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Learning from microorganisms: using new insights in microbial physiology for sustainable nitrogen management

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Diverse nitrogen-transforming microorganisms with a wide variety of physiological properties are employed for biological nitrogen removal from wastewater. There are many technologies that achieve the required nitrogen discharge standards; however, greenhouse gas emissions and energy consumption constitute the bulk of the environmental footprint of wastewater treatment plants. In this review, we highlight current and proposed approaches aiming to achieve more energy-efficient and environment-friendly biological nitrogen removal, discuss whether new discoveries in microbial physiology of nitrogen-transforming microorganisms could be used to reduce greenhouse gas emissions, and summarize recent advances in ammonium recovery from wastewater.

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Introduction

Domestic and industrial wastewaters are a major source of anthropogenic nitrogen deposition into the environment. The main reactive nitrogen species in wastewater is ammonium, and the overall aim of biological nitrogen removal is the conversion of ammonium back into N_2. To reach this aim and meet the strict discharge standards for nitrogen-containing wastewaters, diverse biological nitrogen removal systems have been developed. Furthermore, the continual discovery of new nitrogen-transforming pathways and microorganisms results in relatively less energy- and resource-intensive nitrogen removal strategies [1,2]. Whereas both conventional and novel processes achieve efficient nitrogen removal from wastewater, they still consume considerable amounts of energy and lead to the production of climate-active gases nitric oxide (NO) and nitrous oxide (N_2O), which are turned over by many nitrogen-transforming microorganisms [1,3••,4].

Currently, there are no discharge standards for climate-active gases produced during wastewater treatment; however, the inclusion of wastewater treatment plants (wwtp) as a source of greenhouse gases to the recently refined guidelines for the Intergovernmental Panel on Climate Change [5] and the great public interest in global warming and climate change highlight the growing demand for more efficient biological nitrogen removal that needs to include minimizing greenhouse gas emissions. Furthermore, biological ammonium conversion to N_2 requires up to 91% of the energy (MJ/kg N) that is consumed to convert N_2 to ammonium and, consequently, is an intrinsically unsustainable nitrogen management method, which could theoretically be replaced by ammonium recovery technologies [3••]. In this review, we focus on the recently gained insights into the physiology of nitrogen-transforming microorganisms and the current and prospective application of these for sustainable nitrogen management.

Current applications of nitrogen-transforming microorganisms

Nitrogen removal from wastewater is achieved by taking advantage of physiological properties of nitrogen-transforming microorganisms. To achieve its conversion to N_2, ammonia needs to be oxidized at least to nitrite (nitritation). In wastewater treatment, this is predominantly accomplished by aerobic ammonia-oxidizing bacteria (AOB), although ammonia-oxidizing archaea (AOA) are also observed. Both clades of microorganisms convert ammonium via hydroxylamine and NO to nitrite, which is oxidized further to nitrate (nitratation) by aerobic nitrite-oxidizing bacteria (NOB) [4]. The complete oxidation of ammonium to nitrate is termed nitrification, and is energy-intensive due to the extensive aeration demands for AOB and NOB [6]. In the most commonly used conventional nitrogen removal systems, nitrate is produced by nitrification and then reduced to N_2 via nitrite, NO and N_2O, which comprises four distinct N-oxide reductases (Box 1). This so-called denitrification process mostly involves heterotrophic microorganisms

Box 1 Nitrogen-oxide-reducing enzymes

The biological conversion of nitrate to N_2 involves four distinct reduction reactions that require the activity of four groups of enzyme complexes (for a detailed review see Ref. [33]). Membrane-bound (NAR; *narGH*) or periplasmic nitrate reductases (NAP; *napA*) and nitrite oxidoreductases (NXR; *nrxAB*) can reduce nitrate to nitrite (Eq. (1)). Nitrite is then converted to NO (Eq. (2)) by copper-containing (Cu-NIR; *nirK*) or heme-containing (cd_1-NIR; *nirS*) nitrite reductases. During respiration or detoxification, NO can be transformed to N_2O (Eq. (3)) by many enzymes such as cytochrome c-dependent (cNOR; *norB*) and quinol-dependent nitric oxide reductases (qNOR; *norZ*). Finally, N_2O is reduced to N_2 (Eq. (4)) by the only enzyme known to catalyze this reaction, nitrous oxide reductase (NOS; *nosZ*). The key genes encoding these enzymes, such as *narG, nirS* and *nirK*, are commonly used as genetic markers to trace the denitrification process in wastewater treatment plants and natural environments. However, a wide variety of physiologically diverse microorganisms encode one or more of the four N-oxide reductases [4]. Consequently, the detection of one of these marker genes does not guarantee the presence of other N-oxide reductase enzymes or the capacity to completely reduce nitrate to N_2.

$$NO_3^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O \quad (1)$$

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

$$2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O \quad (3)$$

$$N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O \quad (4)$$

that require the input of internal (e.g. carbon-rich wastewater) or external (e.g. methanol) electron donors, and results in high sludge production and a large resource footprint [1].

As an alternative, aerobic ammonia oxidation to nitrite can be coupled to anaerobic ammonium oxidation (anammox) in partial nitritation anammox (PNA) granules (Figure 1a). By avoiding extensive aeration, electron donor addition, and heterotrophic microorganisms, PNA reduces energy and resource footprints and sludge production considerably [1]. PNA is predominantly applied for treating wastewaters with high ammonium content (800−3000 mg/L NH_4-N) such as effluents of anaerobic digesters [1,7,8], and its potential application in domestic wastewater treatment has been suggested [1]. Whereas considerable strides have been made in laboratory-scale and pilot-scale bioreactors [9,10], bottlenecks such as lower microbial reaction rates at lower temperature (down to 12°C) and ammonium concentration (20−75 mg/L NH_4-N) and reaching required discharge standards still need to be tackled before the feasible application of PNA for full-scale nitrogen removal from domestic wastewater [11].

New nitrogen-transforming microorganisms and their potential application

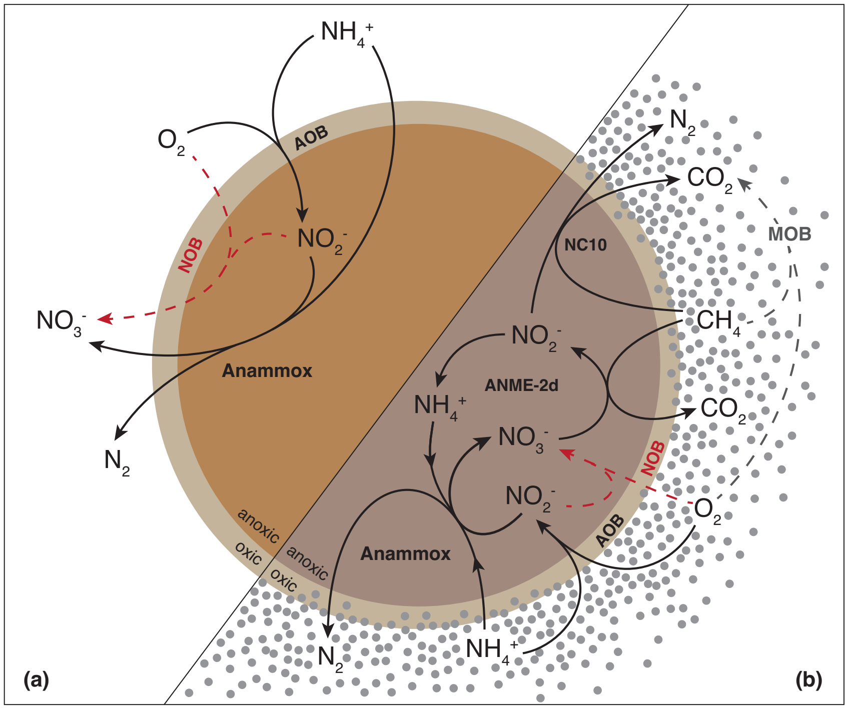
Recently discovered microorganisms that can be potentially applied for nitrogen removal are complete ammonia-oxidizing (comammox) bacteria and nitrite- and nitrate-dependent anaerobic methanotrophs (N-DAMO) [12–15].

Comammox bacteria aerobically oxidize ammonia to nitrate and can fulfil the combined role of AOB and NOB in wwtps. Indeed, these microorganisms are detected in both conventional and PNA systems [16–18]. Physiological experiments [12,19] suggest that, under oxygen limitation, such as in PNA, commamox bacteria would oxidize ammonium only to nitrite, whereas with excess oxygen, such as in conventional systems, they would oxidize ammonia to nitrate. Consequently, these microorganisms appear to be neutral regarding nitrogen removal efficiency in either system. Still, how comammox bacteria would behave under conditions relevant for wastewater treatment (e.g. fluctuating O_2, ammonium, nitrite concentrations) remains unknown. A deeper understanding of their physiology is required to determine whether comammox-based nitrogen removal would be feasible.

On the other hand, a lot of research on the potential application of nitrite-dependent and nitrate-dependent methanotrophs have been performed [20,21]. The former reaction is carried out by NC10 bacteria, which reduce nitrite to N_2 via NO, whereas the latter is performed by ANME-2d archaea that reduce nitrate to ammonium via nitrite [22,23]. The main idea for the application of these microorganisms has been their ultimate integration into PNA granules (Figure 1b) to treat methane-containing and ammonium-containing anaerobic digester effluents [21,24]. Anaerobic digestion is widely used to convert waste sludge to methane, which is collected from the gas phase, purified, and used for heating and electricity generation, but the remaining dissolved methane is eventually emitted to the atmosphere contributing to global warming [25,26].

Instead, dissolved methane could be used as an electron donor for N-DAMO microorganisms, which could improve nitrogen removal efficiency of PNA and mitigate methane emissions. It was demonstrated that stable cocultures of anaerobic ammonium-oxidizing and methane-oxidizing microorganisms are easily formed when ammonium, methane and nitrite (or nitrate) are supplied [20,21]. In contrast to these laboratory-scale bioreactors, in full-scale PNA applications the efficient use of nitrite by anammox and N-DAMO microorganisms would require the suppression of aerobic oxidation of nitrite, which is produced *in situ* by aerobic AOB. This also raises the main problems of integrating N-DAMO into PNA, which are not yet solved. The simultaneous presence of methane and O_2 in anaerobic methane-oxidizing bioreactors promotes the growth of aerobic methane-oxidizing bacteria (MOB), which are detected in many different plants [27,28]. Both MOB and AOB oxidize the substrate of

Figure 1



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Microbial community structure and interactions in a PNA granule **(a)** and in a proposed N-DAMO-PNA granule **(b)**.
(a) In a PNA granule under O_2 limitation, AOB oxidize approximately half of the ammonium to nitrite; subsequently, the produced nitrite and the remaining ammonium is anaerobically converted to N_2 (85%) and nitrate (15%) by the anammox bacteria. The proliferation of NOB has to be suppressed as aerobic nitrate production from nitrite (red arrows) would decrease process efficiency. (b) In the N-DAMO-PNA granule, nitrite and nitrate produced by AOB and anammox bacteria, respectively, would be reduced by NC10 bacteria and ANME-2d archaea, respectively to oxidize methane to CO_2. Ammonium produced by ANME-2d archaea would be used by anammox bacteria. MOB (dashed gray arrows and gray circles) compete for methane and O_2 with N-DAMO microorganisms and AOB, respectively. Here, next to NOB (red arrows), MOB would also need to be suppressed as they cause process inefficiency and instability by growing in bulk liquid and colonizing the granule.

the other clade. However, whereas methane inhibits AOB, many MOB encode the necessary enzymes to detoxify ammonia efficiently into N_2O [4]. Consequently, MOB could easily grow in bulk liquid or colonize the PNA granule, while methane would inhibit AOB, compromising nitrite production, increasing N_2O production, and destabilizing reactor performance (Figure 1b). Next to the complex and intriguing interplay between the involved physiological clades, aeration used in PNA would strip methane from the bulk liquid, resulting in higher direct methane emission and nullifying the targeted benefits. While there are approaches aiming to solve some of these problems [20,21], none of the currently suggested systems addresses all in a manner that would allow feasible full-scale installations.

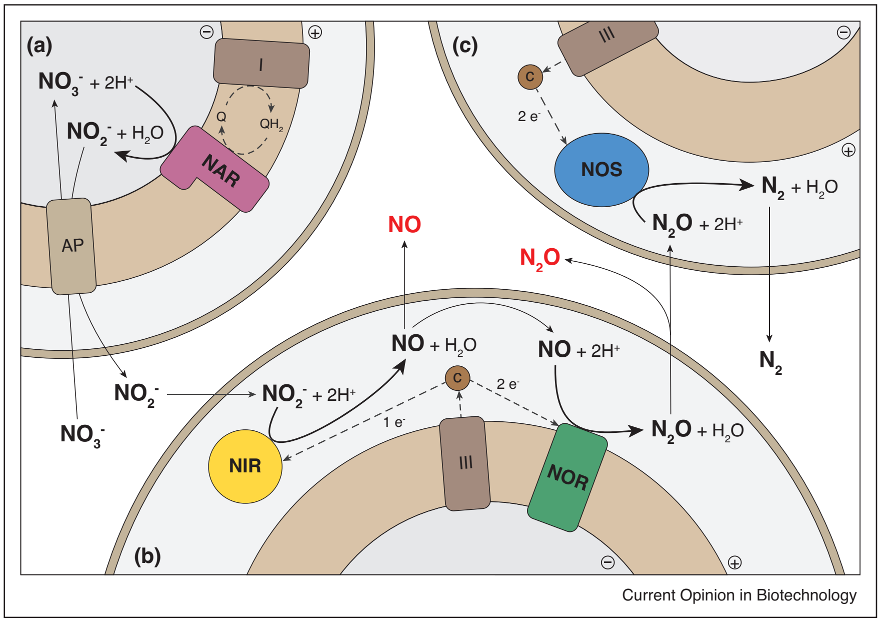
Physiological basis of NO and N_2O emissions from wastewater treatment plants

Physiology and engineering aspects of methane production by methanogenic archaea and their contribution to sustainable wastewater treatment is well understood. Conversely, the physiology of microbial NO and N_2O production is very complex as an infinitely diverse group of microorganisms use several distinct enzymes to produce and consume these two gases. Consequently, many different factors determine the emission of these two climate-active gases, which can reach up to 80% of the operational CO_2 footprint of a wwtp [29]. NO and N_2O emissions from wwtp differ from plant to plant and depend on operational parameters, diversity and different physiologies of N-transforming microorganisms, and even on the methods and duration used to measure these gases [30]. Both in conventional nitrogen removal and PNA, NO and N_2O are emitted through denitrification and ammonium oxidation processes [32]. PNA is suggested to emit less N_2O than conventional systems because anammox bacteria efficiently convert NO into N_2 without producing N_2O [31••]. NO and N_2O are produced as intermediates during the denitrification process, which was traditionally believed to be carried out by single microorganisms encoding all four N-oxide reductases

(Box 1) [4,33]. Whereas this is true for certain cases, in recent years evidence has accumulated demonstrating that a majority of microorganisms encode single N-oxide reductases such as nitrate reductase (NAR) and N_2O reductase (NOS), and denitrification is rather a modular process carried out communally by distinct microorganisms that function together (Figure 2) [33,34,35••,36••,37]. Indeed, microorganisms that encode one or more N-oxide reductases are found throughout the tree of life, and even distinct strains of a single microbial species can harbor completely different combinations of N-oxide reductases [4,38]. Furthermore, ammonia oxidizers use the same nitrite reductase (NIR) and nitric oxide reductase (NOR) enzymes to produce NO and N_2O during so-called 'nitrifier denitrification' [4]. Consequently, N-oxide reducers cannot be collected under umbrella terms such as 'partial denitrifiers' or 'denitrifying microorganisms', and the detection of genetic markers such as nitrite reductases (encoded by *nirS*, *nirK*) or membrane-bound nitrate reductase (encoded by *narG*) (Box 1) cannot be linked directly to complete reduction of nitrate to N_2 or NO and N_2O emission during wastewater treatment. To determine the production and emission of these climate active gases, their microbial turnover and emission from wwtp should be directly measured.

When the denitrification process is performed by a single microorganism, if the activity of NOR or NOS is compromised, NO and N_2O would be released from the cell and result in the emission of these gases. Whereas when the denitrification process is performed by a community of microorganisms (Figure 2), NO and N_2O emissions would occur if the microorganisms that produce these gases stay active, and the microorganisms that reduce them further to N_2 are hampered due to changes in environmental conditions. In such a community, the complete conversion of nitrate to N_2 without NO and N_2O emission requires that the different physiological and growth requirements of all the involved microorganisms are fulfilled. Consequently, we need to gain deeper insight into NO and N_2O turnover. This can be achieved by studying the microbial physiology of NO-reducing and N_2O-reducing specialists in pure as well as natural and synthetic enrichment cultures. Through this approach we can learn how these specialist microorganisms function on their own, and how they interact with the microorganisms that carry out all or distinct steps of the denitrification

Figure 2



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Simplified model of a hypothetical microbial community illustrating the modularity of the denitrification process as a communal effort to reduce nitrate to N_2. Cell A reduces nitrate to nitrite, which Cell B reduces via NO to N_2O. Finally, N_2O is reduced to N_2 by Cell C. In a microbial community, Cell A represents a nitrate reducer. This role can be fulfilled by a wide variety of microorganisms that encode any nitrate reductase, including anammox bacteria or ANME-2d archaea. Cell B can be a so-called 'partial-denitrifier', an AOB or even an MOB, and Cell C is an N_2O-reducing specialist. Here, an imbalance between the activities of different community members, in particular Cell B and Cell C, would result in the accumulation and emission of NO and N_2O. Promoting the activity of Cell C would increase N_2O consumption and counteract N_2O emission. Dashed and thin arrows represent electron transfer and N-compound transport, respectively. NAR: nitrate reductase, NIR: nitrite reductase, NOR: nitric oxide reductase, NOS: nitrous oxide reductase, Q: ubiquinone, QH_2: ubiquinol, AP: nitrate/nitrite antiporter, c: cytochrome c, I: complex I; III: complex III.

process. Consequently, we can determine how the involved microorganisms and the modularity of the denitrification process could be exploited to reduce the emission of climate-active gases from wwtps.

Whereas studies on microbial NO-reducing specialists is scarce [31••], research on N_2O-reducing microorganisms has recently gained traction [39]. Knowledge obtained from environmental studies [36••,40] and growth experiments with enrichment and pure cultures [41,42,43••,44••,45] suggest varying physiological properties for N_2O-reducing specialists, many of which only encode NOS. These differences are an asset, and could be used to promote growth of diverse N_2O-reducing specialists both in conventional systems and PNA to mitigate N_2O emissions under distinct operational conditions.

Nitrogen recovery as an alternative to nitrogen removal

In the last two decades, energy and resource footprints of wwtps have decreased significantly, and considerable strides are being made to reduce greenhouse gas emissions. Nevertheless, energy spent during biological ammonium removal is, ironically, very similar to the energy needed for industrial ammonium production (per kg N) [3••]. With the growing world population, it is predicted that wastewater nitrogen will double by 2050 [46]; consequently, the more sensible option appears to be ammonium recovery from waste streams.

Chemical, physical, and biological processes have been developed to achieve ammonium recovery from wastewater [47–50]. Despite local implementation of some of these [50], currently no sustainable solution exists that allows energy-efficient ammonium recovery from domestic wastewater that is competitive with current and emerging treatment methods or that can be achieved by retro-fitting current wwtp [3••]. Nevertheless, there are promising technologies for ammonium recovery from domestic wastewater.

Microbial electrochemical cells provide an opportunity for simultaneous energy and ammonium recovery [51]. Here, dissolved organic acid oxidation at the anode generates an electrical current that increases pH at the cathode, converting aqueous ammonium to ammonia gas, which is collected and recovered as ammonium via acidification [51]. Whereas initial scaleup attempts appear promising, drawbacks such as low ammonium concentration and conductivity of wastewater need to be addressed for feasible applications [52]. Another biological nitrogen recovery method is ammonia bioaccumulation in phototrophic microalgae, which offers the potential to assimilate nitrogen from wastewater without organic carbon input [47]. After processing, microalgae could be used as protein-rich animal food [53] or to generate ammonium and stable biosolids for land use [2]. However, the growth of microalgae currently requires large bioreactors and intense light supply; consequently, their application would be restricted to areas with intense sunlight and cheap land.

Finally, non-biological ammonium recovery using natural-adsorbent-based processes was shown to have efficient recovery (>98%) with ammonium concentrations similar to domestic wastewater (40−60 mg/L NH_4-N) and should be considered as a viable alternative to microbiological ammonia recovery systems [3••]. However, drawbacks such as high counter ion demand (4:1 salt: ammonium ratio) and difficult continuous operation due to clogging and backwashing hinder the full-scale application of natural adsorbents [3••]. Based on experiences from innovative polymer-based adsorbents (e.g. for micropollutants), development of tailored ammonium-adsorbing polymers and their implementation in currently existing wwtp can be envisaged [54,55]. Whereas it is difficult to determine a timeline for the future availability of next-generation adsorbents, these would offer new opportunities for ammonium recovery from industrial and domestic wastewaters [3••].

Conclusions

Fourteen distinct microbial reactions that transform nitrogen between its redox states form the basis of processes such as nitrification, denitrification and anammox that are applied for biological nitrogen removal. These reactions are carried out by a wide variety of physiologically diverse microorganisms found throughout the tree of life. The in-depth physiological understanding of the involved microorganisms is key to address persistent problems associated with nitrogen removal, in particular emission of climate-active gases and energy consumption. Moving forward, biological and physicochemical ammonia recovery should be considered as a sustainable nitrogen management approach compared to the energy-intensive conversion of industrially produced ammonium back to N_2 during wastewater treatment.

Conflict of interest statement

Nothing declared.

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