into individual PLFA at Station 3 in June 1998. Shown are the PLFA concentrations (A) and the ^{13}C incorporation rates in the dark with (white bars) and without (black bars) nitrification inhibitors (B).

Table 2
Comparison of published nitrification activities (maximal reported values)

Estuary	Nitrification activity (μmol N l^{-1} day^{-1})	NH_4 concentration (μM)	Method	Ref.
Rhone river plume	1–2	1–10	^{14}C, ATU	[13]
Narragansett Bay	up to 11	8	^{14}C, N-serve	[11, 12]
Long Island sound	6.3	6	^{13}N	[46]
Urdaibai estuary	up to 4.6		^{14}C, ATU	[5]
Tamar estuary	3	5	^{14}C, N-serve	[3]
Chesapeake Bay	32	30	^{15}N	[47]
Schelde estuary	80	500	^{14}C, N-serve	[2]
Schelde estuary	up to 45	up to 150	^{14}C,N-serve	This study

If data were reported as amount carbon incorporated we converted to nitrogen using a conversion factor of 8.3 mol N/mol C [23].

There was a contrast between the upper estuary and the lower part with respect to factors that regulated nitrification rate. In the upper estuary, in situ nitrification activity and potential activity were almost alike during May and June 1998 (Fig. 2), when the difference in incubation temperature between in situ and potential measurements was very low. Therefore, nitrification appeared not to be limited by either ammonium or oxygen concentration during this period. This also implies that neither oxygen nor ammonium was limiting in the incubation bottles during the in situ measurements despite the sometimes low concentrations. The difference between potential and in situ activity is largest in autumn months. This is probably due to temperature limited nitrification in autumn. In contrast to the situation in the upstream estuary, there is an indication for ammonium limitation of nitrification activity at the most seaward sample stations, as bacterial activity increases upon addition of ammonium to the incubation bottles (Fig. 3). However, the present data alone are not sufficient to prove ammonium limitation in the downstream estuary. Another explanation of decreasing activity could be the influence of increasing salinity [10]. Furthermore, the lower residence times in the lower estuary play a role in decreased activity. The distribution and activity of the nitrifying population are also influenced by their attachment to particulate material [36]. We found that 57–86% of the nitrifying biomass is attached to particles, which is in accordance with observations in the Elbe estuary [8]. The tendency to attach is a known phenomenon of (nitrifying) bacteria. Free-living bacteria are subject to the residual seaward flow in an estuary, which means that they have little time to adapt to the particular circumstances at a given section in the estuary. Attachment to particles increases their residence time [9].

4.2. Inhibitor experiments and PLFA labeling

CO_2 fixation in the pure culture *N. europaea* was inhibited by 90–96% by all inhibitors. In estuarine samples, inhibition in the ^{14}C assay never exceeded 48%, indicating that more than half of the dark bicarbonate incorporation in natural samples is the result of other chemolithotrophic bacteria or dark fixation by heterotrophic bacteria or phytoplankton [26]. However, incorporation of ^{13}C-bicarbonate into PLFA was almost completely inhibited with N-serve and chlorate. The dark PLFA labelling is specific for chemoautotrophic organisms, as dark fixation by anapleurotic routes in heterotrophic bacteria and algae is not channelled to fatty acid synthesis. The complete inhibition of the PLFA labelling with nitrification specific inhibitors therefore strongly suggests that nitrifying bacteria dominated the chemoautotrophic community. The ^{13}C-PLFA labelling pattern was dominated by 16:0 and 16:1ω7c and is similar to the fatty acids found in most ammonium oxidising bacteria belonging to the β- and γ-subclasses of the Proteobacteria [37]. More detailed identification of the active populations by ^{13}C-PLFA labelling is unfortunately not possible.

The inhibitors acetylene and methyl fluoride had a stimulating effect on ^{14}C incorporation in natural samples, while they almost completely inhibited bicarbonate incorporation in the *N. europaea* culture. Both acetylene and methyl fluoride [38,39] have successfully been tested on natural soil and sediment samples. In combination with ^{14}C incorporation methyl fluoride has been used in natural samples from an alkaline, saline lake [17]. It is unclear what the reason could be for the increased incorporation with methyl fluoride and acetylene in our field sample, but it might be that bacteria able of degrading these inhibitors were stimulated and that these bacteria partially use carbon dioxide for growth, similar to the type II methanotrophs containing the serine pathway [40]. Many of the inhibitors used have the disadvantage of being insoluble in water. The use of a solvent is therefore necessary. During the inhibitor test, we used DMSO. Although DMSO has been described not to have inhibiting effects on *N. europaea* [41], we found an inhibiting effect on bicarbonate incorporation with this species, as well as with natural assemblages of bacteria. Because the gaseous inhibitors failed to work in our natural samples, we used the inhibitor N-serve with ethanol as a solvent during the in situ measurements [24].

Inhibitors were tested at one concentration level only in this study. It has been described earlier that not all species or strains of nitrifying bacteria are similarly affected by a given inhibitor [16]. For example N-serve concentrations

required for complete inhibition ranged from 0.9 to 43 μM for seven strains belonging to three different genera of ammonia oxidisers. The 50 μM we used was on the higher side of this range. The almost complete inhibition as found with the PLFA method suggests that this inhibitor concentration was indeed effective despite the possible decreased availability of the inhibitor by particle adsorption in this turbid estuary.

Acknowledgements

The authors thank captain and crew of the RV *Luctor* for assistance during sampling and Pieter van Rijswijk for assistance during laboratory experiments. Natacha Brion, Free University of Brussels, is acknowledged for practical advice concerning the nitrification measurements. Jack Middelburg is acknowledged for critically reading the manuscript. We thank Prof. Ralf Conrad and two anonymous reviewers for valuable suggestions. This is Publication 3010 NIOO-KNAW Centre for Estuarine and Marine Ecology, Yerseke, The Netherlands.

References

[1] Helder, W. and de Vries, R.T.P. (1983) Estuarine nitrite maxima and nitrifying bacteria (Ems-Dollard estuary). Neth. J. Sea Res. 17 (1), 1–18.
[2] Somville, M. (1984) Use of nitrifying activity measurements for describing the effect of salinity on nitrification in the Scheldt estuary. Appl. Environ. Microbiol. 47, 424–426.
[3] Owens, N.J.P. (1986) Estuarine nitrification: a naturally occurring fluidized bed reaction? Estuar. Coast. Shelf Sci. 22, 31–44.
[4] Pakulski, J.D., Benner, R., Amon, R., Eadie, B. and Whiltledge, T. (1995) Community metabolism and nutrient cycling in the Mississippi River plume: evidence for intense nitrification at intermediate salinities. Mar. Ecol. Prog. Ser. 117, 207–218.
[5] Iriarte, A., Madariaga, I., de Diez-Garagarza, F., Revilla, M. and Orive, E. (1996) Primary plankton production, respiration and nitrification in a shallow temperate estuary during summer. J. Exp. Mar. Biol. Ecol. 208, 127–151.
[6] de Wilde, H.P.J. and de Bie, M.J.M. (2000) Nitrous oxide in the Schelde estuary: production by nitrification and emission to the atmosphere. Mar. Chem. 69, 203–216.
[7] Abril, G., Riou, S.A., Etcheber, H., Frankignoulle, M., Wit, R. and de Middelburg, J.J. (2000) Transient, tidal time-scale, nitrogen transformations in an estuarine turbidity maximum-fluid mud system (The Gironde, South-West France). Estuar. Coast. Shelf Sci. 50, 703–715.
[8] Stehr, G., Böttcher, B., Dittberner, P., Rath, G. and Koops, H.-P. (1995) The ammonia-oxidizing nitrifying population of the River Elbe estuary. FEMS Microbiol. Ecol. 17, 177–186.
[9] Soetaert, K. and Herman, P.M.J. (1995) Estimating estuarine residence times in the Westerschelde (The Netherlands) using a box model with fixed dispersion coefficients. Hydrobiologia 311, 215–224.
[10] Billen, G. (1975) Nitrification in the Scheldt estuary (Belgium and The Netherlands). Estuar. Coast. Mar. Sci. 3, 79–89.
[11] Berounsky, V.M. and Nixon, S.W. (1990) Temperature and the annual cycle of nitrification in waters of Narragansett Bay. Limnol. Oceanogr. 35 (7), 1610–1617.
[12] Berounsky, V.M. and Nixon, S.W. (1993) Rates of nitrification along an estuarine gradient in Narragansett Bay. Estuaries 16, 718–730.
[13] Feliatra, F. and Bianchi, M. (1993) Rates of nitrification and carbon uptake in the Rhone river plume (Northwestern Mediterranean Sea). Microb. Ecol. 26, 21–28.
[14] Bianchi, M., Bonin, P. and Feliatra, F. (1994) Bacterial nitrification and denitrification rates in the Rhône River plume (Northwestern Mediterranean Sea). Mar. Ecol. Prog. Ser. 103, 197–202.
[15] Oremland, R.S. and Capone, D.G. (1988) Use of 'specific' inhibitors in biogeochemistry and microbial ecology. Adv. Microb. Ecol. 10, 285–383.
[16] Bedard, C. and Knowles, R. (1989) Physiology, biochemistry and specific inhibitors of CH^4, NH^4 and CO oxidation by methanotrophs and nitrifiers. Microbiol. Rev. 53 (1), 68–84.
[17] Joye, S.B., Connell, T.L., Miller, L.G., Oremland, R.S. and Jellison, R.S. (1999) Oxidation of ammonia and methane in an alkaline, saline lake. Limnol. Oceanogr. 44, 178–188.
[18] Boschker, H.T.S., Nold, S.C., Wellsbury, P., Bos, D., Graaf, W., de Pel, R., Parkes, R.J. and Cappenberg, T.E. (1998) Direct linking of microbial populations to specific biogeochemical processes by ^{13}C-labelling of biomarkers. Nature 392, 801–805.
[19] Soetaert, K. and Herman, P.M.J. (1995) Nitrogen dynamics in the Westerschelde estuary (SW Netherlands) estimated by means of the ecosystem model MOSES. Hydrobiologia 311, 225–246.
[20] Wollast, R. (1988) The Scheldt estuary. In: Pollution of the North Sea, an Assessment (Salomons, W., Bayne, B.L., Duursma, E.K. and Förstner, U., Eds.), pp. 183–193. Springer Verlag, Berlin.
[21] Billen, G., Somville, M., de Becker, E. and Servais, P. (1985) A nitrogen budget of the Scheldt hydrographical basin. Neth. J. Sea Res. 19 (3–4), 223–230.
[22] Middelburg, J.J. and Nieuwenhuize, J. (1998) Carbon and nitrogen stable isotopes in suspended matter and sediments of the Schelde estuary. Mar. Chem. 60, 217–225.
[23] Somville, M. (1978) A method for the measurement of nitrification rates in water. Water Res. 12, 843–848.
[24] Brion, N. and Billen, G. (1997) Wastewater as a source of nitrifying bacteria in river systems: the case of the river Seine downstream from Paris. In: Etude de Processus de Nitrification à l'Échelle de Grands Réseaux Hydrographiques Anthropisés (Brion, N., Ed.), pp. 26–37. Université Libre de Bruxelles, Brussels.
[25] Belser, L.W. and Mays, E.L. (1980) Specific inhibition of nitrite oxidation by chlorate and its use in assessing nitrification in soils and sediments. Appl. Environ. Microbiol. 39, 505–510.
[26] Kromkamp, J.C. and Peene, J. (1995) Possibility of net phytoplankton primary production in the turbid Schelde Estuary (SW Netherlands). Mar. Ecol. Prog. Ser. 121, 249–259.
[27] Bodelier, P.L.E., Libochant, J.A., Blom, C.W.P.M. and Laanbroek, H.J. (1996) Dynamics of nitrification and denitrification in root-oxygenated sediments and adaptation of ammonia-oxidizing bacteria to low-oxygen or anoxic habitats. Appl. Environ. Microbiol. 62, 4100–4107.
[28] Hyman, M.R. and Arp, D.J. (1987) Quantification and removal of some contaminating gases from acetylene used to study gas-utilizing enzymes and microorganisms. Appl. Environ. Microbiol. 53, 298–303.
[29] Boschker, H.T.S., Graaf, W., de Köster, M., Meyer-Reil, L.-A. and Cappenberg, T.E. (2001) Bacterial populations and processes involved in acetate and propionate consumption in an anaerobic sediment. FEMS Microbiol. Ecol. 35, 97–103.
[30] Volkman, J.K., Jeffrey, S.W., Nichols, P.D., Rogers, G.I. and Garland, C.D. (1989) Fatty acid and lipid consumption of 10 species of microalgae used in mariculture. J. Exp. Mar. Biol. Ecol. 128, 219–240.
[31] TRIPOS (1999) Biomonitoring van fytoplankton in de Nederlandse zoute en brakke wateren 1998. Rapportnummer: 99.Too17-3a. In Dutch. Rijkswaterstaat. Rijksinstituut voor Kust en Zee (RIKZ).

[32] Brinch-Iversen, J. and King, G.M. (1990) Effects of substrate concentration, growth state and oxygen availability on relationships among bacterial carbon, nitrogen and phospholipid phosporus content. FEMS Microbiol. Ecol. 74, 345–356.
[33] Goosen, N.K., Van Rijswijk, P. and Brockmann, U. (1995) Comparison of heterotrophic bacterial production rates in early spring in the turbid estuaries of the Scheldt and the Elbe. Hydrobiologia 311, 31–42.
[34] van Damme, S., Meire, P., Maeckelberghe, H., Verdievel, M., Bourgoing, L., Taveniers, E., Ysebaert, T. and Wattel, G. (1995) De waterkwaliteit van de zeeschelde: evolutie in de voorbije dertig jaar. Water 85, 244–256.
[35] de Bie, M.J.M., Speksnijder, A.G.C.L., Kowalchuk, G.A., Schuurman, T., Zwart, G., Stephen, J.R., Diekmann, O.E. and Laanbroek, H.J. (2001) Shifts in the dominant populations of ammonia-oxidizing β-subclass Proteobacteria along the eutrophic Schelde estuary. Aquat. Microb. Ecol. 23, 225–236.
[36] Prosser, J.I. (1989) Autotrophic nitrification in bacteria. Adv. Microbiol. Physiol. 30, 125–181.
[37] Blumer, M., Chase, T. and Watson, S.W. (1969) Fatty acids in the lipids of marine and terrestrial nitrifying bacteria. J. Bacteriol. 99, 366–370.
[38] Miller, L.G., Coutlakis, M.D., Oremland, R.S. and Ward, B.B. (1993) Selective inhibition of ammonium oxidation and nitrification-linked N_2O formation by methyl fluoride and dimethyl ether. Appl. Environ. Microbiol. 59 (8), 2457–2464.
[39] Caffrey, J.M. and Miller, L.G. (1995) A comparison of two nitrification inhibitors used to measure nitrification rates in estuarine sediments. FEMS Microbiol. Ecol. 17, 213–220.
[40] Hanson, R.S. and Hanson, T.E. (1996) Methanotrophic bacteria. Microbiol. Rev. 60, 439–471.
[41] Powell, S.J. and Prosser, J.I. (1985) The effect of nitrapyrin and chloropicolinic acid on ammonium oxidation by *Nitrosomonas europaea*. FEMS Microbiol. Lett. 28, 51–54.
[42] Roy, R. and Knowles, R. (1995) Differential inhibition by allylsulfide of nitrification and methane oxidation in freshwater sediment. Appl. Environ. Microbiol. 61 (12), 4278–4283.
[43] Juliette, L.Y., Hyman, M.R. and Arp, D.J. (1993) Mechanism-based inactivation of ammonia monooxygenase in *Nitrosomonas europaea* by allylsulfide. Appl. Environ. Microbiol. 59, 3728–3735.
[44] Sloth, N.P., Nielsen, L.P. and Blackburn, T.H. (1992) Nitrification in sediment cores measured with acetylene inhibition. Limnol. Oceanogr. 37 (5), 1108–1112.
[45] Oremland, R.S. and Culbertson, C.W. (1992) Evaluation of methyl fluoride and dimethyl ether as inhibitors of aerobic methane oxidation. Appl. Environ. Microbiol. 58 (9), 2983–2992.
[46] Capone, D.G., Horrigan, S.G., Dunham, S.E. and Fowler, J. (1990) Direct determination of nitrification in marine waters by using the short-lived radioisotope of nitrogen, ^{13}N. Appl. Environ. Microbiol. 56, 1182–1184.
[47] Horrigan, S.G., Montoya, J.P., Nevins, J.L., McCarthy, J.J., Ducklow, H.W., Goericke, R. and Malone, T. (1990) Nitrogenous nutrient transformations in the spring and fall in the Chesapeake Bay. Estuar. Coast. Shelf Sci. 30, 369–391.