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Simultaneous NH_3 oxidation and N_2 production at reduced O_2 tensions by sewage sludge subcultured with chemolithotrophic medium

E.B. Muller, A.H. Stouthamer & H.W. van Verseveld
Department of Microbiology; Biological Laboratory, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands

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Abstract

The ammonia oxidation rate by sewage sludge was determined as a function of the dissolved oxygen tension. Samples of sludge were taken from a domestic waste water treatment pilot plant in which sludge was completely retained by membrane filtration. The samples were subcultured chemolithotrophically in recycling reactors. The gas supplied was a mixture of pure argon and oxygen. The K_{O2} for ammonia oxidation was estimated to be 0.97 (± 0.16) kPa dissolved oxygen. Together with ammonia oxidation and oxygen consumption, dinitrogen gas was produced. So, aerobic denitrification occurred. At dissolved oxygen tensions of 1.25 kPa and higher, the dinitrogen production rate (per N-mole) equalled 20% of the ammonia oxidation rate. This proportion was even 58% at 0.3 kPa dissolved oxygen. At 0.15 kPa dissolved oxygen, however, nitrification hardly proceeded, while dinitrogen production soon stopped. Most likely, a nitrifier concomitantly oxidized ammonia and reduced nitrite to dinitrogen.

Introduction

The removal of nitrogen containing compounds during the aerobic treatment of domestic waste water is traditionally regarded to consist of two distinct processes. At first, in two successive steps ammonia is oxidized to nitrate by autotrophic nitrifiers. Subsequently, if the treatment plant possesses an anoxic zone, nitrate is denitrified to nitrogen gas by heterotrophic bacteria. More recently, however, evidence has been reported that this distinction is not strict.

The autotrophic nitrifiers involved are *Nitrosomonas* spp. and *Nitrobacter* spp. (Koops et al. 1991; Painter 1986). Members of the first genus oxidize ammonia at the respiratory chain, which yields nitrite in two successive steps (see Hooper 1987; Wood 1986 for extensive reviews):

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \quad (1)$$

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^- \quad (2)$$

One pair of the electrons produced in the second reaction is utilized in the first step. The other pair flows to the terminal cytochrome *c* oxidase. This enzyme reduces oxygen according to:

$$0.5O_2 + 2H^+ + 2e^- \rightarrow H_2O \quad (3)$$

Additionally, electrons are used for the generation of reductant power (NAD(P)H) via reversed electron flow, and might flow to a nitrite reductase (DiSpirito et al. 1985; Miller & Nicolas 1985). Regardless these flows, the net reaction is:

$$NH_3 + 1.5O_2 \leftrightarrow NO_2^- + H^+ + H_2O \quad (4)$$

Subsequently, nitrite thus formed is oxidized by *Nitrobacter* spp. according to:

$$NO_2^- + H_2O \leftrightarrow NO_3^- + 2H^+ + 2e^- \quad (5)$$

Again, electrons are involved in NAD(P)H formation. Also, a nitrite reductase has been purified (Ahlers et al. 1990), which might accept electrons at anoxic conditions. If electrons only flow to the terminal oxidase, as in eq. (3), the net reaction is:

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \quad (6)$$

These nitrification reactions, which are needed for energy generation, were considered to be confined to oxic conditions as oxygen is consumed. In agreement with this, the activity of nitrifiers have been shown to be strongly reduced at low oxygen tensions (Hanaki et al. 1990; Laanbroek & Gerards 1993; van Niel et al. 1993).

Denitrification is carried out by many heterotrophic bacteria that use an oxidized nitrogen source as an electron acceptor in stead of oxygen. Electrons released by the oxidation of organic substrates may be used by several subsequent reductases. Complete nitrate respiration is as follows (see Stouthamer 1988; Zumft et al. 1988 for overviews):

$$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O \quad (7)$$

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O \quad (8)$$

$$2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O \quad (9)$$

$$N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O \quad (10)$$

Since the energy gained with denitrification is lower than with oxygen reduction, denitrification was considered only to occur at anoxic conditions.

In contrast to these conventional nitrification and denitrification pathways, other processes have been reported. For instance, ammonia and nitrate can simultaneously be converted to nitrogen gas at anoxic conditions (Van de Graaf et al. 1990). Also, bacteria that denitrify in the presence of oxygen have been isolated. Among them are heterotrophic species, such as *Thiosphaera pantotropha* (Robertson & Kuenen 1989) and *Alcaligenes* sp. (Krul 1976), and autotrophic nitrifiers.

At oxic and anoxic conditions, *Nitrosomonas* spp. are able to form nitric oxide and nitrous oxide by the biological reduction of nitrite (Poth & Focht 1985; Remde & Conrad 1990; Ritchie & Nicholas 1972). Once has dinitrogen production been demonstrated for a *Nitrosomonas* sp. which had been isolated from a stream sediment receiving a lot of nitrogen deposition (Poth 1986); since the widely used *N. europaea* ATCC 19718 formed nitrous oxide instead, complete denitrification is not a universal characteristic of ammonia oxidizers. At aerobiosis, the production rates of nitric and nitrous oxides depend on the nitrite concentration (Anderson & Levine 1986; Bock et al. 1992), whereas most authors have reported an inverse relationship with the dissolved oxygen tension (Goreau et al. 1980; Lipschultz et al. 1981; Poth & Focht 1985; Stüven et al. 1992). At the optimal dissolved oxygen tension, i.e., at 0.5–1.0 kPa, 10% of the nitrite formed was shown to be reduced to nitrous oxide by a marine strain (Goreau et al. 1980), 2.5% to nitrous oxide and 3.8% to nitric oxide by a strain isolated from soil (Lipschultz et al. 1981), and a nitrogen loss of 11% was found with strain *Freitag*, which even increased to 19% in the presence of pyruvate (Stüven et al. 1992). At anoxic conditions nitrite is not respired chemolithotrophically (Abeliovich & Vonshak 1992; Hynes & Knowles 1984; Stüven et al. 1992), but nitric and nitrous oxides may emerge at mixotrophic conditions (Abeliovich & Vonshak 1992; Stüven et al. 1992). So, the denitrification activities of *Nitrosomonas* spp. are substantial at low dissolved oxygen tensions.

The denitrifying capacities of *Nitrobacter* spp. have been studied less extensively. At aerobic conditions most strains are unable to denitrify (Bock et al. 1992; Freitag et al. 1987; Hynes & Knowles 1984). Some strains have been shown to grow in oxygen-depleted batch cultures which were supplied with pyruvate, ammonia and nitrate (Freitag et al. 1987). Then, pyruvate and nitrate were consumed, while ammonia accumulated and nitrous oxide emerged. Nitric oxide, which is sometimes produced at low dissolved oxygen tensions, might be involved in the formation of NADH (Bock et al. 1992; Freitag & Bock 1990).

This study aims to determine the nitrification capacity of sewage sludge as a function of the oxygen tension. For this purpose, sewage sludge was taken from a domestic waste water treatment pilot plant in which sludge was completely retained by membrane filtration. The samples were subcultured chemolithotrophically in gas-tight recycling reactors. The gas supplied was a mixture of pure argon and oxygen. Since nitrification was unexpectedly accompanied by considerable nitrogen gas production, it is additionally aimed at to identify the potential denitrification process. For this purpose, redox balances are used to compare gas exchange rates to nitrification rates.

Materials and methods

Source of sludge

Activated sludge was taken from an experimental domestic waste water treatment plant operated at

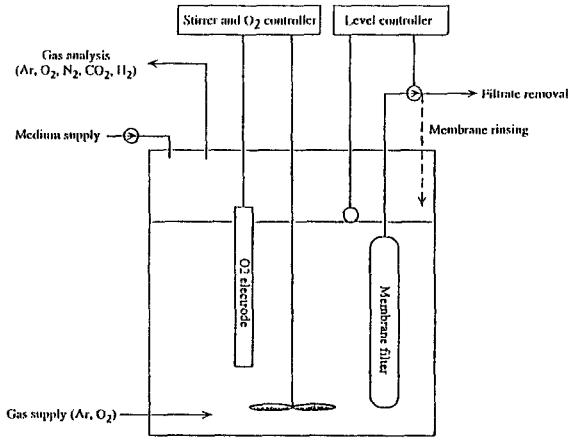

Fig. 1. Configuration of recycling reactors used; temperature and pH controllers are not represented. Before sampling filtrates, membrane units were rinsed by filtrate circulation (dashed arrow).

the Netherlands Organization for Applied Scientific Research (TNO) in Delft. For 9 months sludge had completely been retained by membrane filtration (see Muller et al. 1995a, for a complete description). Operating conditions in the week prior to sampling were: the pH ranged between 6.7 and 6.9; the kjeldahl nitrogen load was 8.5 mmol·l^{-1}·day^{-1}; the temperature was around 20° C; the dissolved oxygen tension near the entrance of influent ranged between 0.4 and 2.6 kPa; the tension furthest from the entrance exceeded 17 kPa (1 kPa = 14 μM dissolved oxygen in pure water at 20° C). The sludge concentration (dry weight) in the plant was 49 $g.^{-1}$; sludge consisted of a dense suspension of free cells, very small flocs (< 50 μm) and floc fragments. Ammonium and nitrite were undetectable in the effluent.

Reactor configuration and culture conditions

Sludge was diluted with effluent to 27–29 g·l^{-1} (dry weight) and 1.2 l samples were cultured in reactors with complete sludge retention (recycling reactors; see Fig. 1). The devices were designed and manufactured by the Electronic and Mechanical Workshop of the Faculty of Biology, Vrije Universiteit, Amsterdam, The Netherlands. In recycling mode, medium was continuously supplied at a fixed rate. An optical level controller regulated the effluent (filtrate) pump such that the volume was kept constant. Sludge was completely retained by Teflon filters that had a poresize of 0.22 μm (type GV, Millipore, Bedford, USA). Filters were sealed and placed over a socket of sintered steel; subsequently, this unit was placed in the reactor vessel (see Schrickx et al. 1993). Process constants were as follows: the pH was kept at 7.0 with 0.6 M Na_2CO_3; the temperature was 30° C; the substrate provision rate was 0.1 l·h^{-1}; the dissolved oxygen tension was polarographically measured (electrode type INPRO 6001, Ingold, Urdorf, Switzerland), and was kept constant by the regulation of the stirrer speed, which always exceeded 700 rpm. Chemolithotrophic medium contained 3 mM KH_2PO_4, 1 mM $CaCl_2$, 15 μM $FeCl_3$, 1 μM Na_2MoO_4, 10 μM $ZnSO_4$, 0.5 mM $MgSO_4$, 2.5 μM $CuSO_4$, 5 μM $CoCl_2$, 30 μM $MnCl_2$, 5 μM H_3BO_3, 0.3 mM HCl, 5 mM H_2SO_4; nitrogen source was 2.7 mM $(NH_4)_2SO_4$.

Gas mixtures were obtained by blending pure argon and pure oxygen with mass flow controllers (type 5878, Brooks Instruments B.V., Veenendaal, The Netherlands). The gas supply rate was 10–15 l·h^{-1}. Special attention was paid to the exchange of gas with the environment. Off-gas from the reactors filled with destilled water and flushed with pure argon did not contain nitrogen and oxygen, while the carbon dioxide and hydrogen content was lower than 0.001% (v/v). The remainder was argon. Off-gas from reactors with sludge not fed with medium and supplied with gas mixtures of argon and oxygen contained argon and oxygen in almost the same ratio as that in the gas inlet; nitrogen was not detected (see Fig. 2). Small deviations in oxygen and carbon dioxide contents resulted from endogenous respiration. Hence, it was concluded that the reactors were gas-tight.

Experimental set-up and analysis

Three sludge samples were cultured in batch mode for 1 day. After endogenous respiration had stabilized, ammonium was added until approximately 5 mM was reached as a final concentration. Then, the supply of chemolithotrophic medium was started, a filter unit was implemented and the controller for filtrate removal was switched on (see Fig. 1). In this way, the reactor configuration was changed to recycling mode, i.e., effluent was removed while sludge was completely retained, which enabled the determination of nitrification rates at a non-inhibitory nitrite concentration (see Muller et al. 1995b). Subsequently, the dissolved oxygen tensions were adjusted to 5, 2.5 and 1.25 kPa, respectively (1 kPa = 12 μM dissolved oxygen in pure water at 30° C). To get these tensions, the ratios of argon and oxygen in the gas supply and gas flow rates were changed. When ammonium and nitrite had become

undetectable with test strips (Merckoquant, Merck, Darmstadt, Germany) and oxygen concentrations in inlet and outlet gas had become equivalent, indicating the nitrification reactions had ceased, the medium supply was switched off. Then, the samples were aerated in batch mode for 4 hours. Subsequently, experiments were repeated at 0.6, 0.3 and 0.15 kPa dissolved oxygen using new filter units.

At regular time intervals, 5 ml samples of effluent were taken to be photometrically analysed on ammonium, nitrite and nitrate (Autoanalyzer II, Technicon Industrial Systems, Tarry Town, NY). To rinse the filter units, filtrate was circulated before sampling effluent (see Fig. 1). The dry weight of reactor contents were determined as described by the American Public Health Association (1980). Gas analysis was done with a mass spectrometer (MM8-80F, VG Gas Analysis Systems Ltd., Middlewich, England); gases determined were N_2, O_2, Ar, CO_2 and H_2.

To determine maximal ammonium consumption rates, it was assumed that only one species was present which oxidized ammonia to nitrite; growth of the nitrifying population was negligible; and kjeldahl-nitrogen was neither consumed nor released as a result of (cryptic) growth, predation, etc. Since culture volumes, medium supply and effluent removal rates were kept constant, the ammonium concentration ($[NH_4]_t$, in mM) is described by (Muller et al. 1995b):

$$[NH_4]_t = [NH_4]_r + ([NH_4]_0 - [NH_4]_r + r_{ns}D^{-1})e^{-Dt} \qquad (11)$$

in which subscript r denotes medium reservoir, D is the effluent removal rate as a fraction of the culture volume in h^{-1}, and r_{ns} is the rate of ammonium consumption in $mmol \cdot l^{-1} \cdot h^{-1}$. When the ammonium concentration was sufficiently high to avoid substrate limitation, i.e., higher than 1 mM, r_{ns} was regarded as a parameter with respect to the ammonium concentration (Prosser 1989; Muller et al. 1995b). To compare ammonium consumption rates, r_{ns} was expressed as a fraction of the sludge concentration (X, in g MLSS$\cdot l^{-1}$), so $q_{ns} \equiv r_{ns} \cdot X^{-1}$.

The dissolved oxygen tension at which the ammonium consumption rate was half that at excess oxygen (saturation constant, K_{O2}) was determined with kinetics equivalent to that of Monod, except that substrate consumption rates were used in stead of growth rates. Data analysis was performed with Kaleidagraph version 2.1 for Macintosh (Abelbeck Software, Redding, PA, USA).

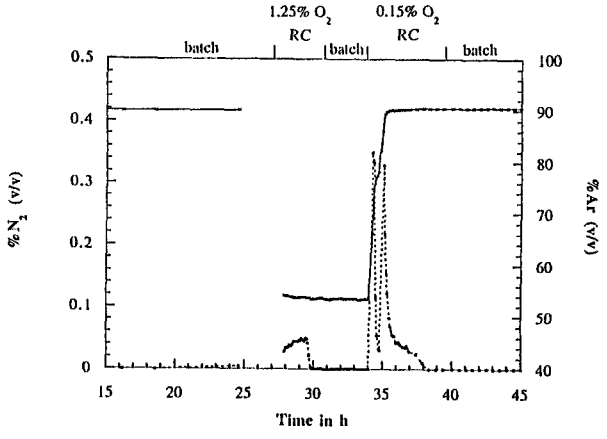

Fig. 2. Ar (x—x) and N_2 (o- - -o) contents (v/v) in outlet gas at 1.25 and 0.15 kPa dissolved oxygen. The contents of gases not shown were O_2 (approx. 50% and 10%, respectively) and CO_2 (< 0.2%). After addition of NH_4^+ to a final concentration of about 5 mM (at t = 27 h and 34 h), the batch cultures were changed to recycling mode (RC; see Fig. 1). Subsequently, the gas flow rate and the ratio of Ar and O_2 in the inlet gas were changed to maintain the dissolved oxygen tensions imposed. N_2 and O_2 concentrations in outlet gas after stabilization of the Ar content were taken for evaluation (see text).

Results

During the first day sludge samples were aerated in controlled batch reactors to let oxidizable compounds still present to be removed. At a dissolved oxygen tension higher than 10 kPa, oxygen consumption rates were initially 44 and decreased till 25 $\mu mol \cdot g^{-1} h^{-1}$. Carbon dioxide production dropped from 39 to 15 $\mu mol \cdot g^{-1} \cdot h^{-1}$. The lower values, which were stable, resulted from heterotrophic endogenous respiration. Nitrogen gas production was not observed (see Fig. 2). When batch cultures were turned into chemolithotrophically operated recycling reactors, which are continuous cultures with complete sludge retention, dissolved oxygen tensions imposed were reached within half an hour.

The change in mineral nitrogen content in the recycling reactors was a function of the dissolved oxygen tension, as is illustrated by the concentration of ammonium, nitrite and nitrate in effluents. Figure 3a, 4a and 5a show the results acquired at 2.5, 0.6 and 0.15 kPa dissolved oxygen, respectively. At 2.5 kPa dissolved oxygen, it took 2 hours before ammonium became exhausted, while at 0.15 kPa, the ammonium concentration after 18 hours had only declined to 2.7 mM. At 5 kPa dissolved oxygen, ammonium dis-

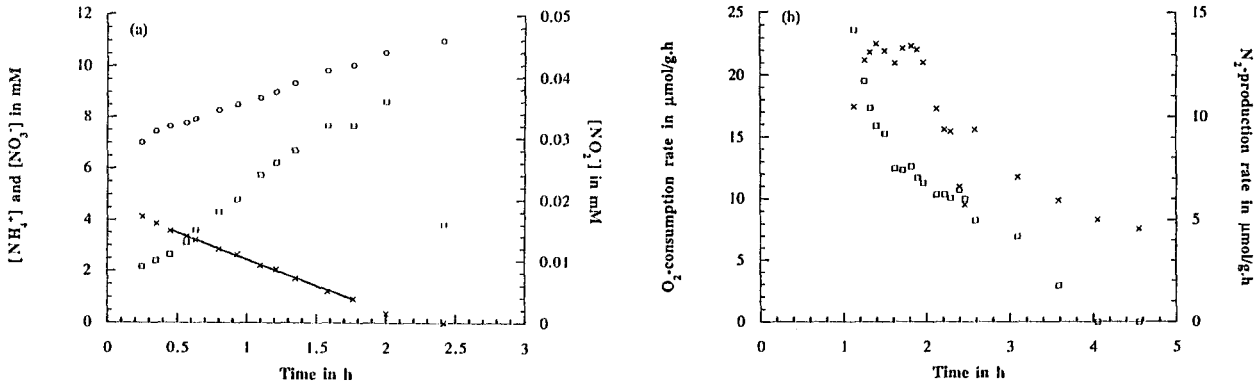

Fig. 3. *a* The courses of $[NH_4^+]$ (x—x), $[NO_2^-]$ (□) and $[NO_3^-]$ (o) in effluent, and *b* the rates of O_2-consumption corrected for endogenous respiration (×) and N_2-production (□) at 2.5 kPa dissolved oxygen. At time zero, $[NH_4^+)$ was shifted to approximately 5 mM; subsequently, chemolithotrophic medium with 5.4 mM NH_4^+ was continuously supplied. Before the shift NH_4^+ and NO_2^- were undetectable. The line in *a* presents the estimated $[NH_4^+]$ according to eq. (11).

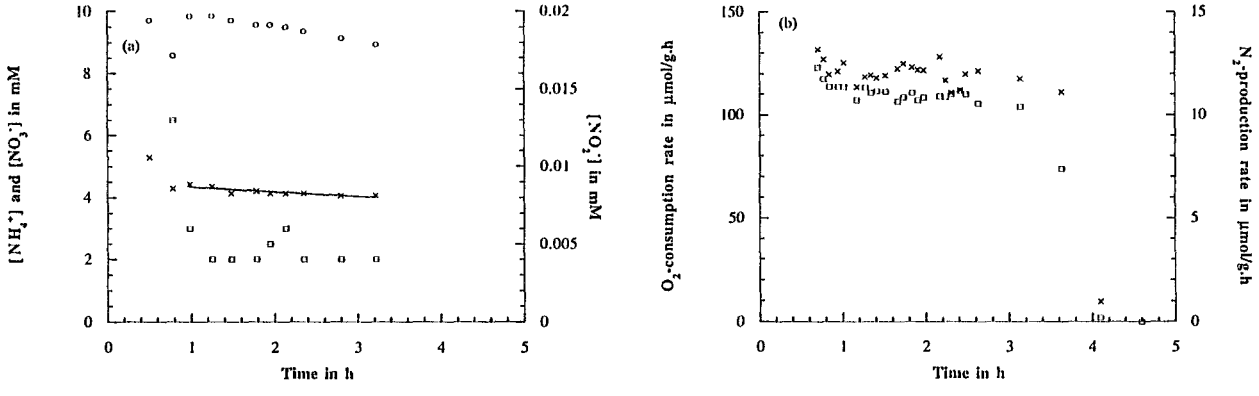

Fig. 4. *a* The courses of $[NH_4^+]$ (x—x), $[NO_2^-]$ (□) and $[NO_3^-]$ (o) in effluent, and *b* the rates of O_2-consumption corrected for endogenous respiration (×) and N_2-production (□) at 0.6 kPa dissolved oxygen.

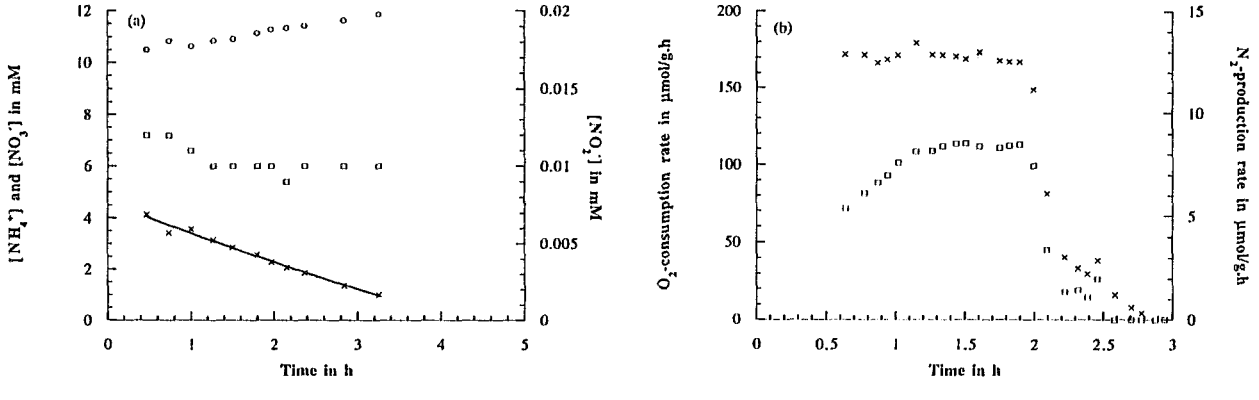

Fig. 5. *a* The courses of $[NH_4^+]$ (x—x), $[NO_2^-]$ (□) and $[NO_3^-]$ (o) in effluent, and *b* the rates of O_2-consumption corrected for endogenous respiration (×) and N_2-production (□) at 0.15 kPa dissolved oxygen.

Table 1. Ammonium consumption rates (q_{ns}) and denitrification characteristics when dinitrogen production rates (q_{N2}) were stable.

Dissolved oxygen tension kPa	q_{ns} $\mu mol \cdot g^{-1} \cdot h^{-1}$	q_{N2} $\mu mol \cdot g^{-1} \cdot h^{-1}$	N_2 produced per NH_4 consumed N-mole per N-mole	a[1]
0.15	8.22 ± 0.95	–	–	–
0.30	25.36 ± 0.55	7.35 ± 0.10	0.58	0.42
0.60	46.33 ± 1.29	10.99 ± 0.25	0.47	0.55
1.25	65.94 ± 1.09	6.90 ± 0.28	0.21	0.72
2.50	84.76 ± 0.83	8.45 ± 0.08	0.20	0.74
5.00	93.23 ± 1.37	10.86 ± 0.11	0.23	0.74

[1] Fraction of electrons used for oxygen reduction at coupled ammonia oxidation and nitrite respiration.

Table 2. Allocation of mineral nitrogen compounds for the period that the dinitrogen production was stable.

Dissolved oxygen tension kPa	Effluent %	Reactor vessel %	Nitrogen gas %	Recovery %
0.15	–	–	–	–
0.30	17.3	84.1	7.1	108.4
0.60	13.2	87.3	8.1	108.5
1.25	10.4	92.2	4.7	107.3
2.50	8.7	94.4	4.5	107.6
5.00	6.9	94.5	4.7	106.1

appeared slightly faster than at 2.5 kPa. At the intermediate tensions, i.e., 1.25 and 0.3 kPa dissolved oxygen, removal times were 3 and 7 hours, respectively. The activity of nitrite oxidizers was also a function of the dissolved oxygen tension, as can be seen from the accumulation of nitrate (see Fig. 3a, 4a and 5a). At high dissolved oxygen tensions, the nitrate concentration increased, whereas this concentration slowly decreased at the lowest dissolved oxygen tension. This decline was slower than expected from the dilution rate of the soluble reactor contents (i.e., 0.08 h^{-1}), so, nitrite was still oxidized. The nitrite concentration, which is a function of nitrification and probably denitrification activities (see Discussion), increased together with a decreasing ammonium concentration at 5, 2.5 and 1.25 kPa dissolved oxygen; the maximal concentrations observed were 60, 40 and 15 μM, respectively. At 0.6 kPa dissolved oxygen, nitrite concentrations were around 10 μM when the ammonium concentration declined. At 0.3 and 0.15 kPa dissolved oxygen, nitrite was still present after 18 hours at a concentration of 9 and 4 μM, respectively.

For the period that the ammonium concentration was sufficiently high to avoid substrate limitation, the ammonium concentration was fitted as a function of time with eq. (11) (see Table 1). Ammonium consumption rates were satisfactorily estimated since standard errors were small. Figure 3a, 4a and 5a show that the fits at 2.5, 0.6 and 0.15 kPa dissolved oxygen were quite acceptable; those at the dissolved oxygen tensions not shown were equally good. The rates were used to estimate the maximal rate and the saturation constant for ammonium consumption (K_{O2}) as function of the dissolved oxygen tension, which were 114.54 (± 6.75) $\mu mol \cdot g^{-1} \cdot h^{-1}$ and 0.97 (± 0.16) kPa dissolved oxygen, respectively (data not shown).

To calculate rates of dinitrogen production and oxygen consumption, the effects of experimental artifacts had to be omitted. Since filters were introduced at the start of the experiments, some gas exchange with the

environment was inevitable. Moreover, flow rates and ratios of oxygen and argon in the gas supply were changed to cope with the oxygen demand at the dissolved oxygen tensions imposed (see Fig. 2 for examples). Data affected by these artifacts were omitted using the argon concentration in outlet gas as this gas is inert. Only those oxygen and nitrogen concentrations were considered for which holds that the argon concentration had stabilized. As a consequence, data were not available until 0.5–1.3 hour after the start of the experiments.

The oxygen consumption rate corrected for endogenous respiration also show that nitrification rates depended on the dissolved oxygen tension. At 5 kPa dissolved oxygen, the oxygen consumption rate corrected for endogenous respiration was maximally 0.2 $mmol \cdot g^{-1} \cdot h^{-1}$ (data not shown). At lower dissolved oxygen tensions, oxygen consumption rates were lower, which is illustrated by the results obtained at 2.5 and 0.6 kPa dissolved oxygen (see Fig. 3b and 4b). At 0.15 kPa dissolved oxygen, the oxygen consumption rate corrected for endogenous respiration dropped rapidly after 2 hours (see Fig. 5b). So, after a period of adaptation, nitrification proceeded slowly at the lowest dissolved oxygen tension.

Dinitrogen production accompanied oxygen consumption at all dissolved oxygen tensions (see Fig. 3b, 4b and 5b). As Fig. 2 exemplifies, dinitrogen was never detected in the outlet gas before ammonium addition and after ammonium exhaustion. At 5, 2.5 and 1.25 kPa dissolved oxygen, the dinitrogen production rate increased for 1–1.5 hour and then stabilized at 7–11 $\mu mol \cdot g^{-1} \cdot h^{-1}$. At 0.6 and 0.3 kPa dissolved oxygen, this rate was initially higher and stabilized after 1 hour at 11 and 8 $\mu mol \cdot g^{-1} \cdot h^{-1}$, respectively. At the lowest dissolved oxygen tension, the production of dinitrogen stopped when the oxygen consumption rate corrected for endogenous respiration dropped (see Fig. 5b). Consequently, the dinitrogen production rate was independent of the dissolved oxygen tension when higher than 0.15 kPa, and was coupled to the consumption of oxygen. In addition, dinitrogen was only produced when ammonium was detectable.

The dinitrogen production rate as a fraction of the ammonium consumption rate was calculated for the period that the gas exchange was stable (see Table 1). At 1.25 kPa dissolved oxygen and higher, the denitrification rate (per N-mole) was about 20% of the ammonium oxidation rate. This percentage increased at lower dissolved oxygen tensions and was largest at 0.3 kPa, when the dinitrogen production rate was almost 60% of the ammonium consumption rate. At 0.15 kPa dissolved oxygen, this fraction could not be determined since dinitrogen production did not stabilize (see Fig. 5b).

The allocation of mineral nitrogen compounds were determined for the period that the nitrogen gas production was stable (see Table 2). Mineral nitrogen initially present plus nitrogen supplied was taken as 100%. The main part, mostly nitrate, remained in the reactor vessel. The part that was removed with effluent depended on the experimental duration. The percentage that was completely denitrified ranged from 4.5 to 8.1%, which is low as a result of the high contribution of nitrate to the balances. Nitrous oxide and nitric oxide were not determined. However, their emergence could not have been substantial as the nitrogen recovery always exceeded 100%. The recovery of mineral nitrogen compounds were between 106% and 109%. This does not indicate a substantial nitrogen release due to endogeneous respiration, since the endogeneous carbon dioxide production and the carbon to nitrogen ratio of sludge (6–7 mol/mol) justified a deviation of less than 1%. So, those deviations should have been a result of experimental inaccuracies.

Discussion

Activated sludge that was taken from a pilot plant with complete sludge retention was chemo-lithotrophically subcultured in recycling reactors. The nitrification rates were determined as a function of the dissolved oxygen tension. In addition to nitrification reactions, considerable dinitrogen production was observed. Hence, nitrification and denitrification proceeded concomitantly. After a discussion on the nitrification rates determined, this section centers to the reduction and oxidation reactions possibly involved in the denitrification activities observed.

The nitrification rates by activated sludge clearly depended on the dissolved oxygen tension. The saturation constant of oxygen consumption by ammonia oxidizers (K_{O2}) was determined at 0.97 kPa dissolved oxygen. This value is in agreement with determinations obtained with cocultures of *Nitrosomonas europaea* and *Nitrobacter agilis* (Laanbroek & Gerards 1993), monocultures of *Nm. europaea* (Niel et al. 1993) and activated sludge from a mixed flow reactor (Hanaki et al. 1990). The maximal nitrite consumption rates could not be determined with first order kinetics, because the nitrite concentrations were lower than the saturation

constants reported (Prosser 1989). It seems, though, that the nitrite consumption rates were less affected by the dissolved oxygen tension than the ammonium consumption rates, since nitrite accumulated more at high than at low dissolved oxygen tensions (see Fig. 3a and 4a). In contrast to this, the activities of *Nitrobacter* spp. have been shown to be more reduced at low dissolved oxygen tensions than that of *Nm. europaea* (Hanaki et al. 1990; Laanbroek & Gerards 1993). This paradox is explained by the substantial nitrite respiration at low dissolved oxygen tensions (see below).

Dinitrogen was produced during ammonia oxidation and oxygen consumption at dissolved oxygen tensions between 0.3 and 5 kPa (see Fig. 3, 4 and 5). So, aerobic denitrification occurred. For this denitrification, an unknown oxidation reaction was the driving force, as organic substrates were not supplied. Four sources of reductant power may have accounted for the denitrification observed: endogeneous substrates; exogeneous substrates, as lysates and excretion products; ammonia; nitrite. It is also unclear whether denitrification started with nitrate or nitrite reduction. As discussed below, it is most likely that a single organism concomitantly oxidized ammonia and reduced nitrite.

The gas exchange rates before ammonium addition and after ammonium exhaustion show that reductant power which was gained from the oxidation of endogeneous and exogeneous substrates might have accounted for the denitrification observed. During those periods, the sum of endogeneous and exogeneous respiration was 25 μmol $O_2 \cdot g^{-1} \cdot h^{-1}$, which allows for the production of 17 and 10 μmol $N_2 \cdot g^{-1} \cdot h^{-1}$ for nitrite and nitrate respiration, respectively (cf. eqs. (3) and (7–10) above). The highest rate observed during nitrification was 11 μmol $N_2 \cdot g^{-1} \cdot h^{-1}$ (see Table 1). So, the gas exchange rates allowed for heterotrophic denitrification.

However, heterotrophic denitrification would be in contrast to other observations. First, it is unclear why oxygen consumption would have been completely replaced by nitrite or nitrate respiration at the higher dissolved oxygen tensions imposed. Aerobic processes should have also proceeded at those oxygen tensions, as commonly observed during wastewater treatment, which should have lead to denitrification rates that are proportionally reduced. Moreover, it cannot explain why dinitrogen was not produced before ammonium addition and after ammonium exhaustion (see Fig. 2, 3, 4 and 5). Nitrate concentrations were always high, and the nitrite concentration was still 9 and 4 μM 18 hours after the dinitrogen evolution had disappeared at 0.3 and 0.15 kPa dissolved oxygen, respectively. These concentrations would have been sufficiently high for heterotrophic nitrite respiration (cf. the experiments done at 5 and 2.5 kPa dissolved oxygen; Fig. 3a and 4a). The final disagreement follows from the results obtained at 0.15 kPa dissolved oxygen. Although electron donors were sufficiently available, as explained above, within 4 hours the dinitrogen production disappeared, which was accompanied by a strong decline of the oxygen consumption (see Fig. 5b). So, the denitrification observed required oxygen consumption. In contrast to this, heterotrophic denitrification proceeds better at anoxic conditions and low dissolved oxygen tensions than at high tensions (see discussions in Kuenen & Robertson, 1987; Robertson & Kuenen 1990; Stouthamer 1988; Zumft et al. 1988). Hence, it is unlikely that organic compounds were oxidized to denitrify.

This leaves the possibilities of ammonia and nitrite oxidation as a source of reduction equivalents, which would be in agreement with the close coupling that was observed between the nitrification and denitrification reactions. Nitrifying bacteria are generally considered to carry out either the first or the second oxidation. Yet, it is difficult to separate these reactions as they were mostly closely linked (see Fig. 3a and 4a). At 0.3 kPa dissolved oxygen, however, dinitrogen production had disappeared together with ammonium exhaustion, while nitrite was still available for a long period thereafter (see above). This suggests that only ammonia oxidation yielded the electrons needed for denitrification. In line with this are the results obtained at 0.15 kPa dissolved oxygen, which show that oxygen consumption was necessary for denitrification (see Fig. 5b). At the first step of ammonia oxidation dioxygen is needed to form hydroxylamin, which is an intermediate in nitrite formation (see eq. (1)). At the oxidation of nitrite to nitrate, however, oxygen incorporated originates from a water molecule (Hooper 1987; Wood 1986, 1988). So, if the terminal oxidase in both organisms would be completely replaced by denitrifying oxidoreductases, the ammonia oxidizer still needs dioxygen, while the nitrite oxidizer grows anaerobically. Hence, it is likely that only ammonium oxidation accounted for the reduction equivalents that were required for denitrification.

The initial substrates for the denitrification may have been nitrite or nitrate, which can be examined by comparing the electron balances. The oxidation of ammonia to nitrite yields 2 electrons (see eqs. (1–

2)); a similar evaluation results when nitrite oxidation is considered (see eq. (5)). Complete nitrite respiration requires 3 electrons, whereas full nitrate reduction takes 5 (see eqs. (7–10)). Hence, the dinitrogen produced as a fraction of ammonium oxidized is maximally 0.67 N-mole per N-mole when nitrite is completely reduced, and is at most 0.4 for nitrate reduction. Since at 0.3 and 0.6 kPa dissolved oxygen, these fractions were 0.58 and 0.44, respectively (see Table 2), nitrite should have been the initial substrate for denitrification.

So, nitrite reduction was most likely coupled to ammonia oxidation. This hypothesis can be tested by linking the common nitrification and denitrification pathways (see eqs. (1–10)). For this purpose, suppose that a is the fraction of electrons net formed by ammonia oxidation that flow to the terminal cytochrome c oxidase. Then, the fraction (1 - a) is used for complete nitrite reduction. So, the redox reactions carried out by the ammonia oxidizer are:

$$\begin{aligned} &NH_3 + O_2 \rightarrow NO_2^- + 3H^+ + 2e^- \\ &a(0.5O_2 + 2e^- + 2H^+ \rightarrow H_2O) \\ &(1 - a)(0.67NO_2^- + 2.67H^+ + 2e^- \rightarrow \\ &0.33N_2 + 1.33H_2O) \end{aligned} \qquad (12)$$

Since nitrite concentrations were always low (see Fig. 3a, 4a and 5a), remaining nitrite (i.e., $0.33 + 0.67a$) mol nitrite per mol ammonia oxidized) was subsequently oxidized to nitrate according to eq. (6). This leads to the overall reaction:

$$\begin{aligned} &NH_3 + (1.17 + 0.83a)O_2 \rightarrow (0.33 + 0.67a)NO_3^- + \\ &(0.33 - 0.33a)N_2 + (0.33 + 0.67a)H^+ + \\ &(1.33 - 0.33a)H_2O \end{aligned} \qquad (13)$$

Thus, with q_{N2} and q_{O2} is the dinitrogen production and oxygen consumption rate, respectively:

$$q_{N2}/q_{O2} = (0.33 - 0.33a)(1.17 + 0.83a)^{-1} \qquad (14)$$

or,

$$a = (q_{O2} - 3.5q_{N2})(2.5q_{N2} + q_{O2})^{-1} \qquad (15)$$

Obviously, a has to be between 0 and 1, which was always the case (see Table 1). A similar derivation for nitrite respiration by nitrite oxidizers gives values for a that are slightly smaller. If the ammonia oxidizer respired nitrate, however, a negative flow to the terminal oxidase would result at 0.3 kPa dissolved oxygen. So, ammonia oxidation could have supplied the electrons for nitrite respiration.

So, it is likely that dinitrogen was produced by a nitrifier that linked ammonia oxidation to nitrite reduction. The best studied nitrifier, *Nitrosomonas* sp., also reduces nitrite at an oxygen limitation, which mostly gives nitric and nitrous oxide as products (Goreau et al. 1980; Hynes & Knowles 1984; Lipschultz et al. 1981; Poth & Focht 1985; Remde & Conrad 1990; Stüven et al. 1992). Once, an isolate has been shown to possess complete denitrification capacities (Poth 1986). So, a *Nitrosomonas* sp. may be involved in this study. If so, however, one wonders why dinitrogen production by this nitrifier has not been demonstrated many times before. Three reasons may account for this. First, the high nitrogen content of air demands the utilization of sophisticated techniques, such as the application of nitrogen isotopes, nitrogen-free carrier gases, gas-tight reactors and a mass spectrometer. In the second place, the nitrifying biomass concentration should be sufficiently high to detect nitrogen production. In this study, the concentration of ammonia oxidizers should have been around 0.3 $g \cdot l^{-1}$, as they supposedly contribute to 1% of a sludge population in a domestic wastewater treatment plant (Painter 1986). The concentration of *Nitrosomonas* sp. in chemostats, however, is usually about 10 $mg \cdot l^{-1}$ (Laanbroek & Gerards 1993; Niel et al. 1993). As a result, the dinitrogen content of outlet gas will be too low to detect. Finally, the species most frequently studied is *Nm. europaea* ATCC 19718, which does not produce dinitrogen (Poth 1986).

In conclusion, ammonia oxidation by sewage sludge is a function of the dissolved oxygen tension. In addition, it is most likely that a nitrifier is present in sewage sludge that concomitantly oxidizes ammonia and reduces nitrite to dinitrogen at low dissolved oxygen tensions. This way of denitrification may benefit the treatment of wastewater, since during conventional treatment, organic substrates and ammonium are simultaneously oxidized. This frequently results in a shortage of organic substrates needed for subsequent denitrification.

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