

Deammonification in biofilm systems: population structure and function

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Abstract For the development of alternative concepts for the cost effective treatment of wastewaters with high ammonium content and low C/N-ratio, autotrophic consortia of micro-organisms with the ability to convert ammonium directly into N_2 are of particular interest. Several full-scale industrial biofilm plants eliminating nitrogen without carbon source for years in a stable process, are suspected for some time to harbor active anaerobic ammonium oxidizers in deeper, oxygen-limited biofilm layers. In order to identify the processes of the single-stage nitrogen elimination (deammonification) in biofilm systems and to allocate them to the responsible micro-organisms, a deammonifying moving-bed pilot plant was investigated in detail.

^{15}N -labelled tracer compounds were used as well as 16S rDNA libraries and *in situ* identification of dominant organisms. The usage of rRNA-targeted oligonucleotide probes (FISH) was particularly emphasized on the ammonium oxidizers of the β -subclass of Proteobacteria and on the members of the order Planctomycetales. The combined application of these methods led to a deeper insight into the population structure and function of a deammonifying biofilm.

Keywords Anaerobic ammonium oxidation; biofilm; deammonification; fluorescence *in situ* hybridization; moving-bed reactor; nitrification; nitrogen removal

Introduction

Wastewater with high nitrogen concentrations, such as sludge liquor, landfill leachate or wastewater after anaerobic pre-treatment, are rather cost-intensive when treated with conventional concepts, as both the oxygen demand for nitrification and the demand for organic substrates for denitrification depend on the respective nitrogen concentration in the wastewater. For the mentioned wastewater qualities, which are characterized by a low C/N-ratio, the demand for organic substrates can only be covered by addition of external carbon sources. Consequently, autotrophic micro-organisms which are able to convert ammonium directly into dinitrogen gas are of particular importance for the development of alternative cost effective treatment concepts of these problematic wastewaters. Nitrogen losses obviously not linked to classical denitrification processes in nitrifying biofilm systems, such as the biological contactor (BC) of the landfill leachate treatment plant at Mechernich, provided first hints on the existence of autotrophic nitrogen eliminating bacteria in wastewater purifying mixed biocoenoses (Hippen *et al.*, 1997, 1998, 2001; Siegrist *et al.*, 1998; Helmer and Kunst, 1998; Helmer *et al.*, 1999, 2001). The single-stage nitrogen conversion from ammonium to N_2 is referred to as deammonification.

In the meantime, ways in the nitrogen metabolism which deviate from the classical patterns of aerobic nitrification and anoxic denitrification have been ascertained for both the classical ammonium oxidizers of the β -subclass of Proteobacteria (Bock *et al.*, 1995; Schmidt and Bock, 1997; Zart and Bock, 1998) and for novel autotrophic members of the order *Planctomycetales*, which were recently identified and preliminarily named as *Candidatus* "Brocadia anammoxidans" (Strous *et al.*, 1999; Strous, 2000) and *Candidatus*

“*Kuenenia stuttgartiensis*” (Schmid *et al.*, 2000). The latter organisms are able to oxidize ammonium under anoxic conditions with nitrite as electron acceptor – with a slight production of nitrate – to N_2 . Enriched cultures of the novel planctomycete genera reach conversion rates for anaerobic ammonium oxidation that are 25 times higher than for pure cultures of ammonium oxidizers of the β -subclass of Proteobacteria (Jetten *et al.*, 1999).

For the anaerobic ammonium oxidation, ammonium and nitrite have to be available as reaction partners. However, nitrogen losses in nitrifying biofilm systems, as in the rotating biological contactor in Mechernich, occur with sole feeding of ammonium via the incoming wastewater. In order to identify the processes and the responsible micro-organisms of the single-stage nitrogen elimination in biofilm systems, a deammonifying moving-bed pilot plant was analyzed in detail. ^{15}N -labelled tracer compounds were used as well as 16S rDNA libraries and *in situ* identification of dominant organism groups using rRNA-targeted oligonucleotide probes for fluorescence *in situ* hybridization (FISH), with particular emphasis on the ammonium oxidizers of the β -subclass of Proteobacteria and on the members of the order Planctomycetales. The combination of *in situ* identification of dominant bacterial groups and the analysis of their physiological importance for the entire system was applied to enhance the principal understanding of the single-stage autotrophic nitrogen elimination, which is the prerequisite for the definition of optimal operation and control strategies for a stable utilization of this process in practice.

Materials and methods

Pilot plant

The moving-bed pilot plant was fed with wastewater from the sludge treatment of a municipal wastewater treatment plant, which is characterized by its high nitrogen concentrations. As growth carriers, Kaldnes material was used, which first was being covered for 6–8 weeks with the deammonifying biomass from the BC unit at Mechernich. The pilot plant consisted of a pre-denitrification reactor, followed by a settlement tank and a nitrification stage consisting of three in-line reactors. During the operation phase with parallel batch tests 75% of the converted ammonium were eliminated by deammonification. For more detailed information about design and operation of the pilot plant, please refer to Hippen *et al.* (1999).

Batch tests

Batch tests were run in two parallel reactors with a working volume of 1.8 l each. On test days, the carriers were taken from that reactor of the continuously working pilot plant that was selected respectively and put into batch reactors until a volumetric filling of 40% was reached. The reactors were equipped with slow stirring implements. The temperature was set at 28°C, the pH-value to 8.0. Over a period of 5 hours, samples were taken every 30 minutes. The samples were filtered and checked for NH_4 -N, NO_2 -N, NO_3 -N and COD. At both the beginning and the end of each test, the dissolved organic nitrogen content was analyzed. The batch reactors could be closed with a gas-tight lid, so that complete nitrogen balances at different points of time could be taken including the gaseous nitrogen compounds. Gas samples were checked for NO, N_2O and N_2 with a gas chromatograph.

Impact of oxygen. In order to investigate the impact of oxygen on the process of deammonification nitrogen conversion rates were measured at oxygen concentrations of 0 mg/l, 0.7 mg/l, 2.0 mg/l and 5.0 mg/l. For this, the carriers were taken in a mineral salts medium (per l: 87,7 mg KH_2PO_4 , 300 mg $MgSO_4 \cdot 7 H_2O$, 5,06 g $NaHCO_3$; the trace elements cobalt, manganese, zinc, copper, nickel, and iron in a concentration of 10 $\mu g/l$ each.). 150 mg/l NH_4 -N were provided in the form of NH_4Cl at the beginning of the tests.

Inhibitor substances as a tool to prove specific reactions of the deammonification. Nitrogen losses in nitrifying biofilm systems, as in the BC unit at Mechernich, occur with sole feeding of ammonium via the incoming wastewater. However, recent research showed that nitrite plays a role in the process of deammonification (Helmer *et al.*, 1999, 2001). In order to definitely prove the assumption that nitrite is produced via nitrification during deammonification, the inhibitor allyl thiourea was used. Allyl thiourea inhibits the function of the ammonium mono-oxygenase (AMO) and therefore the aerobic oxidation of ammonium to nitrite. Allyl thiourea was added in various batch tests at a concentration of 20 mg/l.

¹⁵N-labelled tracer compounds

The tests with ¹⁵N-labelled tracer compounds were run in small gas-tight flasks (volume: 120 ml). The following substrates were added: ¹⁴NH₄-N and ¹⁵NO₂-N for anoxic experiments, ¹⁵NH₄-N and ¹⁴NO₂-N for oxic experiments. The flasks were incubated for 24 hours. NH₄-N, NO₂-N and NO₃-N were analyzed from the liquid phase. From the gaseous phase ²⁹N₂, ³⁰N₂, ⁴⁴N₂O, ⁴⁵N₂O, ⁴⁶N₂O were monitored with a gas chromatograph/mass spectrometer.

Comparative sequence analysis of 16S rDNA

After DNA extraction PCR amplification, cloning, sequencing and phylogenetic analysis of biofilm derived 16S rDNA sequences was performed as previously described (Schmid *et al.*, 2000).

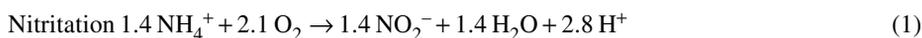
Fluorescence *in situ* hybridization, microscopy, and quantification of probe target bacteria

For *in situ* hybridization samples were fixed in 4% paraformaldehyde for 3 h. Hybridizations were performed as described by Amann (1995) with the following 16S rRNA targeting oligonucleotide probes: (i) Nso 1225, specific for the ammonia-oxidizers of the β -subclass of Proteobacteria (Mobarry *et al.*, 1996); (ii) S^{*}-Amx-0820-a-A-22, specific for anaerobic ammonium oxidizers (Schmid *et al.*, 2000); and (iii) the EUB probe mixture (EUB 338, EUB II, EUB III) specific for all *Bacteria* (Daims *et al.*, 1999). Probes were purchased as Cy3, Cy5 and 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) labeled derivatives from Interactiva (Ulm, Germany). Simultaneous hybridization with probes requiring different stringency was realized by a successive-hybridization procedure (Wagner *et al.*, 1994). For image acquisitions a Zeiss LSM 510 scanning confocal microscope (Zeiss, Jena, Germany) equipped with a UV laser (351 and 364 nm), an Ar ion laser (458 and 488 nm) and two HeNe lasers (543 and 633nm) was used together with the standard software package delivered with the instrument (version 2.1). For quantification of biofilm bacteria related to the anaerobic ammonium oxidizer or β -subclass ammonia oxidizers, Cy3 labeled probes S^{*}-Amx-0820-a-A-22 or Nso1225 were used together with the Cy5 labeled bacterial probe set (EUB338, EUB338-II, EUB338-III) for simultaneous hybridization. After image acquisition the macro R.A.M. (Relative Area Measurement) for the Kontron KS400 software package version 3.0 (Carl Zeiss Vision) was applied according to Schmid *et al.* (2000).

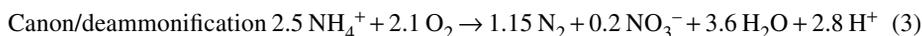
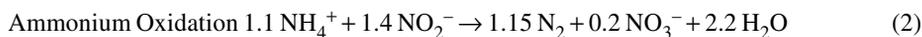
Results and discussion

In batch reactors designed for analyzing conversion rates and nitrogen balances under precisely adjustable conditions, two autotrophic nitrogen converting reactions occurred in the biofilm. The reactors performed under aerobic conditions nitrification and under anoxic conditions the anaerobic ammonium oxidation (Helmer *et al.*, 2001). Autotrophic nitrogen elimination as a sum of aerobic nitrification and anaerobic ammonium oxidation was previously described by Strous (2000) for biofilm aggregates in a laboratory sequencing

batch reactor under oxygen-limiting conditions. The process was named “Canon” (“Completely autotrophic nitrogen removal over nitrite”). From mass balances the stoichiometry of the Canon process was calculated (3), which can be explained by the equations for aerobic nitrification (1) and anaerobic ammonium oxidation (2).



Anaerobic



From a stoichiometrical point of view, these findings fit very well to the reactions occurring during the batch experiments presented in this study, indicating that both processes, Canon and deammonification, describe the same metabolic reactions. Under anoxic conditions ammonium was converted to an electron donor in a stoichiometric ratio of 1:1.37 with nitrite as electron acceptor (Figure 1). This has been equally reported for the anaerobic ammonium oxidation of *Candidatus* “Brocadia anammoxidans” (1:1.31 ± 0.06). As an end product N₂ was yielded. As nitrate could not be used as electron acceptor for the oxygen-independent oxidation of ammonium, nitrite needed to be supplied in each batch test.

The ratio of nitrification and anaerobic ammonium oxidation could be controlled via the oxygen concentration in the biofilm surrounding the medium. At an oxygen concentration of 0.7 mg/l, both processes were outbalanced. In this case a direct, almost complete elimination of ammonium without addition of nitrite was possible (Figure 2).

At a DO concentration of 0.7 mg/l and with ammonium as sole nitrogen source the inhibition of aerobic ammonium oxidation with allyl thiourea caused a halt to the whole deammonification process and the level of all nitrogen compounds was constant (Figure 3). This implies that the nitrification and anaerobic ammonium oxidation are directly linked and the only pathway for N-removal.

With the supply of ¹⁵N-labelled nitrite it was possible to trace and to quantitatively record whether the nitrite reacts with the ammonium in an anaerobic ammonium oxidation to ¹⁴⁻¹⁵N₂ or whether it is classically endogenously denitrified to ¹⁵⁻¹⁵N₂. An anoxic batch test with ¹⁵NO₂-N and ¹⁴NH₄-N proved, that 94% N₂ was formed by anaerobic ammonium oxidation and only 6% N₂ emerged by classical denitrification (Figure 4).

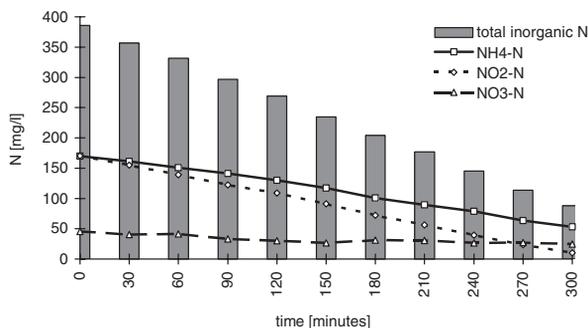


Figure 1 Nitrogen conversion reactions in an anoxic batch test with initial addition of ammonium and nitrite (NH₄-N conversion: 117 mg/l, NO₂-N conversion: 160 mg/l)

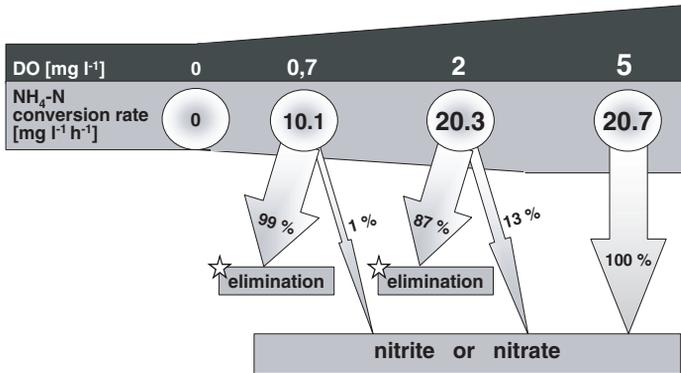


Figure 2 NH₄-N conversion in dependence on DO concentration

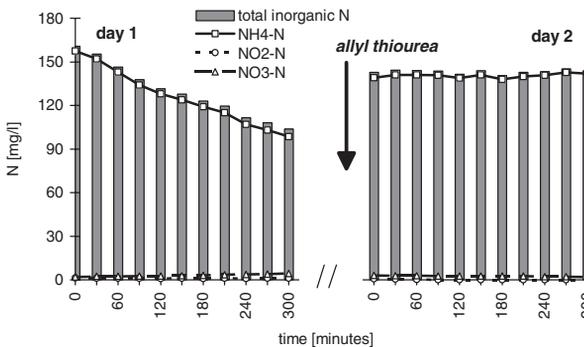


Figure 3 Effects of inhibition of nitritation by allyl thiourea on deammonification in batch tests (DO concentration = 0.7 mg/l)

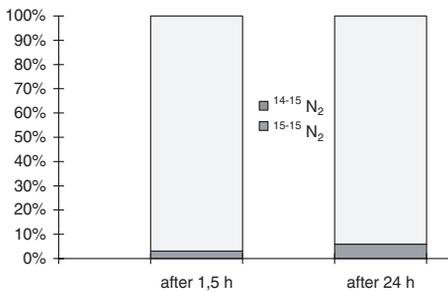


Figure 4 Percentages of N₂ produced by denitrification (¹⁵-¹⁵N₂) and anaerobic ammonium oxidation (¹⁴-¹⁵N₂) under anoxic conditions (addition: ¹⁴NH₄-N and ¹⁵NO₂-N)

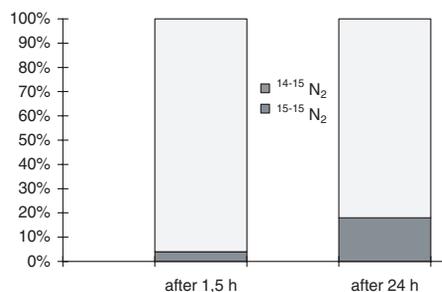


Figure 5 Percentages of N₂ produced by deammonification (¹⁵-¹⁵N₂) and anaerobic ammonium oxidation (¹⁴-¹⁵N₂) under oxic conditions (addition: ¹⁵NH₄-N and ¹⁴NO₂-N)

In oxic batch tests with ¹⁵N-labelled tracer compounds (Figure 5) the process of deammonification could be determined by addition of ¹⁵NH₄-N and ¹⁴NO₂-N. The production of ¹⁴-¹⁵N₂ indicated the anaerobic ammonium oxidation, whereas ¹⁵-¹⁵N₂ was the result of the deammonification. Assuming that ¹⁴NO₂-N was used completely as an electron acceptor for anaerobic ammonium oxidation (with ¹⁵NH₄-N as electron donor in a ratio NO₂-N/NH₄-N = 1.3), it can be calculated from the measured conversion rates of 58 mg NH₄-N/d and 50 mg NO₂-N/d, that 38.5 mg NH₄-N/d were consumed for anaerobic ammonium oxidation, whereas the rest of the 19.5 mg NH₄-N/d had to be deammonified.

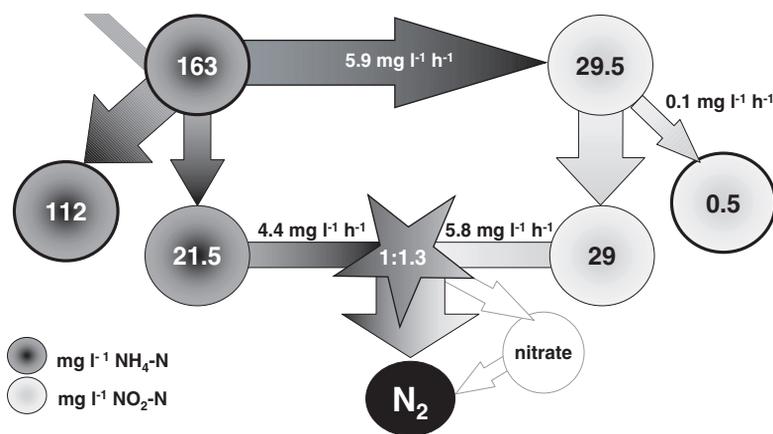


Figure 6 Calculation of nitrogen conversions during deammonification at a DO concentration of 0.7 mg/l (test duration: 5 hours)

From the Eqs (2) and (3) it can be seen that the stoichiometry between ammonium conversion and N_2 production is 1:1.05 for anaerobic ammonium oxidation and 1:0.46 for the whole process of deammonification. The ratio of measured $^{14-15}N_2$ from anaerobic ammonium oxidation and $^{15-15}N_2$ from deammonification was very well within the scope of the ratio expected from the stoichiometry (18% N_2 from deammonification, 82% N_2 from anaerobic ammonium oxidation).

With deammonification considered as two simultaneously performed reactions it is possible to calculate DO concentration dependent on the nitrogen flow for the batch tests. Figure 6 shows the routes of nitrogen conversions with Kaldnes carriers from the deammonifying reactor 2 (Hippen *et al.*, 1999) at a DO concentration of 0.7 mg/l. We added 163 mg/l ammonium at the beginning of the test and measured 112 mg/l ammonium and 0.5 mg/l nitrite after 5 hours test duration. Since it is not possible to measure the nitrite production from nitrification due to the immediate usage of nitrite as electron acceptor for anaerobic ammonium oxidation, the ammonium conversion rate for nitrification was supposed to be the rate of nitrification of reactor 1 (reactor 1 showed only nitrification, no deammonification) minus 10%. This presumed nitrification rate was lowered by 10%, because of batch tests at a DO concentration of 5 mg/l, when carriers from both reactors 1 and 2 only show nitrification (data not shown). For the nitrification rate considered to be 5.9 mg $NH_4-N/(l \cdot h)$, 29.5 mg/l NO_2-N would have been formed within 5 hours. Subtracting the finally remaining 0.5 mg/l nitrite, 29 mg/l nitrite are acting as electron acceptor for 21.5 mg/l ammonium as electron donor. This NO_2-N/NH_4-N -ratio of 1.3 reflected exactly Eq. (1). The whole

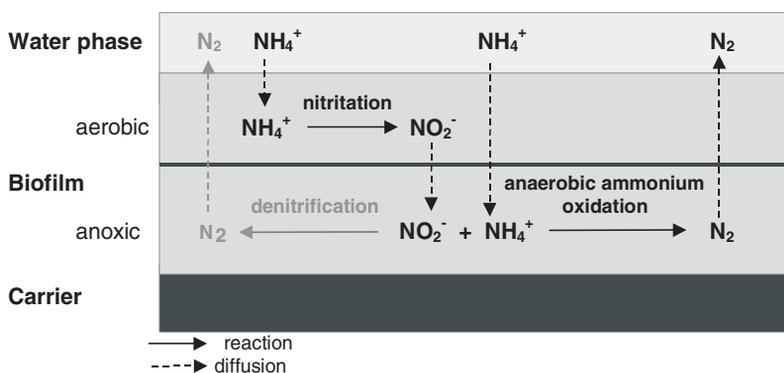


Figure 7 Deammonification consists of two reactions: nitritation + anaerobic ammonium oxidation

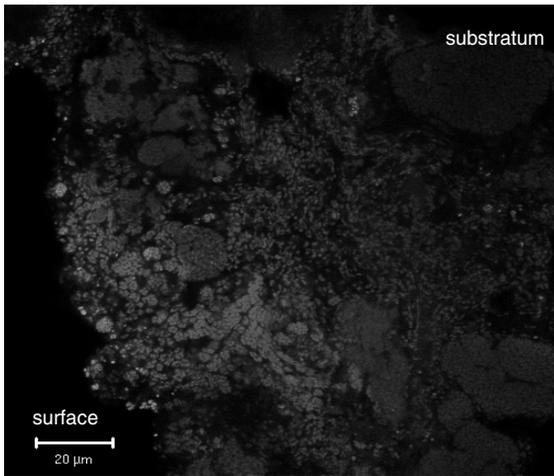


Figure 8 *In situ* identification of classical β -subclass ammonia oxidizers labeled green with probe NSO 1225 and anaerobic ammonium oxidizers labeled red with probe S^{*}-Amx-0820-a-A-22 in biofilm of the moving-bed pilot plant

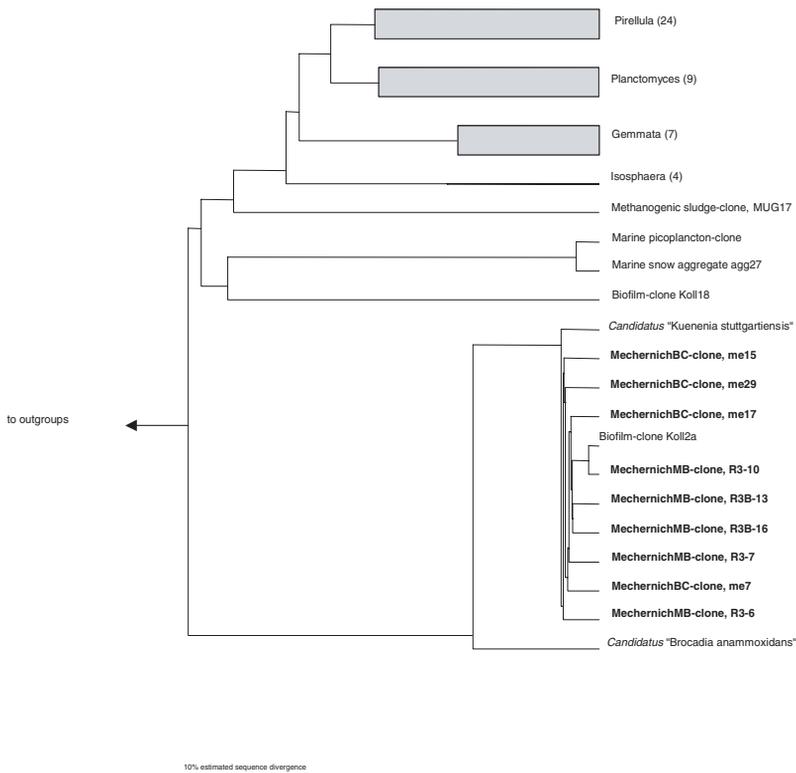


Figure 9 Phylogenetic neighbor joining tree reflecting the relationships of the Mechernich rotating biological contactor 16S rDNA clones (indicated as MechernichBC-clones), the moving-bed pilot plant 16S rDNA clones (indicated as MechernichMB-clones), the previously published microorganisms capable of performing anaerobic ammonium oxidation [*Candidatus* "Brocadia anammoxidans" and *Candidatus* "Kuenenia stuttgartiensis"], the rotating disk biofilm-clone Koll2a (Egli *et al.*, 1999) and other Planctomycetales. The rectangles indicate phylogenetic groups

process of deammonification during the test with a total $\text{NH}_4\text{-N}$ -conversion of 51 mg/l and a utilization of 29 mg/l nitrite yielded a $\text{NO}_2\text{-N}/\text{NH}_4\text{-N}$ -ratio of 0.57. This is also indicated by the Eqs (1) and (2) with a resulting theoretical conversion ratio of 0.56.

A model scheme of deammonification realized by initial nitrification and subsequent anaerobic ammonium oxidation in the biofilm is shown in Figure 7. The model idea is supported by the molecular analyses performed. FISH demonstrated that classical ammonia-oxidizers of the β -subclass of Proteobacteria thrived within the putatively aerobic surface region of the biofilm while the anaerobic ammonium oxidizers dominated the deeper presumably anaerobic biofilm layers (Figure 8). 16S rRNA sequences retrieved from the biofilm demonstrated the presence of micro-organisms closely related to *Candidatus* "Kuenenia stuttgartiensis" capable of performing anaerobic ammonium oxidation (Figure 9).

Conclusions

By combining precise N-balancing, following the route of nitrogen with ^{15}N -labelled tracer compounds, with an *in situ* investigation of the identity and spatial grouping of dominant bacteria, it succeeded to elucidate the deammonifying biofilm of a moving-bed pilot plant in its population structure and function. Under oxygen-limiting conditions the diffusion depth of DO allows the co-existence of two different bacterial groups in one biofilm. In the outer oxygen supplied layers of the biofilm part of the ammonium was converted in an aerobic ammonium oxidation step performed by classical ammonia oxidizers of the β -subclass of Proteobacteria to nitrite. Subsequently the other part of the ammonium and the nitrite diffused into the deeper, oxygen-limited regions of the biofilm, where the ammonium was anaerobically oxidated with nitrite by bacteria closely related to the recently identified *Candidatus* "Kuenenia stuttgartiensis" (Schmid *et al.*, 2000). The combination of different methods allows the direct assignment of certain metabolic abilities to the different identified bacteria, providing a considerably better insight into mixed bacterial bioceenoses, than the exclusive use of only one method.

The fact that the moving-bed pilot plant was inoculated with biomass from the biological contactor of the landfill leachate treatment plant at Mechernich, allows the assumption that deammonification, as described in this paper, is responsible for nitrogen losses in the Mechernich BC unit. Molecular analysis proved the co-existence of classical ammonia oxidizers of the β -subclass of Proteobacteria and bacteria, closely related to *Candidatus* "Kuenenia stuttgartiensis", also for this biofilm system (data not shown). The BC unit of the landfill leachate treatment plant at Mechernich/Germany was the first full-scale biofilm system with deammonification occurring since 1994 (Baumgarten and Seyfried, 1996). What started as a "mystery", is now explained in its microbiological fundamentals, allowing further progress in technical application.

References

- Amann, R. (1995). *In situ* identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In: Akkermans, A.D.C., van Elsas J.D., de Bruijn F.J. (ed.) *Molecular microbial ecology manual*. Kluwer Academic Publishers, Dordrecht, The Netherlands, 1–15.
- Baumgarten, G. and Seyfried, C.F. (1996). Experiences and new developments in biological pre-treatment and physical post-treatment of landfill leachate. *Wat. Sci. Tech.*, **34**(7–8), 445–453.
- Bock, E., Schmidt, I., Stüven, R. and Zart, D. (1995). Nitrogen loss caused by denitrifying *Nitrosomonas* cells using ammonium or hydrogen as electron donors and nitrite as electron acceptor. *Arch. Microbiol.*, **163**, 16–20.
- Daims, H., Brühl, A., Amann, R., Schleifer, K.-H. and Wagner, M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. *Syst. Appl. Microbiol.*, **22**, 434–444.

- Egli, K., Fanger, U., Siegrist, H.R. and van der Meer, J.R. (1999). Genbank direct submission. Accession number: AJ250882.
- Helmer, C. and Kunst, S. (1998). Simultaneous nitrification/denitrification in an aerobic biofilm system. *Wat. Sci. Tech.*, **37**(3–4), 183–187.
- Helmer, C., Kunst, S., Juretschko, S., Schmid, M.C., Schleifer, K.-H. and Wagner, M. (1999). Nitrogen loss in a nitrifying biofilm system. *Wat. Sci. Tech.*, **39**(7), 13–21.
- Helmer, C., Tromm, C., Hippen, A., Rosenwinkel, K.-H., Seyfried, C.F. and Kunst, S. (2001). Single stage biological nitrogen removal by nitrification and anaerobic ammonium oxidation in biofilm systems. *Wat. Sci. Tech.*, **43**(1), 311–321.
- Hippen, A., Baumgarten, G., Rosenwinkel, K.-H. and Seyfried, C.F. (1997). Aerobic deammonification – A new experience in the treatment of wastewaters. *Wat. Sci. Tech.*, **35**(10), 111–120.
- Hippen, A., Scholten, E., Helmer, C., Kunst, S., Seyfried, C.F., Rosenwinkel, K.-H. and Diekmann, H. (1998). Aerobic deammonification in high nitrogen loaded wastewaters. *New Advances in Biological Nitrogen and Phosphorus Removal for Municipal or Industrial Wastewaters*, Narbonne, France, 12–14 October 1998.
- Hippen, A., Helmer, C., Kunst, S., Rosenwinkel, K.-H. and Seyfried, C.F. (1999). Sludge liquor treatment with aerobic deammonification. Conference Proceedings of “Wastewater ’99”, 3rd International Conference of ACE CR, Teplice, 18–20 May 1999.
- Hippen, A., Helmer, C., Kunst, S., Rosenwinkel, K.-H. and Seyfried, C.F. (2001). Six years of practical experiences with aerobic/anoxic deammonification in biofilm systems. *Wat. Sci. Tech.*, **44**(2–3), 39–48.
- Jetten, M.S.M., Strous, M., Van de Pas-Schoonen, K.T., Schalk, J., Van Dongen, L.G.J.M., Van de Graaf, A.A., Logemann, S., Muyzer, G., Van Loosdrecht, M.C.M. and Kuenen, J.G. (1999). The anaerobic oxidation of ammonium. *FEMS Microbiol. Rev.*, **22**, 421–437.
- Mobarry, B.K., Wagner, M., Urbain, V., Rittmann, B.E. and Stahl, D.A. (1996). Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Appl. Environ. Microbiol.*, **62**, 2156–2162.
- Schmid, M., Twachtman, U., Klein, M., Strous, M., Juretschko, S., Jetten, M., Metzger, J.W., Schleifer, K.-H. and Wagner, M. (2000). Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. *System. Appl. Microbiol.*, **23**, 93–106.
- Schmidt, I. and Bock, E. (1997). Anaerobic ammonia oxidation with nitrogen dioxide by *Nitrosomonas europaea*. *Arch. Microbiol.*, **167**, 160–111.
- Siegrist, H., Reithaar, S., Koch, G. and Lais, P. (1998). Nitrogen loss in a nitrifying rotating contactor treating ammonium-rich wastewater without organic carbon. *Wat. Sci. Tech.*, **38**(8–9), 241–248.
- Strous, M. (2000). *Microbiology of Anaerobic Ammonium Oxidation*. PhD thesis, Technical University Delft, Netherlands.
- Strous, M., Fuerst, J.A., Kramer, E.H.M., Logemann, S., Muyzer, G., Van de Pas-Schoonen, K.T., Webb, R., Kuenen, J.G. and Jetten, M.S.M. (1999). Missing lithotroph identified as new planctomycete. *Nature*, **400**, 446–469.
- Wagner, M., Amann, R., Kämpfer, P., Abmus, B., Hartmann, A., Hutzler, P., Springer, N. and Schleifer, K.-H. (1994). Identification and *in situ* detection of gram-negative filamentous bacteria in activated sludge. *Syst. Appl. Microbiol.*, **17**, 405–417.
- Zart, D. and Bock, E. (1998). High rate of aerobic nitrification and denitrification by *Nitrosomonas europaea* grown in a fermenter with complete biomass retention in the presence of gaseous NO₂ or NO. *Arch. Microbiol.*, **169**, 282–286.

