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Current perspectives on the application of N-damo and anammox in wastewater treatment

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The efficient treatment of wastewater for the removal of nitrogen is of key importance to prevent eutrophication and deoxygenation of receiving water bodies. In addition, ineffective wastewater treatment can be a source of greenhouse gasses. The application of newly discovered microbial processes, such as nitrite/nitrate-dependent methane oxidation (N-damo), can make wastewater treatment systems more sustainable; especially when they are combined with anaerobic ammonium oxidation (anammox). A treatment system based on these microbial processes will need oxygen supply for the production of nitrite. This oxygen may inhibit N-damo and anammox and careful regulation of the oxygen supply is of key importance for the success of the application of N-damo in wastewater treatment.

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Introduction

Eutrophication and global warming are severe environmental challenges; the safe boundaries for human interference within the nitrogen cycle and climate change have been greatly exceeded [1]. Wastewater is a large source of nitrogen pollution in receiving water bodies and the efficient removal of nitrogen from wastewater is therefore of key importance to prevent eutrophication and deoxygenation of the environment. However, operational costs and energy requirements are high, and wastewater treatment systems can also be a source of greenhouse gasses (N_2O, NO, CH_4) [2,3]. The current systems will therefore not remain sustainable in the future. In wastewater treatment plants (wwtps), carbon is generally removed first by aerobic microorganisms. Hereafter, very little carbon remains in the wastewater and the main nitrogenous pollutant is ammonium. In conventional nitrogen removal systems, this ammonium is first oxidized to nitrate, which is then converted to dinitrogen gas via denitrification. The first step requires extensive aeration, and the second step requires addition of external carbon (usually methanol) to ensure the availability of electron donor for denitrification. Both steps increase the energy and resource demand, thereby increasing the operational costs and carbon footprint of a wwtp.

In order to reduce the energy and resource needs of wwtps, systems that are not dependent on external carbon and only depend on minimal aeration have to be developed. Combined oxygen-limited and anoxic treatment has several advantages compared to conventional oxic–anoxic wastewater treatment. There is less production of sludge and it has a smaller resource footprint. In these systems, nitrogen removal under anoxic conditions should be achieved without the addition of external electron donors. Methane will be produced under anoxic conditions and can be used as energy source. Not all methane can be removed from wastewater since methane retrieval from large amounts of wastewater is economically not attractive. It was calculated that effluent water of a UASB reactor still contains 20 mg per L methane [4]; stripping of these low concentrations of methane will lead to unintended methane release into the atmosphere which will contribute to global warming [5]. The recent discovery of nitrite/nitrate-dependent anaerobic methane oxidation (N-damo) provides a new way to couple the removal of this residual methane in wastewater to the removal of nitrogen. This coupling would ultimately lead to the simultaneous removal of both methane and ammonium under anoxic conditions without the need for an external electron donor. In the ideal situation, N-damo would be combined with anaerobic ammonia oxidation (anammox) resulting in the simultaneous removal of nitrogen and methane. These two processes together would offer the possibility for the development of sustainable wastewater treatment systems at moderate temperatures (around or below 20°) [6].

N-damo

Anaerobic oxidation of methane can be coupled to both nitrite and nitrate reduction. Nitrite-dependent methane oxidation is performed by bacteria of the NC10 phylum, with *Candidatus* Methylomirabilis oxyfera as their best studied representative [7••]. This bacterium has a unique intra-aerobic pathway which produces oxygen from two molecules of NO (NO dismutation). The formed oxygen is subsequently used for methane oxidation in a manner very similar to aerobic methanotrophs. Nitrate-dependent methane oxidation is performed by archaea belonging to the ANME-2D lineage (*Ca.* Methanoperedens spp) via reversed methanogenesis which is coupled to nitrate reduction to ammonium via nitrite [8••,9–11]. Genome sequencing of a *Methanoperedens* species revealed the presence of genes for the conversion of nitrite to ammonia [10] and it has been shown that N-damo archaea convert approximately 10% of the produced nitrite into ammonia [10,12]. The two groups of anaerobic methanotrophs can be present together in enrichment cultures, since the bacteria can use the nitrite produced by the archaea for the oxidation of methane. Molecular tools have been developed to investigate if N-damo bacteria and archaea are present in wwtps and natural systems [13–16]. Furthermore, it has been shown that both N-damo bacteria and archaea could be enriched in bioreactors using wwtp sludge as inoculum [4,17,18]. In addition, it has been shown that N-damo bacteria be obtained in a coculture with methanogens where they actively remove nitrite [19].

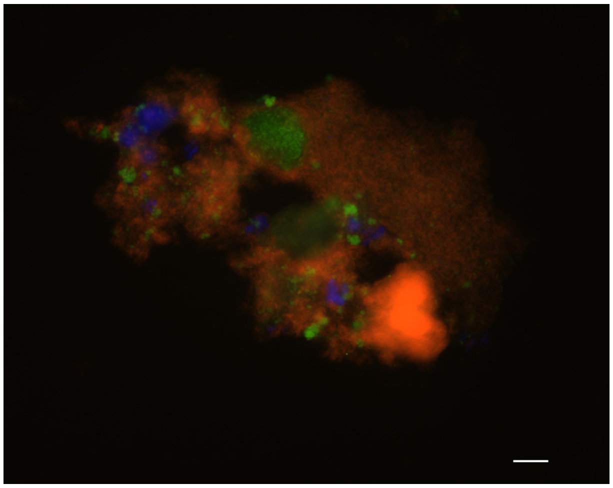
Table 1

Reduction in methane release per mmol of ammonium by combining N-damo and anammox processes.

Process stoichiometry	
Archaea	$CH_4 + 4NO_3^- \rightarrow CO_2 + 4NO_2^-$
Bacteria	$3CH_4 + 8NO_2^- \rightarrow 3CO_2 + 4N_2$
Anammox	$1NH_4^+ + 1.3NO_2^- \rightarrow 1N_2 + 0.3NO_3^-$
Conversions per mM NH_4^+	
Anammox	0.3 M NO_3^-
N-damo archaea	0.08 mM CH_4
N-damo bacteria	0.11 mM CH_4
Total conversion	0.19 mM CH_4
Reduction (%)	15.04% CH_4[a]

[a] Based on the presence of 20 mg/L methane in UASB effluent water [18].

Figure 1



Current Opinion in Biotechnology

FISH picture of a N-damo archaea and bacteria and anammox coculture. N-damo archaea are hybridized with arch915 (Cy5, blue), N-damo bacteria with damo1027 (Cy3, red) and anammox bacteria are hybridized with amx820 (FLUOS, green). The scale bar represents 20 μm.

N-damo/anammox cocultures

A coculture of N-damo and anammox microorganisms can be used to remove residual methane from effluent water. Anammox produces nitrogen gas and nitrate from ammonium and nitrite. The produced nitrate can then be used by N-damo archaea to convert methane. These microorganisms will produce nitrite, which can subsequently be used by N-damo bacteria leading to a further reduction of the amount of methane in the effluent water. Theoretically, combining anammox and N-damo would lead to a 15% decrease in methane in the effluent water without additional aeration (Table 1). The presence of *Ca.* M. oxyfera in anammox granular and sewage sludge has been shown, indicating that anammox and N-damo bacteria can coexist [20,21]. In addition, the presence of both N-damo archaea and bacteria and anammox bacteria was shown in laboratory-scale bioreactors [8••,22] (Figure 1.). Enrichment cultures containing all three groups of microorganisms could be obtained from methanogenic and activated sludge [23]. Both nitrite-dependent anaerobic methanotrophs and anammox bacteria need nitrite for growth. In laboratory conditions, nitrite can be supplied. When nitrite becomes limiting, anammox might outcompete *Ca.* M. oxyfera, as studies indicated that anammox bacteria have a higher affinity for this substrate [24]. On the other hand, accumulation of nitrite can be toxic to *Ca.* M. oxyfera. The activity of N-damo bacteria was reported to be severely inhibited at nitrite levels of 1 mM [25]. Anammox bacteria have been shown to be inhibited by nitrite levels ranging from 7 mM up to above 10 mM [26,27]. However, nitrite concentrations above 1 mM are never observed in wwtps. The partial nitrification of ammonia to nitrite (nitritation) can only be achieved when the oxygen concentration in the system is tightly regulated. Anammox bacteria are reversibly inhibited by low concentrations of oxygen [28]. When oxygen is limited and ammonia is in excess, aerobic and anaerobic ammonia-oxidizing microorganisms can form stable co-cultures in which part of the ammonia is converted into nitrite by the aerobic ammonia oxidizers. In turn, the produced nitrite is used by anammox to convert the remaining ammonia, yielding dinitrogen gas as the main product [29,30].

Oxygen supply for the production of nitrite

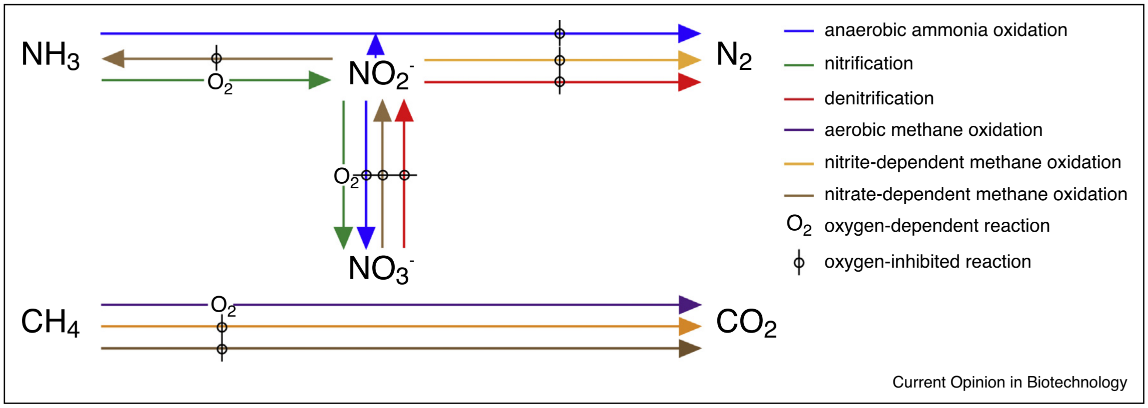
Similar to anammox bacteria, N-damo bacteria have been shown to be inhibited by oxygen. Upon oxygen exposure (experiments with 2 and 8% of oxygen), almost all genes involved in the denitrification pathway were down-regulated whereas genes involved in methane oxidation were constitutively expressed [31]. Although the tested oxygen concentrations are relatively high and the experiments were performed as batch incubations, it is clear from these experiments that N-damo bacteria are not tolerant to oxygen. Anoxic handling of N-damo bacteria and archaea is not essential to maintain activity [32], but there are no experimental data on the effect of oxygen on the activity of N-damo archaea. However, it can be speculated that these microorganisms cannot handle high oxygen concentrations. It is yet unclear what the effect on N-damo microorganisms would be when exposed to dynamic oxygen concentrations, such as the conditions in a wastewater treatment system. Oxygen exposure of the N-damo enrichment culture used by Luesken *et al.* [31] not only led to the inhibition of N-damo bacteria but also to an increase in gene expression of the aerobic methanotrophs that were present in the culture. It can be expected that aerobic methanotrophs will become active in many microbial communities fed with methane once exposed to oxygen [31].

The oxygen supply for the production of nitrite has to be tightly regulated to prevent oxygen inhibition, overproduction of nitrite or complete nitrification to nitrate and growth of heterotrophs. Trace amounts of oxygen, especially when constantly supplied, can be enough to support activity and even growth of aerobic microorganisms. The newly discovered comammox microorganisms [33•,34•], are able to oxidize ammonia under hypoxic conditions (<3.1 μM) [33•]. However, not only nitrifiers can grow under very low oxygen concentrations, also methanotrophs can be abundant and active when little oxygen is available [35•]. In this way aerobic methane oxidation can be coupled to denitrification. Furthermore it is known that it can be combined with fermentation [36]. *Methylomonas denitrificans* needs trace amounts of oxygen to activate the methane while using oxidized nitrogen (nitrate) as an electron acceptor [35•] whereas *Methylomicrobium buryatense* 5GB1 can combine fermentation and aeration leading to the consumption of methane and the excretion of organic carbon [36]. Thus, the introduction of oxygen for nitrite production by ammonia oxidizing microorganisms in wwtps could also trigger methanotrophs to grow. This might result in a complicated interplay between various groups of microorganisms involved in nitrogen and methane removal such as nitrifiers, aerobic and anaerobic methanotrophs and anammox bacteria (Figure 2) which might interfere with the removal of nitrogen and methane from wastewater.

Implications for the application

The successful application of the combination of N-damo and anammox will be dependent on the type of system that will be used. Both anammox and N-damo microorganisms grow slow; thus sufficient biomass retention is crucial. Membrane bioreactors can be successfully used to remove methane using N-damo [37••]. The integration of both anaerobic ammonia and methane oxidation in a single membrane reactor has been shown in several studies [38–41]. In these studies, methane was fed via hollow fibers to a co-culture of anammox and anaerobic methane oxidizing microorganisms. Zhu *et al.* [42] and Luesken *et al.* [21] reported simultaneous removal of ammonia and methane in granular N-damo/anammox cultures; these granules have a fast settling time which ensures biomass retention. In applied systems, limited aeration is needed for the production of nitrite from ammonia. This will lead to stripping of methane from the bulk liquid. Providing air via hollow fiber membranes reduces this risk as suggested by Chen *et al.* [43]. Their model showed that by feeding oxygen via a membrane and other substrates via the bulk liquid, a stratified biofilm will be formed with ammonia oxidizers closest to the membrane (where oxygen is supplied), and anaerobic methane and ammonia oxidizers more towards the anaerobic liquid in the reactor. Optimal functioning of this system will be strongly influenced by the hydraulic retention time and oxygen loading of the system, as the model indicates that these are key regulators for the relative abundance of anammox and N-damo microorganisms. The model further suggests that controlling biofilm thickness is crucial for maintaining high nitrogen and methane removal rates. This is also confirmed by an additional flux analysis [44], which shows that heterotrophs can outcompete anammox and N-damo microorganisms in biofilms under low oxygen and high COD conditions. It might be difficult to keep all aforementioned factors within the optimal range in wwtps, since influent fluxes are not constant and conditions vary over time. In addition, the model of Chen *et al.* [43] proposed a minor role for aerobic methanotrophs in such a system supplied with oxygen. In contrast; modelling of simultaneous ammonia and methane removal in a granular reactor supplied with oxygen, showed that aerobic methanotrophs were the main organisms responsible for methane removal [45]. If the latter is indeed the case for wastewater treatment systems, both oxygen and methane will be used by aerobic methanotrophs instead of by AOB and N-damo microorganisms. Some nitrite can be supplied by N-damo archaea, but this supply will be dependent on the

Figure 2



Schematic overview of a combined N-damo/anammox system. Ammonia, methane and oxygen are supplied and converted to nitrogen gas and carbon dioxide via the combined microbial pathways. Nitrogen conversions and dependence or inhibition by oxygen are shown for the different processes.

amount of methane consumed and on the oxygen sensitivity of these microorganisms. A recent study [20] showed that not only nitrite and oxygen are influencing the abundance of N-damo and anammox bacteria in sewage sludge, but also the pH and the amount of nitrate play a significant role. However, in this study no comparison was made of the activity of the different groups of microorganisms. The successful application of a combined N-damo and anammox treatment is dependent on many different parameters; some of these might even be unknown. Therefore it is very important to further investigate activities of N-damo and anammox microorganisms in mixed cultures under different conditions in both the laboratory and wastewater treatment systems.

Conclusions

In order to apply N-damo for wastewater treatment, the combination with anammox seems to be promising, as was already suggested in Table 1 and two recent review papers [46,47]. This would reduce the amount of methane being released to the atmosphere and reduce the oxygen needed compared to conventional systems, because nitrogen does not have to be fully oxidized to nitrate and would reduce the need for an external electron donor. Oxygen has to be supplied for the production of nitrite and this will lead to competition between different microorganisms that can use oxygen. There is only limited knowledge about the competition between ammonia oxidizers and methanotrophs for oxygen. Furthermore, the effect of low amounts of oxygen on the growth and activity of N-damo microorganisms is not clear. In addition, the production of greenhouse gasses such as N_2O and NO by a combined anammoxi/N-damo system has to be investigated in the future. In these systems, both N-damo bacteria and archaea will be active. N-damo archaea will be able to use the nitrate that will be formed in the system by anammox and nitrification for the conversion of methane and will be able to supply anammox and N-damo bacteria with additional nitrite. The application of nitrogen driven methane oxidation seems to be a promising addition on the already existing wastewater treatment systems to further reduce nitrate discharge in the environment and to reduce the amount of methane that escapes to the atmosphere via wwtps.

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