

# Combined anaerobic ammonium and methane oxidation for nitrogen and methane removal

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## Abstract

Anammox (anaerobic ammonium oxidation) is an environment-friendly and cost-efficient nitrogen-removal process currently applied to high-ammonium-loaded wastewaters such as anaerobic digester effluents. In these wastewaters, dissolved methane is also present and should be removed to prevent greenhouse gas emissions into the environment. Potentially, another recently discovered microbial pathway, n-damo (nitrite-dependent anaerobic methane oxidation) could be used for this purpose. In the present paper, we explore the feasibility of simultaneously removing methane and ammonium anaerobically, starting with granules from a full-scale anammox bioreactor. We describe the development of a co-culture of anammox and n-damo bacteria using a medium containing methane, ammonium and nitrite. The results are discussed in the context of other recent studies on the application of anaerobic methane- and ammonia-oxidizing bacteria for wastewater treatment.

## Introduction

Currently, the majority of the systems designed to remove nitrogen from wastewater employs two sequential reactions: (i) aerobic oxidation of ammonia to nitrate by AOB (ammonia-oxidizing bacteria) and NOB (nitrite-oxidizing bacteria), and (ii) the subsequent anaerobic reduction of nitrate to N<sub>2</sub> via denitrification. The first step requires intensive aeration, whereas, in the second step, external electron donors (i.e. methanol) have to be supplied to the heterotrophic denitrifiers.

In recent years, nitrogen-removal systems based on anammox (anaerobic ammonium oxidation) bacteria emerged as an environment-friendly alternative [1]. These bacteria convert ammonium and nitrite directly into N<sub>2</sub> in the absence of oxygen and they are autotrophic; hence neither aeration nor dosing of electron donors is required [2–4]. However, nitrite rarely occurs in municipal and industrial wastewaters, therefore it has to be supplied to anammox bacteria via partial nitrification or denitrification. Bioreactors where nitrite is produced by AOB and consumed by anammox bacteria have already been developed and optimized [4–6]. Application of this combined process for nitrogen removal results in at least 60% cost reduction (less aeration and no external electron donors) and 90% less CO<sub>2</sub> (a completely autotrophic system) compared with conventional nitrification–denitrification systems [7].

The best results were obtained with granular sludge in which a thin layer of AOB covers the anammox granules and supplies anammox bacteria with nitrite while keeping the O<sub>2</sub> below inhibitory levels [4]. These systems are currently applied to wastewaters with high ammonium load such as anaerobic digester effluents [4]. Another major end-product of anaerobic digestion is methane, which is often collected from the off-gas and used as fuel. Nevertheless, dissolved methane leaves the system with the effluent wastewater, which is eventually released to the atmosphere and contributes to the greenhouse effect.

The presence of methane in an anammox bioreactor would form a perfect niche for the recently discovered n-damo (nitrite-dependent anaerobic methane oxidation) bacteria that convert nitrite into N<sub>2</sub> under anaerobic conditions with methane as the electron donor [8,9]. Under these conditions, anaerobic methane- and ammonium-oxidizing bacteria would have to compete for nitrite. However, in a previous study, we showed that a co-culture oxidizing methane and ammonium simultaneously could be established starting from a methane-oxidizing enrichment culture [10]. We have also demonstrated the presence of anaerobic methane oxidizers of the NC10 phylum in two full-scale anammox wastewater-treatment plants [11] in which these bacteria could already be contributing to nitrite/nitrate and residual methane removal.

In our studies, we used granules from a full-scale anammox bioreactor (Dokhaven, Rotterdam, The Netherlands) to investigate whether n-damo bacteria are active in such wastewater-treatment plants and if it would be feasible to use existing anammox granules as inoculum to enrich these bacteria.

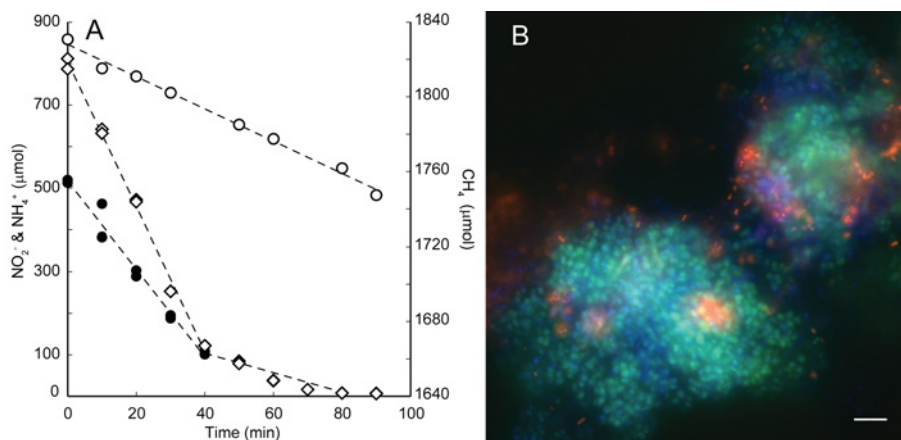
**Key words:** ammonium oxidation, anaerobic ammonium oxidation (anammox), methane oxidation, nitrate-dependent anaerobic methane oxidation (n-damo), wastewater treatment.

**Abbreviations used:** anammox, anaerobic ammonia oxidation; AOB, ammonia-oxidizing bacteria; FISH, fluorescence *in situ* hybridization; n-damo, nitrite-dependent anaerobic methane oxidation; SBR, sequencing batch reactor.

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**Figure 1 | Methane and ammonium oxidation activity and community composition of the enrichment culture**

(A) Methane and ammonium oxidation coupled to nitrite reduction by the whole enrichment culture after 1 year of enrichment. The culture contained  $550 \pm 20$  mg of protein. ●, ammonium; ○, methane; ◇, nitrite. (B) A micrograph of FISH of the co-culture after 1 year. NC10 bacteria hybridize with Cy3 (indocarbocyanine)-labelled probes S<sup>-</sup>-DBACT-0193-a-A-18 and S<sup>-</sup>-DBACT-1027-a-A-18 (red), anammox bacteria hybridize with Fluos-labelled probe S<sup>-</sup>-Amx-0820-a-A-22 (green). DNA was stained with DAPI (4',6-diamidino-2-phenylindole) (blue). Scale bar, 5 μm.

**Bioreactor set-up**

Anammox granules (500 ml) from a full-scale wastewater-treatment plant [6] were used to inoculate a SBR (sequencing batch reactor) (2 litre working volume). The SBR was operated as described previously [10], at room temperature, and the pH was controlled at 7.2. Anaerobic methane- and ammonium-oxidation rates were measured in the whole culture as described in [10,12]. Nitrite, nitrate and ammonium were analysed colorimetrically [12]. Community composition was determined by constructing 16S rRNA genes of anammox (primers Pla46F or Amx368F and Amx 820R) and NC10 phylum bacteria (193F and 1492R, universal reverse) and *pmoA* (cmo182F and cmo682F, specific for anaerobic methane-oxidizing bacteria) gene clone libraries [11,13]. Representative sequences have been submitted to GenBank<sup>®</sup> with the accession numbers JN609383 and JN609384 for 16S and *pmoA* genes respectively. Enrichment of methane- and ammonium-oxidizing bacteria was monitored by FISH (fluorescence *in situ* hybridization) and quantitative PCR [8,14,15].

**Enrichment of an anaerobic ammonium- and methane-oxidizing culture**

Granules from a full-scale anammox bioreactor were incubated in a laboratory-scale SBR. After inoculation, nitrite and ammonium was supplied at a ratio of 4:3, and the concentrations were increased slowly until a stable nitrite reduction activity (21.8 mmol/day) was obtained after 2 months. A clone library ( $n = 8$ ) of anammox 16S rRNA genes revealed that predominantly *Candidatus* 'Brocadia' phylotypes were present (94–99% identity with *Candidatus* 'Brocadia fulgida', DQ459989). Also, one *Candidatus* 'Jettenia'-like sequence was found. The presence

of methane mono-oxygenase (*pmoA*) genes affiliated with n-damo bacteria in the same wastewater-treatment plant [11,16] suggested that the granules may be a suitable inoculum to enrich for this type of bacteria. Nevertheless, they were not sufficiently abundant to be detected by FISH (detection limit of 10 000 cells/ml). After day 60, to create a niche for anaerobic methanotrophs, methane (10 ml/min) and nitrite were supplied to the culture in excess under ammonium limitation. The methane concentration in the headspace was always above 20% (255 μM). Ammonium remained below the detection limit, indicating that anaerobic methanotrophs could consume surplus nitrite, but not out-compete anammox bacteria for it. The activity of both groups of bacteria was measured in several incubations with the entire culture after 1 year of enrichment. To determine the contribution of anaerobic ammonium- and methane-oxidizing bacteria to nitrite reduction, batch incubations were performed under ammonium limitation (Figure 1A). After ammonium depletion, nitrite consumption was coupled to methane oxidation with the 8:3 stoichiometry reported previously [8]. Methane and ammonium consumption rates were 0.7 and 8.4 mmol/l per day (1.6 and 31.6 nmol/mg of protein per min) respectively and in good agreement with previous reports [17,18]. The nitrite reduction rate of 13.9 mmol/l per day in the short-term batch incubations was consistent with the activity of the continuous culture (12.5 mmol/l per day). On the basis of the stoichiometries of 3:8 [19] and 1:1.3 [20] for anaerobic methane and ammonium oxidation respectively, n-damo contributed 13% to total nitrite removal and 80% could be attributed to anammox. The remaining 7% could be due to other electron donors derived from dead biomass and measuring inaccuracies.

The methane-oxidizing activity of the culture could be correlated with the enrichment of NC10 phylum

bacteria. In contrast with the inoculum, bacteria related to *Methylomirabilis oxyfera* could be detected using FISH (Figure 1B). Clone libraries of 16S rRNA and *pmoA* genes revealed that they formed a group distinct from *M. oxyfera* enriched from freshwater sediments (94.5% and 88.1% identity respectively). The abundance of NC10 phylum bacteria were measured with quantitative PCR. In the first 6 months of enrichment, their cell numbers in the reactor did not change [from  $(2.73 \pm 0.19) \times 10^7$  cells when the methane supply started to  $(1.10 \pm 0.05) \times 10^7$  cells on day 180]. After 10 months of enrichment, the population quickly grew from  $(5.19 \pm 0.23) \times 10^7$  cells (day 315) to  $(1.22 \pm 0.18) \times 10^{10}$  cells after 1 year. This corresponds to an activity per cell of  $0.11 \pm 0.02$  fmol/day, within the range 0.09–0.40 fmol/day per cell reported for a solely methane-oxidizing enrichment culture [13]. In our studies, a period of nearly 1 year was necessary to enrich anaerobic methane-oxidizing bacteria starting with intact anammox granules as inoculum. This is more than twice as long than the reversed strategy where anammox bacteria already contributed 77% to the total nitrite-reducing activity in 142 days [10], suggesting that anammox bacteria are better competitors for nitrite.

## Conclusions and outlook

In the present paper, we report that it was also possible to attain a co-culture of anaerobic methane- and ammonium-oxidizing bacteria using granules from a full-scale anammox bioreactor. In the future, such a combined process could be used to remove the excess methane that is present in the effluent of anaerobic digesters together with ammonium and prevent its release to the atmosphere. It is recommended that further research and process optimization is carried out to investigate the feasibility of the combined process. The currently available full-scale systems are run under  $O_2$  limitation to enable nitrite supply for anammox bacteria through aerobic AOB [5,7]. Under these conditions, n-damo bacteria would have to survive oxygen stress; moreover, they would have to compete with aerobic methane-oxidizing bacteria for methane and with anammox bacteria for nitrite. In order to investigate these possibilities, more physiological studies are vital to determine the response of these remarkable bacteria to  $O_2$  and their capacity to compete with other micro-organisms for substrates.

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## References

- 1 Jetten, M.S.M., Horn, S.J. and van Loosdrecht, M.C.M. (1997) Towards a more sustainable municipal wastewater treatment system. *Water Sci. Technol.* **35**, 171–180
- 2 Van de Graaf, A.A., Mulder, A., Debruijn, P., Jetten, M.S.M., Robertson, L.A. and Kuenen, J.G. (1995) Anaerobic oxidation of ammonium is a biologically mediated process. *Appl. Environ. Microbiol.* **61**, 1246–1251
- 3 Strous, M., Van Gerven, E., Zheng, P., Kuenen, J.G. and Jetten, M.S.M. (1997) Ammonium removal from concentrated waste streams with the anaerobic ammonium oxidation (anammox) process in different reactor configurations. *Water Res.* **31**, 1955–1962
- 4 Abma, W.R., Driessen, W., Haarhuis, R. and van Loosdrecht, M.C.M. (2010) Upgrading of sewage treatment plant by sustainable and cost-effective separate treatment of industrial wastewater. *Water Sci. Technol.* **61**, 1715–1722
- 5 Sliemers, A.O., Derwort, N., Gomez, J.L.C., Strous, M., Kuenen, J.G. and Jetten, M.S.M. (2002) Completely autotrophic nitrogen removal over nitrite in one single reactor. *Water Res.* **36**, 2475–2482
- 6 Van der Star, W.R.L., Abma, W.R., Blommers, D., Mulder, J.W., Tokutomi, T., Strous, M., Picoreanu, C. and Van Loosdrecht, M.C.M. (2007) Startup of reactors for anoxic ammonium oxidation: experiences from the first full-scale anammox reactor in Rotterdam. *Water Res.* **41**, 4149–4163
- 7 Kartal, B., Kuenen, J.G. and van Loosdrecht, M.C.M. (2010) Sewage treatment with anammox. *Science* **328**, 702–703
- 8 Ettwig, K.F., Shima, S., van de Pas-Schoonen, K.T., Kahnt, J., Medema, M.H., Op den Camp, H.J.M., Jetten, M.S.M. and Strous, M. (2008) Denitrifying bacteria anaerobically oxidize methane in the absence of Archaea. *Environ. Microbiol.* **10**, 3164–3173
- 9 Ettwig, K.F., Butler, M.K., Le Paslier, D., Pelletier, E., Mangenot, S., Kuypers, M.M.M., Schreiber, F., Dutilh, B.E., Zedelius, J., de Beer, D. et al. (2010) Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* **464**, 543–548
- 10 Luesken, F.A., Sánchez, J., van Alen, T.A., Sanabria, J., Op den Camp, H.J.M., Jetten, M.S.M. and Kartal, B. (2011) Simultaneous nitrite-dependent anaerobic methane and ammonium oxidation. *Appl. Environ. Microbiol.* **77**, 6802–6807
- 11 Luesken, F.A., Zhu, B., van Alen, T.A., Butler, M.K., Diaz, M.R., Song, B., Op den Camp, H.J.M., Jetten, M.S.M. and Ettwig, K.F. (2011) *pmoA* primers for detection of anaerobic methanotrophs. *Appl. Environ. Microbiol.* **77**, 3877–3880
- 12 Kartal, B., Koleva, M., Arsov, R., van der Star, W., Jetten, M.S.M. and Strous, M. (2006) Adaptation of a freshwater anammox population to high salinity wastewater. *J. Biotechnol.* **126**, 546–553
- 13 Ettwig, K.F., van Alen, T., van de Pas-Schoonen, K.T., Jetten, M.S.M. and Strous, M. (2009) Enrichment and molecular detection of denitrifying methanotrophic bacteria of the NC10 phylum. *Appl. Environ. Microbiol.* **75**, 3656–3662
- 14 Schmid, M., Twachtman, U., Klein, M., Strous, M., Juretschko, S., Jetten, M.S.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. *Syst. Appl. Microbiol.* **23**, 93–106
- 15 Schmid, M., Walsh, K., Webb, R., Rijpstra, W.I.C., van de Pas-Schoonen, K., Verbruggen, M.J., Hill, T., Moffett, B., Fuerst, J., Schouten, S. et al. (2003) *Candidatus "Scalindua brodae"*, sp. nov., *Candidatus "Scalindua wagneri"*, sp. nov., two new species of anaerobic ammonium oxidizing bacteria. *Syst. Appl. Microbiol.* **26**, 529–538
- 16 Luesken, F., van Alen, T., van der Biezen, E., Frijters, C., Toonen, G., Kampman, C., Hendrickx, T., Zeeman, G., Temmink, H., Strous, M. et al. (2011) Diversity and enrichment of nitrite-dependent anaerobic methane oxidizing bacteria from wastewater sludge. *Appl. Environ. Microbiol.* **92**, 845–854
- 17 Strous, M., Kuenen, J.G. and Jetten, M.S.M. (1999) Key physiology of anaerobic ammonium oxidation. *Appl. Environ. Microbiol.* **65**, 3248–3250

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- 18 Kartal, B., Kuypers, M.M.M., Lavik, G., Schalk, J., Op den Camp, H.J.M., Jetten, M.S.M. and Strous, M. (2007) Anammox bacteria disguised as denitrifiers: nitrate reduction to dinitrogen gas via nitrite and ammonium. *Environ. Microbiol.* **9**, 635–642
- 19 Raghoebarsing, A.A., Pol, A., van de Pas-Schoonen, K.T., Smolders, A.J.P., Ettwig, K.F., Rijpstra, W.I.C., Schouten, S., Sinninghe Damsté, J.S., Op den Camp, H.J.M., Jetten, M.S.M. and Strous, M. (2006) A microbial consortium couples anaerobic methane oxidation to denitrification. *Nature* **440**, 918–921
- 20 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**, 446–449
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