PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B

Phil. Trans. R. Soc. B (2012) **367**, 1265–1277
doi:10.1098/rstb.2011.0317

Review

Nitrous oxide emissions from wastewater treatment processes

Yingyu Law, Liu Ye, Yuting Pan and Zhiguo Yuan*

Advanced Water Management Centre (AWMC), The University of Queensland, St Lucia, Queensland 4072, Australia

Nitrous oxide (N_2O) emissions from wastewater treatment plants vary substantially between plants, ranging from negligible to substantial (a few per cent of the total nitrogen load), probably because of different designs and operational conditions. In general, plants that achieve high levels of nitrogen removal emit less N_2O, indicating that no compromise is required between high water quality and lower N_2O emissions. N_2O emissions primarily occur in aerated zones/compartments/periods owing to active stripping, and ammonia-oxidizing bacteria, rather than heterotrophic denitrifiers, are the main contributors. However, the detailed mechanisms remain to be fully elucidated, despite strong evidence suggesting that both nitrifier denitrification and the chemical breakdown of intermediates of hydroxylamine oxidation are probably involved. With increased understanding of the fundamental reactions responsible for N_2O production in wastewater treatment systems and the conditions that stimulate their occurrence, reduction of N_2O emissions from wastewater treatment systems through improved plant design and operation will be achieved in the near future.

Keywords: emissions; greenhouse gases; nitrous oxide; nitrogen removal; wastewater treatment

1. INTRODUCTION

Nitrous oxide (N_2O) is a potent greenhouse gas, which accounts for 7.9 per cent of the global anthropogenic greenhouse gas emissions in 2004 [1]. It is also predicted to be the most dominant ozone-depleting substance in the twenty-first century [2]. Since 1750, the atmospheric N_2O concentration has increased by about 16 per cent, from around 270 ppb, to 319 ppb in 2005. Human activity has been responsible for 40–50% of the annual increase in N_2O emissions over its pre-industrial levels [1]. While agriculture is the major contributor accounting for 80 per cent of the anthropogenic N_2O source, other contributors include biomass and fossil combustion, manure management, adipic acid and nitric acid production and waste management [1,3].

Since the first published data by Czepiel *et al.* [4], reporting N_2O emissions from a wastewater treatment plant, awareness and concern of N_2O emissions during wastewater treatment have grown significantly among urban water authorities. Owing to the complexity involved in measuring N_2O emissions from full-scale plants and the lack of standardized measurement methods, N_2O emissions for the wastewater sector have been estimated based on models without the input of measured data. The Environmental Protection Agency of the United States [5] reported that N_2O from the wastewater sector accounts for about 3 per cent of N_2O emissions from all sources and ranks as the sixth largest contributor. Similarly, the Intergovernmental Panel on Climate Change also reports that N_2O emissions from wastewater account for approximately 2.8 per cent of the total anthropogenic sources [1]. Global N_2O emissions from wastewater treatment are expected to increase by approximately 13 per cent between 2005 and 2020.

N_2O is mainly released during biological nitrogen removal in biological nutrient removal (BNR) plants. There are various configurations of BNR plants that can achieve high levels of nitrogen removal from wastewater by promoting nitrification and denitrification in different reaction zones. N_2O is a known obligatory intermediate in the heterotrophic denitrification pathway and is also produced by autotrophic nitrifying bacteria, mainly ammonia-oxidizing bacteria (AOB) [6], as a by-product.

The microbial nitrogen transformation processes in a wastewater treatment plant are fundamentally the same as in other environments such as soil, marine and freshwater habitats. However, unlike most other environments, wastewater treatment plants are engineered systems designed to achieve high nitrogen conversion rates. There are several key features that distinguish these plants from other environments:

— Domestic wastewater usually contains relatively high concentrations of nitrogen, around 20–70 mg l^{-1} total nitrogen as N. In order to attain almost complete nitrogen removal within 3–8 h, high nitrogen loading rates are applied, incurring relatively high nitrification and denitrification rates [7]. These are expected to impact on the rate of N_2O production.

* Author for correspondence (zhiguo@awmc.uq.edu.au).

One contribution of 12 to a Theo Murphy Meeting Issue 'Nitrous oxide: the forgotten greenhouse gas'.

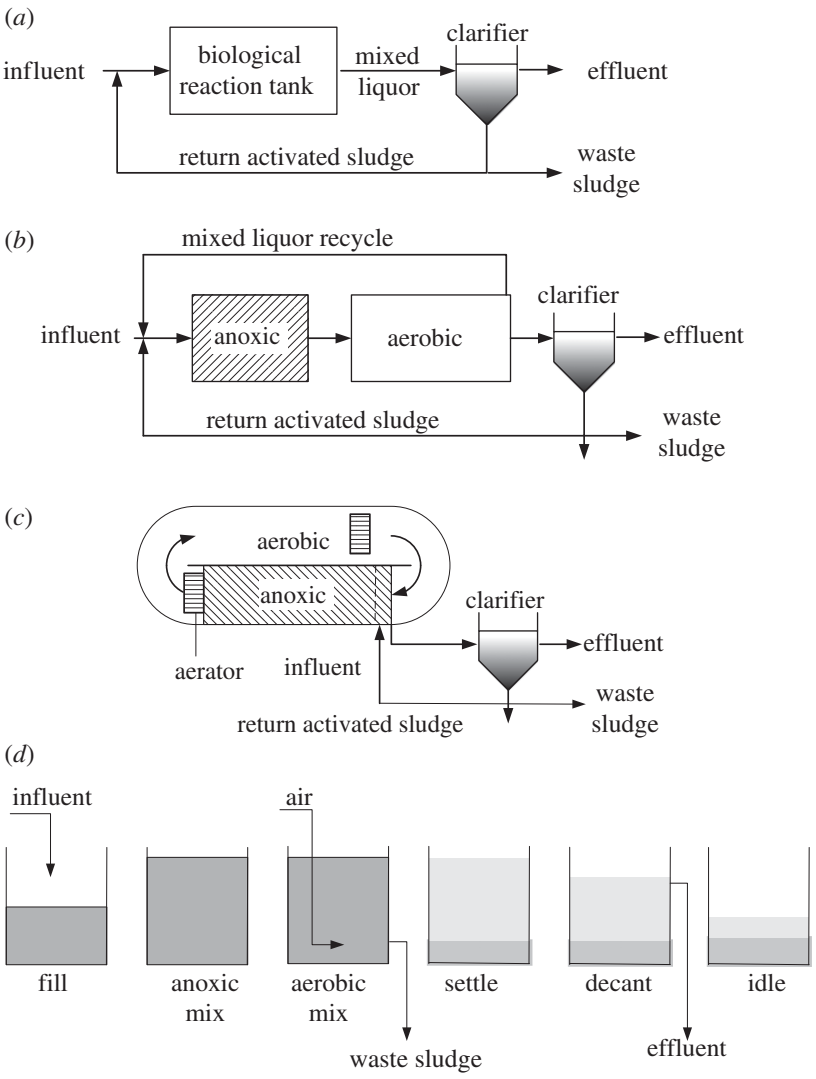

Figure 1. Diagram of (*a*) a conventional activated sludge system; (*b*) a modified Ludzack–Ettinger system; (*c*) an oxidation ditch; and (*d*) a sequencing batch reactor system.

— Bacterial communities in the plants are subjected to rapid changes in process conditions that are applied to promote aerobic or anoxic microbial reactions. Such rapid changes in environmental conditions probably cause physiological stress to both the nitrifying and denitrifying communities, with the potential to induce transient behaviours.
— Active aeration is used to induce aerobic conditions. The aeration systems are engineered to efficiently provide oxygen to the bioreactor, which also enables efficient transfer of N_2O from the liquid phase to the gas phase. Therefore, any temporary imbalance between N_2O production and consumption could result in accumulation and then stripping of N_2O during aeration.
— Given that wastewater treatment systems are highly engineered systems, there are opportunities to mitigate N_2O emissions by improving process design and/or operational conditions.

In this paper, we review the key outcomes arising from the research on N_2O production and emissions from wastewater systems. Following a brief description of the design and operation of wastewater treatment systems, the methods for measuring N_2O in wastewater systems and the emission rates thus far measured are summarized. This is followed by discussions on the key metabolic pathways contributing to N_2O production, and the most important influencing factors. Finally possible mitigation strategies are discussed.

2. DESIGN AND OPERATION OF BIOLOGICAL WASTEWATER TREATMENT PLANTS

(a) *Activated sludge systems for biological wastewater treatment*

Activated sludge is the most widely used process for biological treatment of wastewater. This process uses a microbial community suspended in wastewater to metabolize the biodegradable organic and inorganic components. The microbial community usually clumps together forming three-dimensional aggregates or flocs, known as activated sludge. The sludge and wastewater mixture is called the mixed liquor and the treatment process takes place in a biological reaction tank (bioreactor). At the end of the biological treatment process, the mixed liquor is passed into the clarifier where the sludge is settled and separated from the treated water (figure 1*a*) [7]. The latter is discharged as the effluent. Most of the settled sludge is returned to the bioreactor, with a hydraulic flow rate

that is comparable with that of the influent flow. A small fraction of the sludge, called the waste activated sludge, is removed and disposed of after several steps of sludge treatment (figure 1*a*). The rate of sludge wastage determines the average amount of time that the sludge is retained in the activated sludge system, and is termed the solids retention time. The key components typically removed during the activated sludge treatment process are solids, organic carbon compounds, nitrogen and phosphorus.

(b) *Biological processes for nitrogen removal*

Nitrogen in wastewater is present in the form of complex organic nitrogen compounds, ammonium (NH_4^+), and low (often negligible) levels of nitrite (NO_2^-) and nitrate (NO_3^-) [7]. The organic nitrogen fraction such as amino acids, amino sugars and proteins are readily converted to NH_4^+ by microbial degradation in sewer systems and in the bioreactor. In conventional BNR plants, NH_4^+ is first converted to NO_2^- and NO_3^- via autotrophic nitrification, which is followed by the reduction of NO_3^- and NO_2^- by heterotrophic denitrification to form N_2.

The bioreactor used for nitrogen removal provides conditions enabling both nitrification and denitrification. Aerobic conditions are required for nitrification, whereas a sufficient amount of organic carbon compound is required to support denitrification under anoxic conditions. To achieve this, conventional BNR plants are usually configured as continuous systems whereby wastewater flows through the denitrification and nitrification processes, which are separated into either different compartments or zones.

In a typical modified Ludzack–Ettinger configuration, an anoxic compartment/zone precedes the aerobic compartment (figure 1*b*) [7]. At the end of the aerobic compartment, the nitrified wastewater containing NO_3^- is re-circulated back to the anoxic compartment with a flow rate that is a few times that of the wastewater influent. Wastewater is also fed to the anoxic compartment, which provides the organic carbon for denitrification. A wide range of solids retention time (10–30 days) depending on treatment needs can be applied. There are many variants of this configuration. For example, another pair of anoxic and aerobic compartments can be added to the end of the bioreactor shown in figure 1*b*, to form a four-stage anoxic–aerobic–anoxic–aerobic process. With this design, wastewater is often fed to both the first and second anoxic compartments, thus resulting in a step-feed process. In all cases, the sludge, where micro-organisms reside, is passed between anoxic and aerobic conditions frequently (in hours and in many cases even less than an hour). The dissolved oxygen (DO) concentration in the aerobic compartment is typically within the range of 0.5–2 mg l^{-1}. Although, in some cases could be outside of this range, particularly when the DO is not controlled automatically. In comparison, DO in the anoxic compartment is usually not detectable. However, a limited amount of oxygen is brought into the anoxic compartment through the recirculation stream(s) and through natural surface oxygen transfer.

Figure 1*c* shows an oxidation ditch system, which is also commonly used for BNR [7]. Oxidation ditches are usually equipped with horizontal brush aerators to provide aeration and also to move the mixed liquor along the ditch at a relatively high velocity (0.25–0.35 m s^{-1}) [8]. Each pass of mixed liquor in the ditch typically lasts for several minutes. A relatively high DO concentration is obtained at or close to the aerator and anoxic conditions develop further away from the aerator. The high recirculation flow and the large tank volume dampen the load variations, giving rise to more stable operating conditions in comparison with the modified Ludzack–Ettinger configuration (figure 1*b*). A further feature of an oxidation ditch is that the DO is typically low (e.g. around 0.5 mg l^{-1}), favouring simultaneous nitrification and denitrification. Low DO conditions allow a buildup of an oxygen concentration gradient within the microbial flocs as a result of diffusion limitation. Nitrifiers reside at the outer layer of the flocs where there is sufficient oxygen supply, whereas denitrifiers can remain active in the anoxic zone of the flocs allowing nitrification and denitrification to occur simultaneously [9].

Unlike continuous flow systems outlined above, sequencing batch reactors can also be used to achieve the removal of nitrogen and organic carbon. Aerobic and anoxic conditions are separated by time instead of space [10] (figure 1*d*). All the phases in continuous systems that are spatially separated are provided in a single reactor. A sequencing batch reactor mimics a plug-flow continuous system producing significant concentration gradients of substrates and products with time. This clearly contrasts with the operational conditions found in an oxidation ditch. When a low DO (e.g. 0.5 mg l^{-1}) is provided during an aerobic period of a sequencing batch reactor cycle, simultaneous nitrification and denitrification can also be encouraged.

3. NITROUS OXIDE MEASUREMENT IN WASTEWATER TREATMENT PROCESSES

(a) *Gas-phase nitrous oxide measurement*

In full-scale wastewater treatment plants, the N_2O emitted from activated sludge tanks is usually captured using a closed floating chamber. This technique was originally adapted from emission measurements of solid surfaces. It was first used to measure N_2O flux from liquid surfaces in a municipal wastewater treatment plant located in Durham, New Hampshire in USA [4]. During aeration, dissolved N_2O was stripped from the liquid phase into the gas; during non-aerated phases, air was blown into the headspace of the chamber for sampling. Owing to the lack of online N_2O measurement at that time, samples were grabbed from the headspace of the chamber into 20 ml nylon syringes at specific time intervals. Analysis for N_2O was accomplished using a gas chromatograph (GC) with an electron capture detector. A similar approach was applied in full-scale studies [11] of an intermittent activated sludge process in Japan. An air pump was used to collect part of the emitted gas from a capture chamber into a gas sampling bag. During the anoxic period, argon was supplied into the chamber as a sweeping gas.

Although the emitted N_2O can be captured through the floating chamber, the off-line sampling (grab samples) do not capture the dynamic changes in the N_2O emission profiles, as will be further discussed. This can result in over- or underestimation of the N_2O emissions. Therefore, online, continuous monitoring of N_2O has been employed in recent years for accurate quantification of N_2O emissions from wastewater treatment systems. The types of online sensors include an infrared analyser [12–15], chemiluminescence [6], a Fourier transform infrared analyser [16] and mass spectrometry [17,18]. Among these, the infrared analyser with a broad N_2O measurement range of up to 2000 ppm is the most commonly used method. However, chemiluminescence has a higher sensitivity with a detection limit at parts per trillion levels.

In addition to temporal variations, spatial variations in N_2O emissions should also be considered, especially for continuous processes (figure 1*b*,*c*). Ideally, multiple hoods should be used to measure N_2O emissions from all zones simultaneously. Although not desirable, variations could also be reasonably captured by moving the single hood between zones. For sequencing batch reactor systems (figure 1*d*), a single location is theoretically adequate, although in practice multiple locations are also preferred to cover possible spatial variation of fluxes.

The N_2O emission factor is typically represented as the ratio between the mass of emitted N_2O-N (kg-N d^{-1}) and the amount of influent total Kjeldahl nitrogen load (kg-N d^{-1}). In some cases, the emission factors are represented as the ratio between the mass of N_2O-N emitted and the amount of N removed through nitrification and denitrification in the treatment plant. The mass of emitted N_2O-N is calculated from the measured N_2O concentration, the gas flow rate out of the chamber and the covered cross-sectional area [19]. For aerated zones, the gas flow out of the chamber is equal to the air flow for aeration and is usually recorded by each plant. For non-aerated zones, the gas flow through the chamber can be recorded with a rotameter.

(b) *Liquid-phase nitrous oxide measurement*

Measurement of liquid-phase N_2O using off-line grab samples followed by GC analysis has been used in both laboratory scale reactors and full-scale plants [4,15,20–22]. A liquid sample containing N_2O is injected into a vacuum vial and allowed to reach liquid–gas equilibrium. The gas-phase N_2O concentration (C_{gas}) in the vial is then measured and the liquid-phase N_2O (C_{liquid}) concentration is calculated based on Henry's law. The total N_2O concentration in the sample is obtained by dividing the total amount of N_2O in both the gas and liquid phases by the total liquid volume.

Continuous monitoring of the dissolved N_2O concentration can be done using N_2O microsensors. Kampschreur *et al.* [6] used a modified Clark electrode (Unisense, Denmark) to measure the liquid-phase N_2O in two laboratory scale reactors. Foley *et al.* [23] measured the liquid-phase N_2O in seven full-scale plants in Australia using the same type of microsensor. The Clark-type sensor has an internal reference and a guard cathode. During measurement, N_2O penetrates through the sensor tip membrane and is reduced at the metal cathode surface. The sensor is connected to a high-sensitivity pico-ammeter, which converts the resulting reduction current to a signal. The online signal can be recorded on a laptop. The response of the electrochemical microsensor is known to be linear in the range of 0–1.2 mM [24].

While N_2O microsensors have a low detection limit, the high sensitivity can render it susceptible to interferences especially in full-scale measurements. Combining the analyses of both the microsensor and the GC-vial methods significantly increases the reliability of data.

Similar to the gas-phase analysis, liquid-phase detection at multiple locations is needed to capture the spatial variation in N_2O concentration.

N_2O flux is determined using the liquid-phase measurement [23]. However, this requires the estimation of the mass transfer coefficient between the liquid and gas phases, which is not a straightforward task in full-scale plants [23]. Consequently, the liquid-phase N_2O data are primarily used for understanding N_2O production and emission processes rather than for quantification purposes.

Other parameters such as pH, DO, temperature, total suspended solids and volatile suspended solids (VSS) are often measured at sampling locations and at the wastewater influent for mass balance, correlation analysis of N_2O emission fluxes and for model development.

4. FULL-SCALE EMISSION DATA

The N_2O emission factor (amount of N_2O-N emitted relative to the nitrogen load) reported thus far for full-scale plants varies substantially, ranging from 0 to 25% (table 1). It should be noted that an emission factor of 1 per cent would already increase the carbon footprint of a wastewater treatment plant by approximately 30 per cent [29]. The large variation in N_2O emissions among the investigated plants was probably owing to the different configurations and operational conditions applied. Additionally, the different monitoring and quantification methods used could have been a contributing factor. The large variation also implies that N_2O emissions from a treatment plant can be reduced through proper plant design and operation. Foley *et al.* [23] concluded that plants achieving high-level nitrogen removal would emit less N_2O in comparison with nitrifying plants achieving no or low levels of nitrogen removal. This implies that improved water quality and reduced N_2O emissions can be achieved simultaneously.

Many studies show that N_2O is primarily emitted from the aerated zones [14]. Although N_2O is an obligatory intermediate in denitrification, N_2O formed in anoxic zones will largely be dissolved in the liquid phase and this is converted to N_2 through N_2O reduction before it is transferred to the gas phase. In contrast, N_2O formed in aerobic periods is found to be stripped quickly owing to intensive aeration, forming the primary source of N_2O emitted from wastewater treatment systems [14].

Table 1. Nitrous oxide (N_2O) emission factors reported for several full-scale wastewater treatment plants.

type of plant	N_2O emission (% of N-influent)	sampling method	remarks	reference
activated sludge plant—primary and secondary treatment (aeration only; 4 ml d^{-1})	0.035–0.05	weekly grab samples for 15 weeks	N_2O was emitted in aerated areas, low N_2O flux at non-aerated areas	New Hampshire, USA [4]
activated sludge plant	0.001	grab samples in alternate weeks for 1 year	N_2O emissions increased with nitrite and nitrate concentrations	Germany [25]
anoxic–aerobic activated sludge plant (78 Ml d^{-1})	0.001–0.04	grab samples	N_2O emission was dependent on COD:N	Germany [26]
intermittent activated sludge plant (0.2 Ml d^{-1})	0.01–0.08	collecting gas-phase N_2O samples using air bags during four aeration cycles (2 h)	N_2O emission decreased with shorter aeration periods	Japan [11]
intermittent activated sludge treatment of municipal sewerage (2.5 and 31 Ml d^{-1})	0.47 (0.01)	—	—	France [27]
nitritation–anammox sludge digestion liquor treatment	2.3	online measurement during 4 days	N_2O emissions increased with decreasing oxygen concentration (aerated stage) and increasing nitrite concentration (anoxic stage)	Netherlands [22]
seven BNR plants	0.6–25 (3.5 + 2.7% average)	grab samples	correlation between N_2O emissions and nitrite accumulation was observed	Australia [23]
four treatment plants (completely mixed, plug-flow, membrane bioreactor)	0–0.3	online measurement	NH_4-N and DO had impact on N_2O emission	France [28]
partial nitritation–anammox sequencing batch reactor (three plants, five reactors)	0.4–0.6	online measurement	N_2O emissions were slightly higher than in conventional nitrogen-removal systems	Switzerland [16]
12 BNR plants	0.003–2.59	online measurement	aerobic zones contributed substantially more to N_2O fluxes than anoxic zones	USA [14]
four-stage floc-based partial nitritation and anammox process	5.1–6.6	online measurement	high N_2O emissions may be partly inherent to a separate nitritation step	Belgium [15]

5. NITROUS OXIDE PRODUCTION PATHWAYS

N_2O is produced in BNR systems during autotrophic nitrification and heterotrophic denitrification. Although the nitrification step involves both AOB and nitrite-oxidizing bacteria (NOB), it is widely accepted that NOB does not contribute to N_2O production. The key metabolic pathways involved in N_2O production by AOB and denitrifying bacteria in BNR systems are reviewed in this section.

(a) *Nitrifier denitrification*

Nitrifier denitrification involves the reduction of NO_2^- to NO, N_2O and N_2 by autotrophic AOB. However, only genes encoding NO_2^- and NO reductase (*nirK* and *nor*) are found in the genome of AOB but not N_2O reductase [30–36]. This suggests that N_2O rather than N_2 is the endproduct of the nitrifier denitrification pathway (figure 2). Hydroxylamine (NH_2OH) [38], hydrogen (H_2) [38] and ammonia (NH_3) [39,40] can serve as electron donors for NO_2^- and NO reduction by AOB.

The nitrifier denitrification pathway plays a key role in N_2O production by AOB, especially under anoxic to suboxic conditions [6,22,41]. Experiments conducted with full-scale sludge show that nitrifier denitrification can contribute up to 83 per cent of the N_2O emissions and this depends on the DO level [42]. Kim *et al.* [37] also report that the denitrification activity by AOB is the predominant source of N_2O in an activated sludge under nitrifying conditions and they detected simultaneous expression of *nirK* by AOB.

(b) *Autotrophic ammonia oxidation*

NH_3 rather than NH_4^+ is shown to be the true substrate for AOB [43]. Aerobic NH_3 oxidation to NO_2^- is a two-step process. NH_3 is first converted to

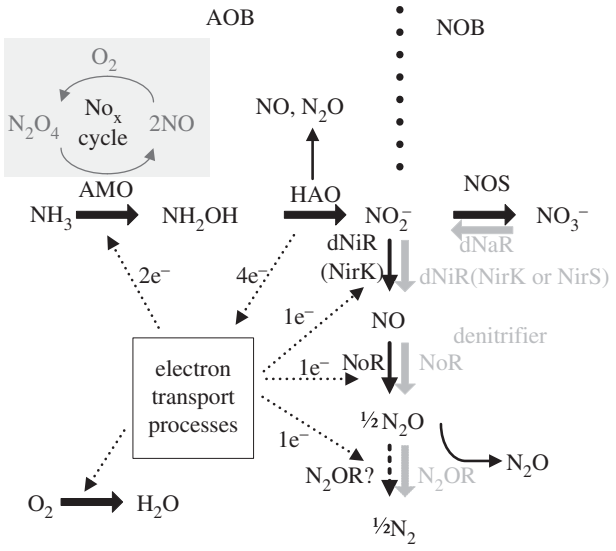

Figure 2. Nitrogen transformation pathways of ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB) and denitrifying bacteria (modified from Kim *et al.* [37]). AOB and NOB pathways divided by large dotted line and denitrifying pathway shown in grey.

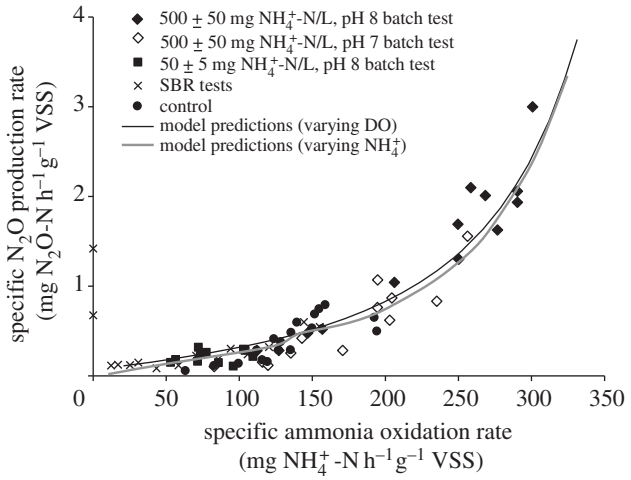

Figure 3. Correlation between the specific N_2O production rate and the specific ammonia oxidation rate. Symbols represent experimental data under various conditions. Solid lines are predictions by a model based on the NOH pathway (adapted from Law *et al.* [50]).

NH_2OH catalysed by a membrane-bound ammonia mono-oxygenase (AMO). This first step requires molecular oxygen and a pair of electrons. The subsequent step is carried out by hydroxylamine oxidoreductase (HAO) in the periplasm to form NO_2^-, generating two pairs of electrons. One pair is used to support the first step of NH_3 oxidation and the remaining pair is used for energy generation [44].

Extended studies by Igarashi *et al.* [45], to characterize the structure and function of HAO, suggest that the NH_2OH oxidation is further split into two reactions to allow two electrons to be accepted and transferred simultaneously. The concurrent reaction involves: (i) conversion of NH_2OH to a nitrosyl radical (NOH); and (ii) conversion of NOH to NO_2^- [46].

N_2O and NO can be formed from the activity of HAO through the unstable NOH intermediate (figure 2). NO is generated as an intermediate during the enzymatic splitting of NOH to NO_2^- [44,47], whereas N_2O is produced through the unstable breakdown of NOH [46].

Despite the fact that this pathway had been postulated for a long time, its relevance to wastewater treatment processes has not been fully confirmed. However, strong evidence demonstrating the potentially significant contribution of this pathway to N_2O production during nitrification is emerging. Increased N_2O production induced by the transition from anoxic to aerobic conditions [48] and high pH [49] are attributed to an increase in NH_3 oxidation rate by AOB. The relationship between the NH_3 oxidation rate and N_2O production rate by AOB was further characterized by Law *et al.* [50], whereby N_2O production rate was shown to be exponentially correlated to the NH_3 oxidation rate (figure 3). This exponential correlation could be represented by a metabolic model based on N_2O production through the chemical degradation of NOH. This provides evidence that N_2O is produced during increased NH_3 oxidation rates and is most likely produced from the unstable breakdown of NOH during NH_2OH oxidation. This suggestion requires confirmation with further experimental studies.

In addition to the chemical breakdown of NOH, biological reduction of NO generated during NH_2OH oxidation could also be a potential source of N_2O. Two molecules of cytochrome *c* are expressed in AOB for transfer of electrons during NH_2OH oxidation to the electron transport chain [51]. One of the two cytochromes, c_{554}, can also act as an NO reductase *in vitro* [52] and is suggested to produce N_2O from NO generated by the enzyme HAO [53]. NO generated during NH_2OH oxidation could also be reduced by homologue NO reductases (NOR), namely NorS [53]. Indeed, genes encoding NorS are detected in the genome of most AOB [53].

As wastewater treatment systems feature high nitrogen conversion and high nitrogen loading, N_2O production during NH_2OH oxidation either through direct decomposition of NOH or the subsequent reduction of the generated NO could play a crucial role in full-scale systems. In addition, sudden process perturbations leading to transiently increased NH_3 oxidation rates may potentially cause increased N_2O production. However, the relevance of this pathway to the natural environment remains to be verified.

Besides aerobic NH_3 oxidation by AOB, dinitrogen tetroxide (N_2O_4)-dependent NH_3 oxidation is proposed as an alternative pathway for *Nitrosomonas* to oxidize NH_3 [54]. Catalysed by AMO, NH_3 oxidation to NH_2OH is coupled to N_2O_4 reduction (figure 2) [55,56]. Two moles of NO are formed and released from the cell per mole of N_2O_4 reduced. This enables NH_3 oxidation to proceed under complete anoxic conditions. Similarly, NH_2OH oxidation is also catalysed by HAO, using NO_2^- as an electron acceptor to form N_2 as a final product [57]. Under aerobic conditions, molecular oxygen is postulated to have an indirect role by re-oxidizing the NO to form N_2O_4. This NO to N_2O_4/NO_2 conversion is called the NO_x cycle

[56]. It is proposed that nitrifier denitrification under oxic conditions plays a role in supplying NO for the NO_x cycle [54].

(c) *Heterotrophic denitrification*

Heterotrophic denitrification is an enzyme-mediated sequential reduction of NO_3^- to N_2 coupled to the oxidation of organic substrates (figure 2). N_2O is an obligate intermediate of heterotrophic denitrification. Under typical denitrifying conditions found in a biological wastewater treatment process, NO and N_2O reductases have higher maximum nitrogen turnover than NO_3^- and NO_2^- reductases [58]. Wicht [59] estimates that the maximum N_2O reduction rate is almost four times faster than the NO_3^- and NO_2^- reduction rates. This indicates that N_2O could be completely reduced under anoxic/anaerobic conditions without the occurrence of its accumulation or emission.

However, fluctuations in environmental conditions have been found to lead to inhibition of the N_2O reductase and accumulation of N_2O (see §6). Also, denitrification enzymes are induced during exposure to anaerobic conditions. Under most circumstances, the induction of N_2O reductase appears to lag behind the others resulting in transient accumulation of N_2O [60]. In addition, N_2O has been found to be the principal product for some denitrifiers as there is only approximately a 20 per cent difference in energy loss if denitrification does not proceed to completion [61,62].

The accumulation of N_2O has been found not to result in significant emissions of N_2O because of the lack of active aeration in the anoxic zones. Under such conditions, the air–liquid interface is limited to the surface area of the reactor, which would lead to limited N_2O emission (table 1) given the relatively high solubility of N_2O. However, the accumulated N_2O that is carried over into the aerobic zone will be stripped quickly [14,63]. This emission can be minimized by providing enough anoxic time to allow the temporarily accumulated N_2O to be removed.

6. KEY PROCESS CONDITIONS LEADING TO NITROUS OXIDE EMISSIONS

The key process conditions affecting the N_2O production from full-scale wastewater treatment plants are summarized in table 1. These, and a range of other process conditions leading to N_2O emissions are further discussed in this section.

(a) *Stripping owing to aeration*

In contrast to freshwater, marine or soil environments, N_2O emission from wastewater treatment plants is substantially enhanced owing to the stripping that is induced by active aeration. N_2O is a relatively soluble gas in water with a Henry's law constant of 24 mM atm^{-1} (at 25°C and 0% salinity) [64] in comparison with 1.3 mM atm^{-1} (at 25°C and 0% salinity) for oxygen [65]. This implies that N_2O could accumulate to relatively high levels in the liquid phase in the absence of active stripping. For example, Law *et al.* [49] observed negligible N_2O emission from a nitrifying reactor in non-aerated periods despite its accumulation to 0.5 mg N l^{-1} in the liquid phase. In contrast, the liquid-phase N_2O was in the range of 0.01–0.03 mg N l^{-1} in the aerated periods. Here, vigorous aeration employed to promote the activity of nitrifying bacteria resulted in stripping of the dissolved N_2O. Gas-phase N_2O measurements in full-scale plants also show that N_2O emissions are two to three orders of magnitude higher in aerated zones than in non-aerated zones [66]. The emitted N_2O can be either produced under aerobic conditions or accumulated during anoxic conditions preceding the aeration.

(b) *Transition between anoxic and aerobic conditions*

As described in §2, anoxic and aerobic compartments/periods are engineered in a wastewater treatment system to achieve nitrification and denitrification, respectively. However, a single sludge process consisting of both nitrifiers and denitrifiers is normally employed. The activated sludge is re-circulated between anoxic and aerobic compartments/periods and this would result in exposure of the mixed bacterial community to repeatedly changing conditions. Fluctuations within a compartment can also occur, for example, the DO concentration may decrease owing to increased loading or limitation of the aeration capacity [12,13,67]. Transient changes in DO concentration are shown to cause immediate increase in N_2O production especially from AOB [68–70].

(i) *Imposition of anoxia on nitrifying bacteria*

It is widely reported that N_2O production from nitrifying cultures is significantly increased during oxygen limitation. Maximum N_2O production rates are observed between DO concentrations of 0.1 and 0.3 mg O_2 l^{-1} [41,42,71,72]. The response of a nitrifying culture to the transition from aerobic to anoxic conditions was demonstrated by Kampschreur *et al.* [68]. The N_2O and NO production rates increased instantly upon the imposition of anoxia from fully aerobic condition. The NO production also increased immediately when tested with NO_2^- and NH_4^+ pulsing under both aerobic and anoxic conditions [68]. Nitrifier denitrification by AOB is suggested to be the main pathway contributing to the production of N_2O and NO as both NH_4^+ and NO_2^- were required to be present.

Yu & Chandran [73] further investigated the response of AOB to low DO coupled to NO_2^- accumulation at the gene expression and transcription level. During the exponential growth phase of the *Nitrosomonas europaea* batch culture, mRNA concentrations for ammonia monooxygenase (*amoA*) and hydroxylamine oxidoreductase (*hao*) were higher in cultures cultivated at lower DO. In addition, the presence of 280 mg NO_2^--N l^{-1} resulted in elevated concentrations of *nirK* and *norB* mRNA for NO_2^- reductase and NO reductase, respectively. They postulate that *N. europaea* increases the efficiency to metabolize NH_3 and NH_2OH under oxygen limitation and also promote the reduction of NO_2^- for detoxification purposes when NO_2^- accumulates. However, such responses are not observed in stationary phase cells suggesting that the efficiency to metabolize substrate and to

detoxify is probably dependent on the physiological growth state of the *N. europaea* culture.

(ii) *Recovery of nitrifying bacteria from anoxic condition*
In contrast to the above study, Yu *et al.* [70] report that it is the recovery from anoxia rather than the transition to anoxia that causes N_2O production from AOB. Such observation was also reported in various full-scale wastewater treatment plants [66]. In an *N. europaea* pure culture grown in chemostat, NO accumulated under anoxic conditions, however N_2O was produced only during the recovery from anoxic to aerobic conditions [70]. The N_2O production during the transient recovery period correlated positively to the accumulation of NH_4^+ during anoxia, and the oxygen concentration upon recovery. In addition, the increased N_2O production during the recovery period did not correlate with changes in the gene-expression level. It was therefore concluded that the tendency of nitrifying cultures to produce N_2O is owing to a shift in metabolism from a low specific activity ($q < q_{max}$) towards the maximum specific activity (q_{max}).

Various other studies also report increased N_2O production during increased aeration rate. Sümer *et al.* [25] found that increased N_2O production coincides with increased oxygen concentration in the activated sludge process. Kampschreur *et al.* [68] also observed that N_2O production by AOB in a nitritation–anammox process decreased with decreased DO concentrations. However, the mechanisms leading to these observations were not identified.

(iii) *Nitrous oxide reduction by denitrifying bacteria during transient aerobic and anaerobic conditions*
Similar to nitrifier denitrification, N_2O emission from heterotrophic denitrification is also shown to be the highest under low DO concentrations of around 0.1–0.3 mg $O_2\,l^{-1}$ [18,42,74]. Therefore, transient and dynamic aerobic and anaerobic conditions will likely increase N_2O emission from heterotrophic denitrification. Oxygen inhibits both the synthesis and activity of denitrifying enzymes of *Alcaligenes faecalis*, in particular the N_2O reductase [18]. The synthesis of the N_2O reductase has a longer lag phase compared with the NO_2^- reductase synthesis after the transition from aerobic to anaerobic conditions. In addition, N_2O reductase activity stops immediately during the transition from anaerobic to aerobic conditions, while the activity of NO_2^- reductase continues at a lower rate for several hours.

(c) *The effect of nitrite, free nitrous acid and pH*

(i) *Nitrifying bacteria*
Hynes & Knowles [75] demonstrate that addition of exogenous NO_2^- does not cause an increase in N_2O production from a fully aerobic *N. europaea* culture. In addition, the optimum pH for the production of NO_2^- and N_2O is approximately 8.5, in the investigated pH range of 5.4–9.5, further suggesting that a high free nitrous acid (HNO_2) concentration, the true substrate for NO_2^- reduction [76], is not required for higher N_2O production. As the aerobic N_2O production is completely inhibited by acetylene (C_2H_2), the authors suggest that N_2O is predominantly produced through degradation of NOH under aerobic conditions [75]. Increased N_2O production rate of an enriched AOB culture at pH 8.0 when compared with pH 6.0 is also reported by Law *et al.* [49].

However, contradictory evidence is produced in some recent studies that report elevated N_2O production rates by AOB in the presence of NO_2^-. Correlation between N_2O production and high NO_2^- concentration by AOB is reported in several full-scale studies [15,25,63,67,77]. In laboratory scale studies, NO_2^- pulses of 10 mg NO_2^--N l^{-1} are shown to increase N_2O production by a nitrifying mixed culture especially at higher DO concentrations, with eightfold and fourfold increases occurring at DO concentrations of 1.0 and 0.1 mg $O_2\,l^{-1}$, respectively [42]. Kampschreur *et al.* [68] also reveal that NO_2^- pulsing increases N_2O production by an enriched AOB culture under aerobic conditions. The contrasting observations on the effect of NO_2^- on N_2O production by AOB are yet to be resolved.

(ii) *Denitrifying bacteria*
The presence of NO_2^- has been shown to affect the activity of N_2O reductase in a denitrifying bacterial culture leading to increased N_2O emission. NO_2^- accumulation of up to 10 mg NO_2^--N l^{-1} was identified as a possible cause of N_2O production in denitrifying sludge [78]. However, the effect of NO_2^- addition on N_2O accumulation is seen to be highly inconsistent [79]. Schulthess *et al.* [80] suggest that NO rather than NO_2^-, which accumulates upon NO_2^- addition, is the true inhibitor of N_2O reductase.

Zhou *et al.* [81] show that HNO_2 rather than NO_2^- is responsible for inhibiting the N_2O reductase in an enriched denitrifying biological phosphorus removal system. N_2O reductase activity was inhibited by 50 per cent at a HNO_2 concentration of 0.0007–0.001 mg HNO_2-N l^{-1} (equivalent to 3–4 mg NO_2^- N l^{-1} at pH 7). However, an internal storage polymer was the sole carbon source available as shown in the study of Zhou *et al.* [81], which is suggested to be a factor affecting N_2O production (further discussed in §6*d*(iii)). Since the concentration of NO in the study was not reported, it is unclear whether HNO_2 could have triggered transient NO accumulation to affect the N_2O reductase activity. The high sensitivity of N_2O reductase to low pH (<6.5) [82] also renders it difficult to distinguish the effect of pH and HNO_2 in denitrifying cultures.

(d) *Effect of carbon sources*

(i) *Availability of carbon source*
The lack of biodegradable organic carbon is an important factor governing N_2O production during denitrification [83,84]. The availability of organic carbon is typically measured as chemical oxygen demand (COD). For complete denitrification, a COD to N ratio above 4 is required. Under conditions of limited carbon sources, the various denitrification enzymes (NO_3^- reductase, NO_2^- reductase, NO

reductase and N_2O reductase) compete for electrons, potentially resulting in incomplete denitrification.

In an intermittently aerated laboratory scale reactor, approximately 20–30% of influent N was emitted as N_2O when the COD to N ratio was less than 3.5 [78]. Similar observations have also been reported by Kishida *et al.* [85]. A pure culture study with *A. faecalis* shows that when carbon sources are limiting, N_2O formation increases by 32–64%, while N_2 production decreases significantly [84]. When excess carbon was supplied to remove electron competition, N_2O formation decreased immediately. On the contrary, it is reported in full-scale studies that only little N_2O generation and emission is observed regardless of carbon deficiency or sufficiency in anoxic zones or aerobic zones [14]. The nitrogen or helium sparging used to induce anoxia in the laboratory scale studies [84,85] may have contributed to the discrepancy between the observation in laboratory scale and full-scale studies. The continuous sparging may have stripped off the dissolved N_2O to render it unavailable for further reduction to N_2. This requires further investigation and verification.

In theory, N_2O and NO are expected to accumulate during COD-limited denitrification as the NO_3^- and NO_2^- reductases have relatively higher affinity for electrons than the NO and N_2O reductases [86]. However, this may not be generalized for all types of carbon sources as different metabolic pathways are employed for different carbon sources.

(ii) *Types of carbon sources*

The availability of different types of carbon sources may enrich different groups of bacteria and have different impacts on denitrification efficiency [87,88]. Methanol, ethanol and acetate, and to a lesser extent glycerol or sludge fermentates, are widely used as supplemented carbon sources for enhancing denitrification in BNR plants. While a COD/N of lower than 1.5 resulted in N_2O production in a denitrifying culture fed with acetate and yeast extract [89,90], a COD limitation did not have an apparent impact on ethanol- and methanol-fed denitrifying cultures [19]. In addition, the methanol-fed denitrifiers are shown to have higher susceptibility to oxygen inhibition when compared with the ethanol-fed denitrifiers [19].

On the contrary, pure culture studies with *A. faecalis* indicate that N_2O production is independent of the energetics of the substrate or the turnover rates of the enzymes. The type of supplemented carbon source (acetate versus butyrate) and the growth rate of the bacteria do not have any impact on overall N_2O production [84]. The discrepancy between different studies may be attributed to the enrichment of different denitrifying populations. Therefore, the types of carbon sources used would affect the types of denitrifiers enriched which potentially have different susceptibility to other operational variables (e.g. NO_2^- and O_2 inhibition).

(iii) *Consumption of internal storage compound*

Systems operated to achieve simultaneous nitrification, denitrification and phosphorus removal can encourage the growth of denitrifiers, such as polyphosphate-accumulating organisms and glycogen-accumulating organisms that are capable of storing organic carbon in the form of polyhydroxybutyrate (PHB). Laboratory scale studies on such systems show that denitrification by glycogen-accumulating organisms leads to increased N_2O emission [17,91,92]. During anaerobic periods, these microorganisms take up organic carbon for storage and subsequently degrade the PHB stored during aerobic/anoxic periods. Since PHB consumption is the rate-limiting step in these organisms [93], high N_2O emission is possible by organisms growing on storage compounds owing to a slow supply of electrons, resulting in competition for electrons between denitrifying enzymes. Schalk-Otte *et al.* [84] observed that N_2O accumulation coincides with the onset of storage compound usage upon COD depletion.

On the contrary, in a PHB-degrading denitrifying pure culture, no accumulation of N_2O or nitrite was detected when PHB was used as the sole carbon source [94]. Further confirmation on the relationship between internal storage compounds and N_2O production is essential as the dynamic conditions employed in treatment plants, such as in P-removal processes and bioselectors, are operated to select for organisms that are able to store carbon sources.

(e) ***Availability of copper ions***

Copper is essential for the biosynthesis of N_2O reductase and its availability affects N_2O production in soil and marine environments [62]. Deficiency in copper supply is found to shift the endproduct of heterotrophic denitrification from N_2 to N_2O, whereas replenishing the copper supply reduces N_2O production and increases N_2 production [95,96]. Although copper is demonstrated to increase the N_2O reductase activity and to reduce N_2O production in an activated sludge [97], the availability of copper in wastewater systems and its subsequent effect on N_2O production has thus far not been investigated.

7. POSSIBLE MITIGATION STRATEGIES

Although the exact triggers for N_2O production by nitrifying and denitrifying sludge are yet to be fully revealed, and the predominant pathway relating to N_2O production by AOB remains to be elucidated, it is generally observed that sudden process perturbations such as rapid shifts in reactor pH, DO and NH_4^+ or NO_2^- spikes lead to immediate increases in N_2O emissions [68,90,98]. In fact, N_2O emission has been recommended to be used as an indication of biological nitrification failure owing to toxic shock loads or insufficient aeration [13].

It is postulated that full-scale plants that are designed and configured to operate under more stable process conditions, such as oxidation ditches with uniform DO concentrations, produce less N_2O when compared with those that are subject to frequent transitions (such as a modified Ludzack–Ettinger plant; figure 1) [70]. Full-scale studies also report that treatment plants designed and operated to achieve low total nitrogen in their effluents are equipped with

design features that result in relatively low N_2O emission levels [67]. These design features include influent flow balancing, high recycle rates, large bioreactor volumes and long solids' retention time. Large bioreactor volumes and influent flow-balancing facilities equip the system with the ability to buffer loadings and reduce the risk of transient oxygen depletion. High recycle rates also tend to dilute the concentrations of NH_4^+ and nitrogen intermediates which lessens the effects of nitrification and denitrification, preventing the buildup of NO_2^- and NH_4^+ to levels that may increase N_2O production [67,99,100].

Several mitigation strategies have been trialled in laboratory scale studies to minimize N_2O emissions. Yang *et al.* [21] demonstrate that NH_4^+ and NO_2^- concentrations in the reactor can be maintained at low concentrations through step feeding, resulting in a 50 per cent reduction in N_2O production. Avoiding transient pH changes under aerobic conditions by slow feeding rather than pulse feeding is shown to significantly reduce N_2O production by an enriched AOB culture [49]. Applying longer solids retention time to increase the AOB biomass concentration (greater than 5 days) and higher DO (>0.5 mg $O_2\,l^{-1}$) is also proposed to minimize N_2O production from nitrification [71]. Pellicer-Nàcher *et al.* [101] demonstrate the possibility of minimizing N_2O emission through sequential aeration in a membrane-aerated biofilm reactor. Here, the N_2O produced by AOB within the membrane bundle is consumed by heterotrophic bacteria outside the bundle. To minimize N_2O production during denitrification, methanol addition prevented N_2O accumulation by eliminating electron competition from other denitrifying enzymes [90].

These mitigation strategies have so far only been demonstrated in laboratory scale systems. Their effectiveness is yet to be verified through full-scale trials. The research community is making steady progress in gaining understanding of the mechanisms involved in N_2O emission in wastewater treatment systems, which will enable the development of effective mitigation strategies.

8. CONCLUSIONS

Despite their relatively small contribution to the overall global greenhouse gas emissions, N_2O emissions from BNR wastewater treatment plants can be very significant in terms of their contribution to the overall carbon footprint of wastewater treatment systems, and should be understood, accounted for and mitigated.

N_2O emissions from wastewater treatment processes vary substantially between plants depending on the design and operation of the plants, and the flow and characteristics of wastewater. These variations indicate that N_2O may be mitigated through proper process design and operation. Indeed, preliminary strategies have been developed but remain to be verified through full-scale applications.

In contrast with many other systems (e.g. soil), where denitrification is revealed to be the primary source of N_2O, autotrophic NH_3 oxidation is found to make relatively more contributions than heterotrophic denitrification in most wastewater treatment plants. This is probably related to the fact that AOB produce N_2O under aerated conditions; and most of the N_2O produced is stripped instantly by aeration. In contrast, denitrifiers produce N_2O primarily under non-aerated conditions. N_2O can remain dissolved in the absence of stripping, giving time for its subsequent reduction to N_2. However, N_2O carried over from non-aerated zones/periods to the aerated zones/periods will probably be stripped there.

The detailed mechanisms involved in N_2O production by AOB remain to be fully elucidated. Both nitrifier denitrification and the breakdown or degradation of nitrification intermediates probably contribute to the overall N_2O production. However, the level of contribution by each of these two processes is unclear and contradictory evidence has been produced. Indeed, their relative contributions could be dependent on process conditions.

Various factors have been reported in the wastewater literature to induce N_2O emissions by AOB and denitrifiers. A detailed understanding of the factors is currently missing.

Future research in the field will focus on both the quantification and reduction of N_2O emissions from various full-scale wastewater treatment plants. Additionally, future studies will reveal the fundamental processes involved in N_2O production by both nitrification and denitrification.

The authors would like to thank the Australian Research Council (ARC) for funding this work through projects LP0991765 and DPO0987204. The Western Australian Water Corporation also supported the research through project LP0991765. Y.L. is an Australian Postgraduate Award recipient. Y.P. acknowledges the scholarship support by the University of Queensland.

REFERENCES

1 IPCC. 2007 *Climate change 2007: synthesis report. Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change* (eds C. W. Team, R. K. Pachauri, A. Reisinger). Geneva, Switzerland: IPCC.

2 Ravishankara, A. R., Daniel, J. S. & Portmann, R. W. 2009 Nitrous oxide (N_2O): the dominant ozone-depleting substance emitted in the 21st century. *Science* **326**, 123–125. (doi:10.1126/science.1176985)

3 Dalal Ram, C., Robertson, W. W., Philip, G. & Parton, W. J. 2003 Nitrous oxide emission from Australian agricultural lands and mitigation options: a review. *Aust. J. Soil Res.* **41**, 165–195. (doi:10.1071/SR02064)

4 Czepiel, P., Crill, P. & Harriss, R. 1995 Nitrous oxide emissions from municipal wastewater treatment. *Environ. Sci. Technol.* **29**, 2352–2356. (doi:10.1021/es00009a030)

5 United States Environmental Protection Agency. 2006 *Global anthropogenic non-CO_2 greenhouse gas emissions: 1990 to 2020*. Washington, DC: US-EPA.

6 Kampschreur, M. J., Tan, N. C. G., Kleerebezem, R., Picioreanu, C., Jetten, M. S. M. & Van Loosdrecht, M. C. M. 2008 Effect of dynamic process conditions on nitrogen oxide emission from a nitrifying culture. *Environ. Sci. Technol.* **42**, 429–435. (doi:10.1021/es071667p)

7 Tchobanoglous, G., Burton, F. & Stensel, H. D. 2002 *Wastewater engineering treatment and reuse*, 4th edn. New York: McGraw-Hill.

8 Seviour, R. J., Lindrea, K. C., Griffiths, P. C. & Blackall, L. L. 1999 The activated sludge process. In *The microbiology of activated sludge* (eds R. J. Seviour & L. L. Blackall). Boston, MA: Kluwer Publishing.

9 Münch, E. V., Lant, P. & Keller, J. 1996 Simultaneous nitrification and denitrification in bench-scale sequencing batch reactors. *Water Res.* **30**, 277–284. (doi:10.1016/0043-1354(95)00174-3)

10 Irvine, R. L., Ketchum, L. H. & Asano, T. 1989 Sequencing batch reactors for biological wastewater treatment. *Crit. Rev. Environ. Sci. Technol.* **18**, 255–294. (doi:10.1080/10643388909388350)

11 Kimochi, Y., Inamori, Y., Mizuochi, M., Xu, K.-Q. & Matsumura, M. 1998 Nitrogen removal and N_2O emission in a full-scale domestic wastewater treatment plant with intermittent aeration. *J. Ferment. Bioeng.* **86**, 202–206. (doi:10.1016/S0922-338X(98)80114-1)

12 Burgess, J. E., Colliver, B. B., Stuetz, R. M. & Stephenson, T. 2002 Dinitrogen oxide production by a mixed culture of nitrifying bacteria during ammonia shock loading and aeration failure. *J. Indust. Microbiol. Biotechnol.* **29**, 309–313. (doi:10.1038/sj.jim.7000286)

13 Butler, M. D., Wang, Y. Y., Cartmell, E. & Stephenson, T. 2009 Nitrous oxide emissions for early warning of biological nitrification failure in activated sludge. *Water Res.* **43**, 1265–1272. (doi:10.1016/j.watres.2008.12.027)

14 Ahn, J. H., Kim, S. P., Park, H. K., Rahm, B., Pagilla, K. & Chandran, K. 2010 N_2O Emissions from activated sludge processes, 2008–2009: results of a national monitoring survey in the United States. *Environ. Sci. Technol.* **44**, 4505–4511. (doi:10.1021/es903845y)

15 Desloover, J., De Clippeleir, H., Boeckx, P., Du Laing, G., Colsen, J., Verstraete, W. & Vlaeminck, S.E. 2011 Floc-based sequential partial nitration and anammox at full scale with contrasting N_2O emissions. *Water Res.* **45**, 2811–2821. (doi:10.1016/j.watres.2011.02.028)

16 Joss, A. *et al.* 2009 Full-scale nitrogen removal from digester liquid with partial nitration and anammox in one SBR. *Environ. Sci. Technol.* **43**, 5301–5306. (doi:10.1021/es900107w)

17 Zeng, R. J., Lemaire, R., Yuan, Z. & Keller, J. 2003 Simultaneous nitrification, denitrification, and phosphorus removal in a lab-scale sequencing batch reactor. *Biotechnol. Bioeng.* **84**, 170–178. (doi:10.1002/bit.10744)

18 Otte, S., Grobben, N., Robertson, L., Jetten, M. & Kuenen, J. 1996 Nitrous oxide production by *Alcaligenes faecalis* under transient and dynamic aerobic and anaerobic conditions. *Appl. Environ. Microbiol.* **62**, 2421–2426.

19 Lu, H. & Chandran, K. 2010 Factors promoting emissions of nitrous oxide and nitric oxide from denitrifying sequencing batch reactors operated with methanol and ethanol as electron donors. *Biotechnol. Bioeng.* **106**, 390–398. (doi:10.1002/bit.22704)

20 Garrido, J. M., Moreno, J., Mendez-Pampn, R. & Lema, J. M. 1998 Nitrous oxide production under toxic conditions in a denitrifying anoxic filter. *Water Res.* **32**, 2550–2552. (doi:10.1016/S0043-1354(97)00433-8)

21 Yang, Q., Liu, X., Peng, C., Wang, S., Sun, H. & Peng, Y. 2009 N_2O production during nitrogen removal via nitrite from domestic wastewater: main sources and control method. *Environ. Sci. Technol.* **43**, 9400–9406. (doi:10.1021/es9019113)

22 Kampschreur, M. J., van der Star, W. R. L., Wielders, H. A., Mulder, J. W., Jetten, M. S. M. & van Loosdretch, M. C. M. 2008 Dynamics of nitric oxide and nitrous oxide emission during full-scale reject water treatment. *Water Res.* **42**, 812–826. (doi:10.1016/j.watres.2007.08.022)

23 Foley, J., De Haas, D., Yuan, Z. & Lant, P. 2009 Nitrous oxide generation in full scale BNR wastewater treatment plants. *Water Res.* **44**, 831–844. (doi:10.1016/j.watres.2009.10.033)

24 Andersen, K., Kjaer, T. & Revsbech, N. P. 2001 An oxygen insensitive microsensor for nitrous oxide. *Sens. Actuat. B-Chem.* **81**, 42–48. (doi:10.1016/S0925-4005(01)00924-8)

25 Sümer, E., Weiske, A., Benckiser, G. & Ottow, J. C. G. 1995 Influence of environmental conditions on the amount of N_2O released from activated sludge in a domestic waste water treatment plant. *Cell. Mol. Life Sci.* **51**, 419–422. (doi:10.1007/BF01928908)

26 Benckiser, G., Eilts, R., Linn, A., Lorch, H. J., Sümer, E., Weiske, A. & Wenzhöfer, F. 1996 N2O emissions from different cropping systems and from aerated, nitrifying and denitrifying tanks of a municipal waste water treatment plant. *Biol. Fertility Soils* **23**, 257–265. (doi:10.1007/BF00335953)

27 Peu, P., Beline, F., Picard, S. & Heduit, A. 2006 Measurement and quantification of nitrous oxide emissions from municipal activated sludge plants in France. In *Proceedings of the 5th IWA World Water Congress, 10–14 September 2006, Beijing, China*. International Water Association.

28 Foley, J., Yuan, Z., Keller, J., Senante, E., Chandran, K., Willis, J., Shah, A. & van Loosdrecht, M. 2011 N_2O and CH_4 emission from wastewater collection and treatment systems. Technical report, Global Water Research Coalition, London, UK.

29 de Haas, D. & Hartley, K. 2004 Greenhouse gas emission from BNR plants: do we have the right focus? *Proceeedings of EPA Workshop: Sewage Management: Risk Assessment and Triple Bottom Line, 5–7 April 2004, Cairns, Australia*.

30 Casciotti, K. L. & Ward, B. B. 2001 Dissimilatory nitrite reductase genes from autotrophic ammonia-oxidizing bacteria. *Appl. Environ. Microbiol.* **67**, 2213–2221. (doi:10.1128/AEM.67.5.2213-2221.2001)

31 Casciotti, K. L. & Ward, B. B. 2005 Phylogenetic analysis of nitric oxide reductase gene homologues from aerobic ammonia-oxidizing bacteria. *FEMS Microbiol. Ecol.* **52**, 197–205. (doi:10.1016/j.femsec.2004.11.002)

32 Beaumont, H., Lens, S., Reijinders, W., Westerhoff, H. & van Spanning, R. 2004 Expression of nitrite reductase in *Nitrosomonas europaea* involves NsrR, a novel nitrite-sensitive transcription repressor. *Mol. Microbiol.* **54**, 148–158.

33 Beaumont, H. J. E., Lens, S. I., Westerhoff, H. V. & van Spanning, R. J. M. 2005 Novel nirK cluster genes in *Nitrosomonas europaea* are required for NirK-dependent tolerance to nitrite. *J. Bacteriol.* **187**, 6849–6851. (doi:10.1128/JB.187.19.6849-6851.2005)

34 Cantera, J. J. L. & Stein, L. Y. 2007 Molecular diversity of nitrite reductase genes (nirK) in nitrifying bacteria. *Environ. Microbiol.* **9**, 765–776. (doi:10.1111/j.1462-2920.2006.01198.x)

35 Shaw, L. J., Nicol, G. W., Smith, Z., Fear, J., Prosser, J. I. & Baggs, E. M. 2006 *Nitrosospira* spp. can produce nitrous oxide via a nitrifier denitrification pathway. *Environ. Microbiol.* **8**, 214–222. (doi:10.1111/j.1462-2920.2005.00882.x)

36 Garbeva, P., Baggs, E. M. & Prosser, J. I. 2007 Phylogeny of nitrite reductase (nirK) and nitric oxide reductase (norB) genes from *Nitrosospira* species isolated from soil. *FEMS Microbiol. Lett.* **266**, 83–89. (doi:10.1111/j.1574-6968.2006.00517.x)

37 Kim, S.-W., Miyahara, M., Fushinobu, S., Wakagi, T. & Shoun, H. 2010 Nitrous oxide emission from nitrifying activated sludge dependent on denitrification by

ammonia-oxidizing bacteria. *Bioresource Technol.* **101**, 3958–3963. (doi:10.1016/j.biortech.2010.01.030)
38 Bock, E., Schmidt, I., Stüven, R. & 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. (doi:10.1007/BF00262198)
39 Poth, M. & Focht, D. 1985 ^{15}N Kinetic analysis of N_2O production by *Nitrosomonas europaea*: an examination of nitrifier denitrification. *Appl. Environ. Microbiol.* **49**, 1134–1141.
40 Ritchie, G. A. F. & Nicholas, D. J. D. 1972 Identification of the sources of nitrous oxide produced by oxidative and reductive processes in *Nitrosomonas europaea*. *Biochem. J.* **126**, 1181–1191.
41 Goreau, T. J., Kaplan, W. A., Wofsy, S. C., McElroy, M. B., Valois, F. W. & Watson, S. W. 1980 Production of NO_2^- and N_2O by nitrifying bacteria at reduced concentrations of oxygen. *Appl. Environ. Microbiol.* **40**, 526–532.
42 Tallec, G., Garnier, J., Billen, G. & Gousailles, M. 2006 Nitrous oxide emissions from secondary activated sludge in nitrifying conditions of urban wastewater treatment plants: effect of oxygenation level. *Water Res.* **40**, 2972–2980. (doi:10.1016/j.watres.2006.05.037)
43 Suzuki, I., Dular, U. & Kwok, S. C. 1974 Ammonia or ammonium ion as substrate for oxidation by *Nitrosomonas europaea* cells and extracts. *J. Bacteriol.* **120**, 556–558.
44 Andersson, K. K. & Hooper, A. B. 1983 O_2 and H_2O are each the source of one O in NO_2^- produced from NH_3 by *Nitrosomonas*: ^{15}N-NMR evidence. *FEBS Lett.* **164**, 236–240. (doi:10.1016/0014-5793(83)80292-0)
45 Igarashi, N., Moriyama, H., Fujiwara, T., Fukumori, Y. & Tanaka, N. 1997 The 2.8 A structure of hydroxylamine oxidoreductase from a nitrifying chemoautotrophic bacterium, *Nitrosomonas europaea*. *Nat. Struct. Mol. Biol.* **4**, 276–284. (doi:10.1038/nsb0497-276)
46 Poughon, L., Dussap, C. G. & Gros, J. B. 2001 Energy model and metabolic flux analysis for autotrophic nitrifiers. *Biotechnol. Bioeng.* **72**, 416–433. (doi:10.1002/1097-0290(20000220)72:4<416::AID-BIT1004>3.0.CO;2-D)
47 Bock, E., Koop, H. P., Harms, H. & Ahlers, B. 1991 The biochemistry of nitrifying organisms. In *Variations in autotrophic life* (eds J. M. Shively & L. L. Barton), pp. 171–199. New York, NY: Academic Press.
48 Yu, R., Kampschreur, M. J., Loosdrecht, M. C. M. V. & Chandran, K. 2010 Molecular mechanisms and specific directionality of autotrophic nitrous oxide and nitric oxide generation during transient anoxia. *Environ. Sci. Technol.* **44**, 1313–1319. (doi:10.1021/es902794a)
49 Law, Y., lant, P. & Yuan, Z. 2012 The effect of pH on N_2O production under aerobic conditions in a partial nitration system. *Water Res.* **45**, 5934–5944. (doi:10.1016/j.watres.2011.08.055)
50 Law, Y., Ni, B.-J., lant, P. & Yuan, Z. Submitted. Nitrous oxide (N_2O) production rate of an enriched culture of ammonia oxidising bacteria exponentially correlated to its ammonia oxidation rate.
51 Arp, D., Chain, P. & Klotz, M. 2007 The impact of genome analyses on our understanding of ammonia-oxidizing bacteria. *Annu. Rev. Microbiol.* **61**, 503–528. (doi:10.1146/annurev.micro.61.080706.093449)
52 Upadhyay, A. K., Hooper, A. B. & Hendrich, M. P. 2006 NO reductase activity of the tetraheme cytochrome *c*554 of *Nitrosomonas europaea*. *J. Am. Chem. Soc.* **128**, 4330–4337. (doi:10.1021/ja055183+)
53 Stein, L. 2011 Surveying N_2O-producing pathways in bacteria. *Methods Enzymol.* **486**, 131–152. (doi:10.1016/B978-0-12-381294-0.00006-7)
54 Schmidt, I. & Jetten, M. S. M. 2004 Anaerobic oxidation of inorganic nitrogen compounds. In *Strict and facultative anaerobes: medical and environmental aspects* (eds M. M. Nakano & P. Zube), pp. 283–303. Berlin, Germany/UK: Springer/Horizon Scientific Press.
55 Schmidt, I. & Bock, E. 1998 Anaerobic ammonia oxidation by cell-free extracts of *Nitrosomonas eutropha*. *Antonie Van Leeuwenhoek* **73**, 271–278. (doi:10.1023/A:1001572121053)
56 Schmidt, I., Zart, D. & Bock, E. 2001 Gaseous NO_2 as a regulator for ammonia oxidation of *Nitrosomonas eutropha*. *Antonie Van Leeuwenhoek Int. J. Gen. Mol. Microbiol.* **79**, 311–318. (doi:10.1023/A:1012038314206)
57 Schmidt, I., Enrique, C. & Lester, P. 2008 Nitric oxide: interaction with the ammonia monooxygenase and regulation of metabolic activities in ammonia oxidizers. *Methods Enzymol.* **440**, 121–135. (doi:10.1016/S0076-6879(07)00807-5)
58 Von Schulthess, R., Wild, D. & Gujer, W. 1994 Nitric and nitrous oxide from denitrifying activated sludge at low oxygen concentrations. *Water Sci. Technol.* **30**, 123–132.
59 Wicht, H. 1996 A model for predicting nitrous oxide production during denitrification in activated sludge. *Water Sci. Technol.* **34**, 99–106.
60 Holtan-Hartwig, L., Dörsch, P. & Bakken, L. R. 2000 Comparison of denitrifying communities in organic soils: kinetics of NO_3^- and N_2O reduction. *Soil Biol. Biochem.* **32**, 833–843. (doi:10.1016/S0038-0717(99)00213-8)
61 Brettar, I. & Hofle, M. G. 1993 Nitrous oxide producing heterotrophic bacteria from a water column of the central Baltic: abundance and molecular identification. *Mar. Ecol. Prog. Ser.* **94**, 253–265. (doi:10.3354/meps094253)
62 Richardson, D., Felgate, H., Watmough, N., Thomson, A. & Baggs, E. 2009 Mitigating release of the potent greenhouse gas N_2O from the nitrogen cycle—could enzymic regulation hold the key. *Trends Biotechnol.* **27**, 388–397. (doi:10.1016/j.tibtech.2009.03.009)
63 Kampschreur, M. J., Temmink, H., Kleerebezem, R., Jetten, M. S. M. & van Loosdrecht, M. C. M. 2009 Nitrous oxide emission during wastewater treatment. *Water Res.* **43**, 4093–4103. (doi:10.1016/j.watres.2009.03.001)
64 Weiss, R. F. & Price, B. A. 1980 Nitrous oxide solubility in water and seawater. *Mar. Chem.* **8**, 347–359. (doi:10.1016/0304-4203(80)90024-9)
65 Dean, J. A. 1992 *Lange's handbook of chemistry.* New York: McGraw-Hill, Inc.
66 Ahn, J. H., Kim, S., Park, H., Rahm, B., Pagilla, K. & Chandran, K. 2010 N_2O Emissions from activated sludge processes, 2008–2009: results of a national monitoring survey in the United States. *Environ. Sci. Technol.* **44**, 4505–4511. (doi:10.1021/es903845y)
67 Foley, J., de Haas, D., Yuan, Z. & Lant, P. 2009 Nitrous oxide generation in full-scale biological nutrient removal wastewater treatment plants. *Water Res.* **44**, 831–844. (doi:10.1016/j.watres.2009.10.033)
68 Kampschreur, M. J., Tan, N. C. G., Kleerebezem, R., Picioreanu, C., Jetten, M. S. M. & van Loosdrecht, M. C. M. 2008 Effect of dynamic process conditions on nitrogen oxides emission from a nitrifying culture. *Environ. Sci. Technol.* **42**, 429–435. (doi:10.1021/es071667p)
69 Kester, R. A., De Boer, W. & Laanbroek, H. J. 1997 Production of NO and N_2O by pure cultures of nitrifying and denitrifying bacteria during changes in aeration. *Appl. Environ. Microbiol.* **63**, 3872–3877.
70 Yu, R., Kampschreur, M. J., Loosdrecht, M. C. M. V. & Chandran, K. 2010 Mechanisms and specific directionality of autotrophic nitrous oxide and nitric oxide generation during transient anoxia. *Environ. Sci. Technol.* **44**, 1313–1319. (doi:10.1021/es902794a)

71 Zheng, H., Hanaki, K. & Matsuo, T. 1994 Production of nitrous oxide gas during nitrification of wastewater. *Water Sci. Technol.* **30**, 133–141.

72 Chuang, H.-P., Ohashi, A., Imachi, H., Tandukar, M. & Harada, H. 2007 Effective partial nitrification to nitrite by down-flow hanging sponge reactor under limited oxygen condition. *Water Res.* **41**, 295–302. (doi:10.1016/j.watres.2006.10.019)

73 Yu, R. & Chandran, K. 2010 Strategies of *Nitrosomonas europaea* 19718 to counter low dissolved oxygen and high nitrite concentrations. *BMC Microbiol.* **10**, 70. (doi:10.1186/1471-2180-10-70)

74 Tallec, G., Garnier, J., Billen, G. & Gousailles, M. 2008 Nitrous oxide emissions from denitrifying activated sludge of urban wastewater treatment plants, under anoxia and low oxygenation. *Bioresour. Technol.* **99**, 2200–2209. (doi:10.1016/j.biortech.2007.05.025)

75 Hynes, R. K. & Knowles, R. 1984 Production of nitrous oxide by *Nitrosomonas europaea*: effects of acetylene, pH, and oxygen. *Can. J. Microbiol.* **30**, 1397–1404. (doi:10.1139/m84-222)

76 Shiskowski, D. M. & Mavinic, D. S. 2006 The influence of nitrite and pH (nitrous acid) on aerobic-phase, autotrophic N_2O generation in a wastewater treatment bioreactor. *J. Environ. Eng. Sci.* **5**, 273–283. (doi:10.1139/s05-034)

77 Kampschreur, M. J., van der Star, W. R. L., Wielders, H. A., Mulder, J. W., Jetten, M. S. M. & van Loosdrecht, M. C. M. 2008 Dynamics of nitric oxide and nitrous oxide emission during full-scale reject water treatment. *Water Res.* **42**, 812–826. (doi:10.1016/j.watres.2007.08.022)

78 Itokawa, H., Hanaki, K. & Matsuo, T. 2001 Nitrous oxide production in high-loading biological nitrogen removal process under low COD/N ratio condition. *Water Res.* **35**, 657–664. (doi:10.1016/S0043-1354(00)00309-2)

79 Betlach, M. R. & Tiedje, J. M. 1981 Kinetic explanation for accumulation of nitrite, nitric oxide, and nitrous oxide during bacterial denitrification. *Appl. Environ. Microbiol.* **42**, 1074–1084.

80 Schulthess, R. V., Kühni, M. & Gujer, W. 1995 Release of nitric and nitrous oxides from denitrifying activated sludge. *Water Res.* **29**, 215–226. (doi:10.1016/0043-1354(94)E0108-I)

81 Zhou, Y., Pijuan, M., Zeng, R. J. & Yuan, Z. 2008 Free nitrous acid inhibition on nitrous oxide reduction by a denitrifying-enhanced biological phosphorus removal sludge. *Environ. Sci. Technol.* **42**, 8260–8265. (doi:10.1021/es800650j)

82 Ghosh, S., Gorelsky, S. I., George, S. D., Chan, J. M., Cabrito, I., Dooley, D. M., Moura, J. J., Moura, I. & Solomon, E. I. 2007 Spectroscopic, computational, and kinetic studies of the μ4-sulfide-bridged tetranuclear CuZ cluster in N_2O reductase: pH effect on the edge ligand and its contribution to reactivity. *J. Am. Chem. Soc.* **129**, 3955–3965. (doi:10.1021/ja068059e)

83 Chung, Y. C. & Chung, M. S. 2000 BNP test to evaluate the influence of C/N ratio on N_2O production in biological denitrification. *Water Sci. Technol.* **42**, 23–27.

84 Schalk-Otte, S., Seviour, R. J., Kuenen, J. G. & Jetten, M. S. M. 2000 Nitrous oxide (N_2O) production by *Alcaligenes faecalis* during feast and famine regimes. *Water Res.* **34**, 2080–2088. (doi:10.1016/S0043-1354(99)00374-7)

85 Kishida, N., Kim, J. H., Kimochi, Y., Nishimura, O., Sasaki, S. & Sudo, R. 2004 Effect of C/N ratio on nitrous oxide emission from swine wastewater treatment process. *Water Sci. Technol.* **49**, 359–371.

86 Knowles, R. 1982 Denitrification. *Microbiol. Rev.* **46**, 43–70.

87 Christensson, M., Lie, E. & Welander, T. 1994 A comparison between ethanol and methanol as carbon sources for denitrification. *Water Sci. Technol.* **30**, 83–90.

88 Hallin, S. & Pell, M. 1998 Metabolic properties of denitrifying bacteria adapting to methanol and ethanol in activated sludge. *Water Res.* **32**, 13–18. (doi:10.1016/S0043-1354(97)00199-1)

89 Hanaki, K., Hong, Z. & Matsuo, T. 1992 Production of nitrous oxide gas during denitrification of wastewater. *Water Sci. Technol.* **26**, 1027–1036.

90 Park, K. Y., Inamori, Y., Mizuochi, M. & Ahn, K. H. 2000 Emission and control of nitrous oxide from a biological wastewater treatment system with intermittent aeration. *J. Biosci. Bioeng.* **90**, 247–252.

91 Lemaire, R., Meyer, R., Taske, A., Crocetti, G. R., Keller, J. & Yuan, Z. 2006 Identifying causes for N_2O accumulation in a lab-scale sequencing batch reactor performing simultaneous nitrification, denitrification and phosphorus removal. *J. Biotechnol.* **122**, 62–72. (doi:10.1016/j.jbiotec.2005.08.024)

92 Zeng, R. J., Yuan, Z. & Keller, J. 2003 Enrichment of denitrifying glycogen-accumulating organisms in anaerobic/anoxic activated sludge system. *Biotechnol. Bioeng.* **81**, 397–404. (doi:10.1002/bit.10484)

93 Murnleitner, E., Kuba, T., Loosdrecht, M. C. M. V. & Heijnen, J. J. 1997 An integrated metabolic model for the aerobic and denitrifying biological phosphorus removal. *Biotechnol. Bioeng.* **54**, 434–450. (doi:10.1002/(SICI)1097-0290(19970605)54:5<434::AID-BIT4>3.0.CO;2-F)

94 Tabrez Khan, S. & Hiraishi, A. 2001 Isolation and characterization of a new poly(3-hydroxybutyrate)-degrading, denitrifying bacterium from activated sludge. *FEMS Microbiol. Lett.* **205**, 253–257. (doi:10.1111/j.1574-6968.2001.tb10957.x)

95 Matsubara, T., Frunzke, K. & Zumft, W. 1982 Modulation by copper of the products of nitrite respiration in *Pseudomonas perfectomarinus*. *J. Bacteriol.* **149**, 816.

96 Granger, J. & Ward, B. B. 2003 Accumulation of nitrogen oxides in copper-limited cultures of denitrifying bacteria. *Limnol. Oceanogr.* **48**, 313–318. (doi:10.4319/lo.2003.48.1.0313)

97 Zhu, X. & Chen, Y. 2011 Reduction of N_2O and NO generation in anaerobic–aerobic (low dissolved oxygen) biological wastewater treatment process by using sludge alkaline fermentation liquid. *Environ. Sci. Technol.* **45**, 2137–2143. (doi:10.1021/es102900h)

98 Sinha, B. & Annachhatre, A. 2007 Partial nitrification—operational parameters and microorganisms involved. *Rev. Environ. Sci. Biotechnol.* **6**, 285–313. (doi:10.1007/s11157-006-9116-x)

99 Casey, T. G., Wentzel, M. C. & Ekama, G. A. 1999 Filamentous organism bulking in nutrient removal activated sludge systems. Paper 9: Review of biochemistry of heterotrophic respiratory metabolism. *Water S.A.* **25**, 409–424.

100 Casey, T. G., Wentzel, M. C. & Ekama, G. A. 1999 Filamentous organism bulking in nutrient removal activated sludge systems. Paper 10: Metabolic behaviour of heterotrophic facultative aerobic organisms under aerated/unaerated conditions. *Water S.A.* **25**, 425–442.

101 Pellicer-Nàcher, C., Sun, S., Lackner, S., Terada, A., Schreiber, F., Zhou, Q. & Smets, B. F. 2010 Sequential aeration of membrane-aerated biofilm reactors for high-rate autotrophic nitrogen removal: experimental demonstration. *Environ. Sci. Technol.* **44**, 7628–7634. (doi:10.1021/es1013467)