BACTERIAL CIRCUITS OF NITROUS OXIDE PRODUCTION CHARACTERIZED USING METABOLIC NETWORK MODELLING AND METABOLOMICS

O. Perez-Garcia and N. Singhal
Department of Civil and Environmental Engineering, The University of Auckland, New Zealand

ABSTRACT

Metabolic network modelling and metabolomics are computational and analytical techniques used to characterize the flow of compounds and energy within metabolic pathways of microbes. This paper illustrates the application of such techniques to explain how different environmental conditions of biological nitrogen removal (BNR) processes trigger the production and emission of nitrous oxide (N_2O) - a greenhouse gas and ozone depletion substance - by nitrifying and denitrifying microbes.

The research approach is exemplified by analysing N_2O production in laboratory scale BNR systems by: (i) pure nitrifying species and (ii) mixed nitrifying cultures. The pure cultures (Nitrosomonas europaea) simulations shows that N_2O is produced due to electron flow imbalances in nitrifying cells, and that electron carriers play a key role by distributing electron equivalents to N_2O and NO formation reactions. The mixed culture simulations reveal two key aspects of N_2O formation in nitrifying microbial communities: (i) microbes can lower N_2O emissions by dissipating NO (a N_2O precursor molecule); and (ii) the structure (i.e. the richness and abundance of species) of the microbial community influences the amount of N_2O produced and emitted.

This study concludes that operational conditions that promote imbalances between the cell's electron donors and electron acceptors cause N_2O formation. Specifically, in nitrification processes, a build-up of electron donors leads to N_2O formation. This paper demonstrates the unique features of metabolic modelling procedures and metabolomics by applying these to obtain insight into microbial functioning in wastewater treatment processes.

KEYWORDS

Nitrous oxide, biological nitrogen removal, metabolic network modelling, metabolomics

1 INTRODUCTION

Nitrous oxide (N_2O) is an atmospheric trace gas that influences the atmosphere's chemistry and the greenhouse gas effect. Biological and chemical processes naturally produce N_2O on the Earth's surface (U.S. EPA, 2010). However, anthropogenic activities over the last 100 years have increased the amount of atmospheric N_2O concentrations by 20%, from 270ppbv to more than 324ppbv (IPCC, 2001; IPCC, 2013), contributing to two negative impacts on the atmosphere (Wuebbles, 2009): a) the greenhouse effect and b) the ozone (O_3) depletion effect. The capacity of one molecule of N_2O to trap heat (i.e. infrared radioactive forcing) is 206 times that of one carbon dioxide (CO_2) molecule (IPCC, 2001). This, together with the long atmospheric lifetime of N_2O (118±25 years), results in the global warming potential of N_2O being 310 times higher than that of CO_2 on a per molecule basis (Schreiber et al., 2012; U.S. EPA, 2014). Aside from its greenhouse gas effect, N_2O has been categorized by the International Panel for Climate Change (IPCC) as the "dominant ozone-depleting substance emitted in the 21st Century" (Ravishankara et al., 2009). Because of this, monitoring and reduction of N_2O emissions are now part of the legally binding commitments entered into by the governments of many countries, including New Zealand, through international agreements like the Kyoto Protocol (N.Z. MfE, 2013) within the United Nations Framework Convention on Climate Change (UNFCCC).

Nitrous oxide (N_2O) can be produced and directly emitted from wastewater treatment plants (WWTP) (Ahn et al., 2010; Foley et al., 2010; Kampschreur et al., 2009). N_2O in particular is produced during biological nitrogen removal (BNR), where nitrification and denitrification processes are used to reduce excessive nitrogen concentration in wastewater (Foley et al., 2010; Wunderlin et al., 2012). The amount of N_2O emitted from BNR reactors in wastewater treatment is highly variable. N_2O emission fraction in full scale WWTP ranges between 0 to 25% of the influent N-load, according to the IPCC (Kampschreur et al., 2009). The amount of emission thus expressed is the percentage of nitrogen load in a BNR system, emitted as N_2O. For example, a BNR system with N-load of 50 mg-N/L emitting 1% of N-load as N_2O is releasing 0.5 mg-N of N_2O per litre treated into the atmosphere. Full scale BNR systems have a lower N-load emission percentage (ranging from 0 to 25%) than lab scale systems (ranging from 0 to 95%) (Kampschreur et al., 2009; Rassamee et al., 2011). Extensive measurements from BNR bioreactors in the United States and Australia WWTP indicate an average of between 0.03% and 1.8% of N-load emitted as N_2O (Ahn et al., 2010; Foley et al., 2010). These values may appear low, but emissions as low as 0.5-1% of oxidized nitrogen are of a similar magnitude to greenhouse gases emitted during energy production for aeration (Ni et al., 2013a), which are now acknowledged by water utilities as significant contributors to the carbon footprint of wastewater treatment plants (Ni et al., 2013a).

Nitrifying and denitrifying microbes are the sources N_2O emissions in BNR, specifically autotrophic ammonia oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB) and heterotrophic denitrifiers organism (DEN) (Schreiber et al., 2012; Stein, 2010; U.S. EPA, 2010). The microbial metabolic pathways that lead to the production of N_2O during nitrification and denitrification in AOB, NOB and DEN are summarized in *Figure 1*. Catalytic enzymes known to mediate the reaction from one nitrogenous compound to another are indicated was follows: AMO, ammonia monooxygenase; HAO, hydroxylamine oxidoreductase; NXR, nitrite oxidoreductase; NAR, membrane bound nitrite reductase; NirK, copper containing nitrite reductase; NirS, cytochrome cd_1 nitrite reductase; cNor, nitric oxide reductase that accept electrons from c-type cytochromes; qNor, nitric oxide reductase that accept electrons from ubiquinols; Nos, nitrous oxide reductase; C_{P460}, cytochrome P460; C_{c554}, cytochrome c554; Hmp, flavohemoglobins; (Hooper et al., 1997; Schreiber et al., 2012; Stein, 2010; Stein, 2011; Whittaker et al., 2000). The four main N_2O production pathways known to occur in biological nitrifying and denitrifying microbes are listed in *Table 1* (Stein, 2011; Wunderlin et al., 2012).Such pathways are i) Aerobic hydroxylamine mediated pathway by AOB; ii) Dissimilatory nitrite reduction pathway in AOB; iii) Dissimilatory nitrite reduction pathway in DEN; and iv) NO detoxification pathways in AOB, NOB and DEN.

The most important environmental factors influencing N_2O emissions in BNR nitrification and denitrification processes are dissolved oxygen, nitrite, ammonium and organic carbon concentrations. Low dissolved oxygen and high nitrite accumulation are in effect environmental conditions that trigger N_2O emission during nitrification, Similarly, a low carbon to nitrogen ratio (COD/N), increased nitrite concentration and high dissolved oxygen are operational parameters leading to N_2O emission during denitrification (Kampschreur et al., 2008; Kampschreur et al., 2009; Rassamee et al., 2011). Quantification of N_2O producing pathway activity in regard to specific operational parameters of BNR has been done only in a few studies (Ahn et al., 2011; Pan et al., 2013a; Wunderlin et al., 2013; Yu et al., 2010). It follows therefore that quantitative relationships between N_2O emission, production pathway rates and BNR operational parameters are necessary to be able to define guidelines for emission prevention (Kampschreur et al., 2009).Consequently, the aim of this research is to

establish the relationships between the activity of N_2O production pathways and environmental conditions (e.g. electron donor and acceptor availability) in BNR bioreactors by using stoichiometric metabolic network (SMN) modelling and metabolite profiling of nitrifying and denitrifying microbes. The research approach is exemplified by analysing N_2O production in laboratory scale BNR systems by: (i) pure nitrifying species and (ii) mixed nitrifying cultures.

Figure 1. Microbial metabolic pathways for NO and N_2O production in nitrification and denitrification processes

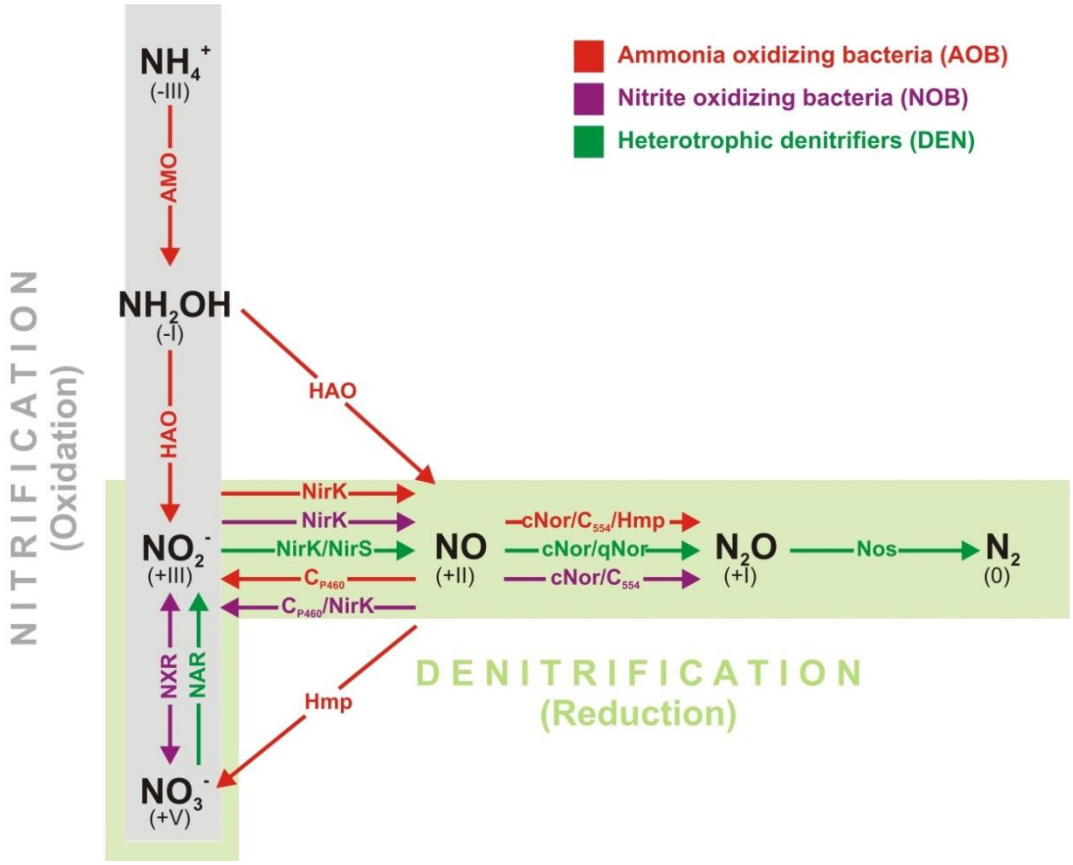
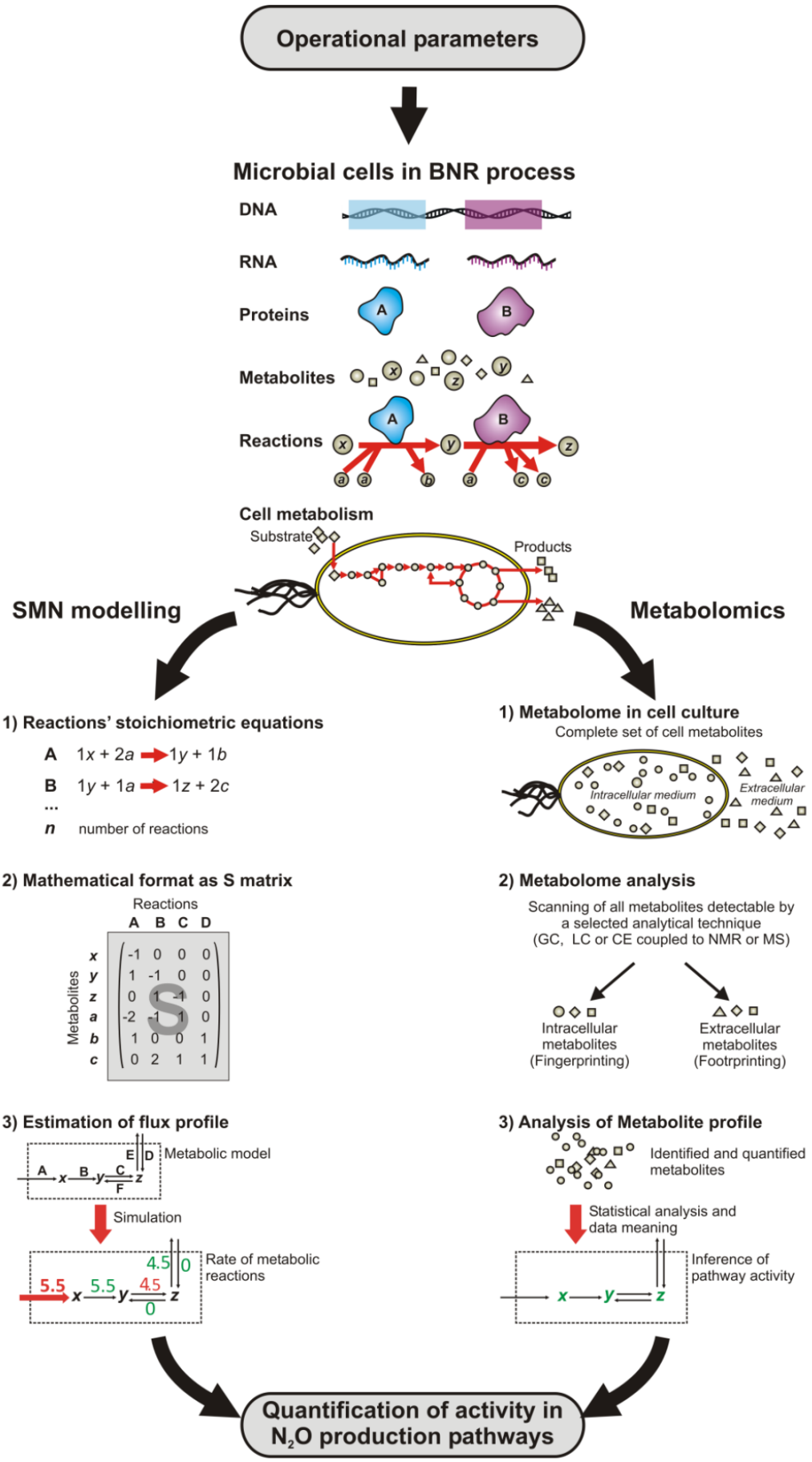


Table 1. The four main N_2O production pathways in nitrifying and denitrifying microbes

Pathway name	Key mediating enzyme(s)
Aerobic hydroxylamine oxidation pathway in ammonia oxidizers (also known as HAO mediated NO production pathway)	Hydroxylamine oxidoreductase (HAO) and nitric oxide reductase (cNor)
Dissimilatory nitrite reduction pathway in ammonia oxidizers (also known as nitrifier denitrification pathway)	Nitrite reductase (NirK) and nitric oxide reductase (cNor)
Dissimilatory nitrite reduction pathway in denitrifiers (also known as incomplete denitrification)	Nitrite reductases (NirK and NirS) and nitric oxide reductases (cNor and qNor)
NO detoxification pathway	Cytochromes (C_{554} and C_{P460}) and flavohemoglobins (Hmp)

Figure 2. Linking operational parameters in BNR process to activity of microbial N_2O production pathway through SMN modelling and Metabolomics



Characterization of microbial metabolism in BNR processes through SMN modelling and metabolomics is an ideal way to establish quantitative relationships between observed N_2O emission, reaction rates of microbial N_2O production pathways and BNR operational parameters. By fitting SMN models to data from N_2O producing-BNR bioreactors is possible to develop quantitative hypothesis of the actual molecular mechanisms occurring in nitrifying and denitrifying cells when producing N_2O. Then those hypotheses can be experimentally validated so that the generated knowledge used to design operational guidelines of BNR reactors in order to avoid N_2O emissions. As shown in *Figure 2*, SMN modelling is a computational method within the Bioinformatics and Systems Biology disciplines that uses stoichiometric equations for biochemical reactions taking place in a target organism (or microbial community) to reconstruct a pathway level - or even full cell level - metabolic model (Feist et al., 2009; Thiele and Palsson, 2010). This model can be used to estimate metabolic reaction rates (also called fluxes) simultaneously occurring in cells under a given environmental condition (Becker et al., 2007; Feist et al., 2009). Metabolomics, on the other hand, is an analytical technology that aims to identify and quantify the set of metabolites present in a biological sample from an organism grown under defined conditions (Villas-Bôas et al., 2005; Weckwerth and Morgenthal, 2005) (See *Figure 2*). Flux and metabolite profiles of cell cultures (such as those of BNR processes) provide the most physiologically relevant description of a cells' metabolism, because they represent the final functional output of interactions of all the genetic, transcriptional, protein, enzymatic and signalling activities within the cells (Cascante and Marin, 2008; Chubukov et al., 2014; Kohlstedt et al., 2010; Lee et al., 2006).

2 N_2O PRODUCTION PATHWAYS IN PURE AOB CULTURES

2.1 OVERVIEW

AOB produce NO and N_2O during nitrification and autotrophic denitrification in wastewater treatment BNR processes (Ahn et al., 2010; Foley et al., 2010; Kampschreur et al., 2009). The operational conditions of wastewater treatment processes that lead to NO and N_2O production by AOB are related to changes in the concentration of electron donors (NH_4^+ and NH_2OH) and acceptors (O_2 and NO_2^-) (Chandran et al., 2011; Kampschreur et al., 2008). However, despite the availability of extensive information on nitrogen respiration and energy production in AOB, the metabolic triggers and modulatory mechanisms controlling NO and N_2O production are not well understood.

NO/N_2O production in AOB occurs via two pathways (Cabail and Pacheco, 2003; Stein, 2010; Stein, 2011; Wunderlin et al., 2012) (*Figure 1*): (i) The aerobic hydroxylamine oxidation pathway mediated by hydroxylamine oxidoreductase (HAO); and (ii) The nitrifier denitrification pathway mediated by nitrite reductase (NirK) and nitric oxide reductase (cNor) enzymes From pure cultures of *Nitrosomonas europaea*, a model AOB species constantly and abundantly detected in full scale nitrification processes (Wagner et al., 2002), it is known that these two pathways are part of the nitrogen respiration, electron transport chain, and energy generation mechanism of AOB ((Hooper et al., 1997; Whittaker et al., 2000; Yu et al., 2010).

Activated Sludge (ASM) models have been modified to dynamically predict NO and N_2O production under different environmental conditions by linking NO and N_2O production to the respiratory activity and the responsible metabolic pathway (Kampschreur et al., 2007; Ni et al., 2011; Ni et al., 2013b; Pan et al., 2013b; Yu et al., 2010). This approach however does not clarify why these gases are produced, as NO and N_2O production has largely been described as being decoupled from the cell's energy metabolism. In this study, we constructed a SMN model based on *Nitrosomonas europaea* energy production metabolism and enzymology, and used it in combination with Flux Balance Analysis (FBA) to quantify the metabolic rates of NO and N_2O production pathways during oxic-anoxic-oxic transitions of *N. europaea* cultures. The obtained metabolic rates were used to infer the physiological mechanisms responsible for the modulation of pathways leading the production of these gases.

2.2 METHODS

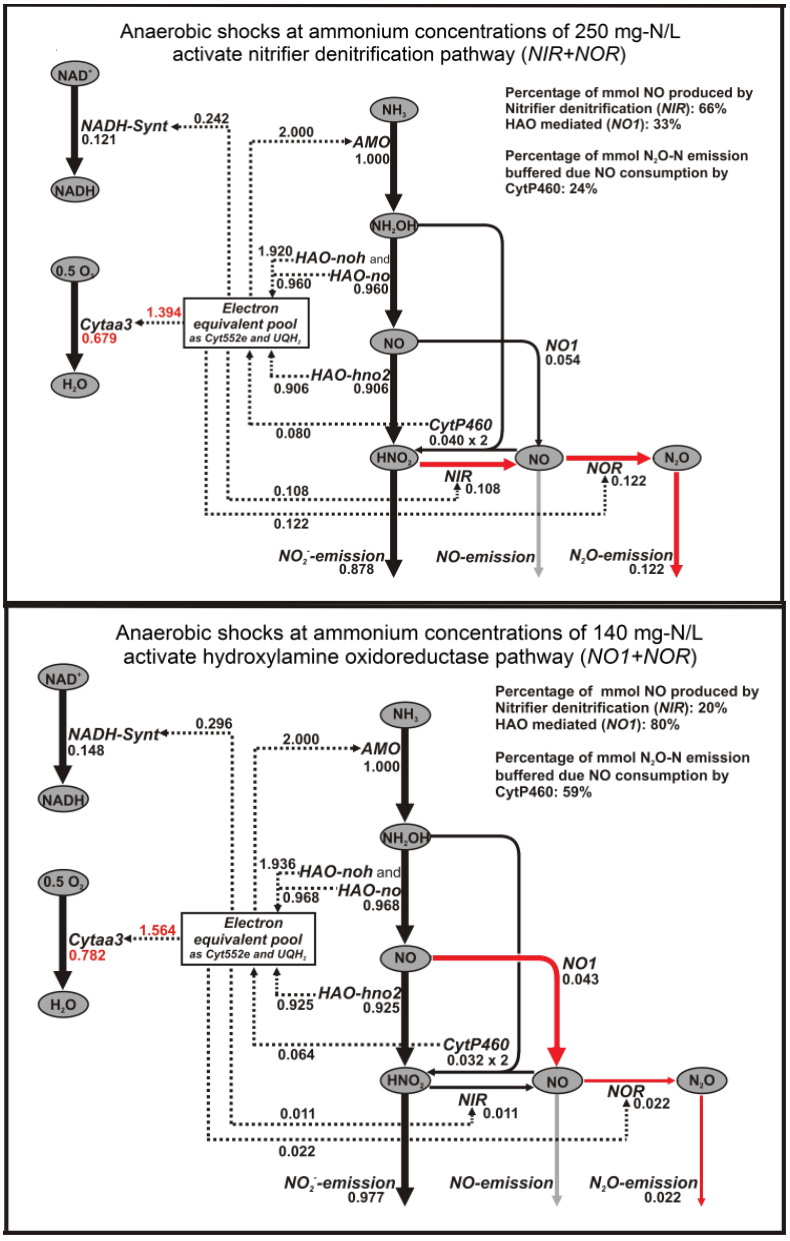
A metabolic network model for biochemical reactions and metabolites formed during *N. europaea* energy production metabolism was constructed by following the procedure described by (Thiele and Palsson, 2010) using organism-specific genomic and biochemical information from literature and the metabolic pathway databases KEGG and MetaCyc (respectively accessible at http://www.genome.jp/kegg/ and http://metacyc.org/). Flux balance analysis (FBA) was applied to estimate the unknown rates of network reactions by using values of consumption rates of substrates (i.e. oxygen and ammonium) as model input. The unknown rates (or fluxes) were found by optimizing with linear programing an objective function (Z) subject to the specified substrate uptake

rates (Becker et al., 2007; Orth et al., 2010; Varma and Palsson, 1994). The obtained metabolic rates were used to infer the physiological mechanisms responsible for the modulation of pathways leading the production of these gases.

2.3 RESULTS

Flux balance analysis (FBA) of *Nitrosomonas europaea* pure cultures revealed that N_2O production and emission occur as an electron sink mechanism in ammonia oxidizing cells. The unlimited availability of electron donors (NH_4^+ concentration = 250 mg-N/L) combined with a lack of electron acceptors (O_2) triggers nitrifier denitrification as an electron sink pathway (*Fig. 3*). When electron donor depletion (NH_4^+ concentration = 140 mg-N/L) is accompanied with a decrease in electron acceptor concentration such that the intracellular electron equivalents generated are not enough to activate nitrifier denitrification pathway, NO/N_2O production through the hydroxylamine oxidoreductase pathway can be expected (*Fig. 3*). In the transition to anoxic conditions, a leak of NO from the HAO-mediated reaction occurs due to limited availability of electron acceptors to completely oxidize NO to HNO_2. The transition from anoxic to oxic conditions results in baseline N_2O production via the hydroxylamine oxidoreductase pathway, but the total amount of N_2O emission is dependent upon activation of the nitrifier denitrification pathway. The FBA of N. europaea metabolism also indicated that NO and N2O emissions are partially mitigated by the NO oxidation to NO_2^- reaction catalysed by cytochrome P460.

Figure 3. Electron flux distribution estimated for N. europaea *metabolism producing N_2O during ammonium oxidation to nitrite*



3 N_2O PRODUCTION IN NITRIFYING MIXED MICROBIAL CULTURES

3.1 OVERVIEW

The contribution of the putative reactions for NO oxidation by AOB and NO reduction by NOB to overall N_2O production and eventual emission from nitrifying mixed cultures has not been investigated. To assess this contribution the rates of redox reactions of the nitrogen respiratory pathways in ammonia oxidizers and nitrite oxidizers need to be quantified.

The objective of this research was to develop and use a multispecies SMN model to evaluate the effect of NO oxidation by AOB and NO reduction by NOB on the amount of N_2O produced in nitrifying processes operated under different ammonium and oxygen concentrations and microbial community structures. This effect was assessed by estimating specific N_2O production rates (sN_2OPR) and metabolic describing variables using different model variants (i.e. including and excluding NO oxidation and NO reduction by NOB reactions and using various microbial community structures) and comparing those estimations to data observed in nine experiments of N_2O production by nitrifying mixed cultures. Model variant that gave a better fit to experimental observations was considered to more accurately represent the mass and energy balance occurring within the microbial community metabolism, therefore providing insights of the effect of NO oxidation; NO reduction by NOB and operational conditions on N_2O production during nitrification.

3.2 METHODS

The nine analysed experiments were reported on publications by Ahn et al., (2011); Law et al., (2012); Wunderlin et al., (2013) and corresponded to one following nitrification processes: i) ammonium oxidation to nitrate by AOB and NOB (full nitrification); ii) ammonium oxidation to nitrite by AOB (nitritation); iii)nitrite oxidation to nitrate by NOB (nitratation) and; iv) hydroxylamine (NH_2OH) oxidation to nitrite and nitrite (NOx) by AOB and NOB (*Fig 4*). The nine analysed experiments (each referred in this research using a letter from A to I) had different operational concentration of ammonium and dissolved oxygen among them, as well as different microbial community structure. The specific oxygen and ammonium uptake rates (sOUR and sAUR respectively) and N_2O production rates (sN_2OPR) observed when the experiments had their highest N_2O productivity where used as model input parameters

These rate values were normalized by the total amount of biomass in bioreactors (expressed as grams of chemical oxygen demand (COD), a standard variable to measure biomass and organic carbon in wastewater treatment). Experiments' nitrifying bacterial community structure is expressed in terms of fraction (f) of species (k) per unit of biomass. f^k values were calculated according to the community composition and species concentrations reported on each experiment publication. The values were calculated only for those AOB and NOB species with highest biomass dry weight percentages on each analysed experiment. The fraction of biomass composed by heterotrophic species was not included on analysis due the null nitrifying activity of these species.

3.2.1 MULTISPECIES METABOLIC NETWORK MODEL

The metabolism of the microbial community detected in the analysed experiments was modelled using a multispecies stoichiometric metabolic network (SMN). The SMN model was formulated to simultaneously capture nitrogen respiration and energy production (ATP and NADH) by AOB and NOB, as well as the exchange of nitrogenous compounds between different AOB and NOB species in a given nitrifying community. Eight microbial species –four AOB and four NOB– were included in the multispecies model to cover the diversity of respiratory redox reactions involving nitrogenous compounds within experiments' nitrifying microbial communities. The species were selected on the basis of: (i) species abundance in experimental nitrifying microbial communities; and (ii) availability of genome data. The four AOB species selected were *Nitrosomonas europaea*, *Nitrosomonas eutropha*, *Nitrosospira multiformis* and *Nitrosococcus oceani*, and the four NOB species selected were *Candidatus* Nitrospira defluvii, *Nitrobacter winogradskyi*, *Nitrobacter hamburgensis* and *Nitrospina gracilis*.

3.3 RESULTS

The reconstruction of eight SMN models (four for AOB and four for NOB species) highlighted that genomes of all the species modelled contain copies of the *nirK*, *cyp*, *hmp* genes, which encode for enzymes with nitric oxide oxidase activity (NO oxidation to NO_2^-), suggesting that this reaction is an important mechanism to deal with NO accumulation in nitrifying microbial communities. Monte Carlo random sampling simulations using the

community model indicated that activation of NO oxidation reactions satisfy the mass and energy balance of nitrifying mixed cultures (Fig 4). The fitness between experiments' observed and model estimated data was measured using mean relative errors (MRE), where a MRE score of one represent a perfect fit. M values indicate the number of variables fitted. Reactions for NO oxidation to NO_2^- were included in the model structure to estimate the experimental values depicted in *Figure 4*. Model variants excluding NO oxidation to NO_2^- had lower fitness (higher lowe MRE scores) than variants including such reactions (data not showed).

Figure 4. Fitness between experimental and estimated datasets for the nine analysed experiments

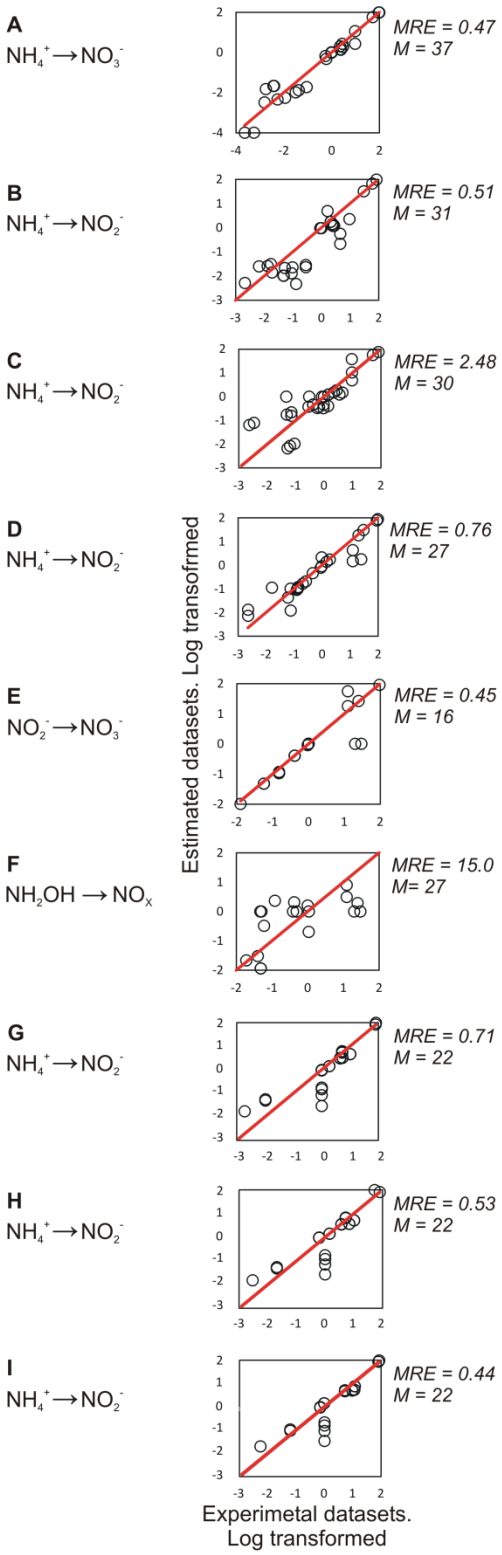
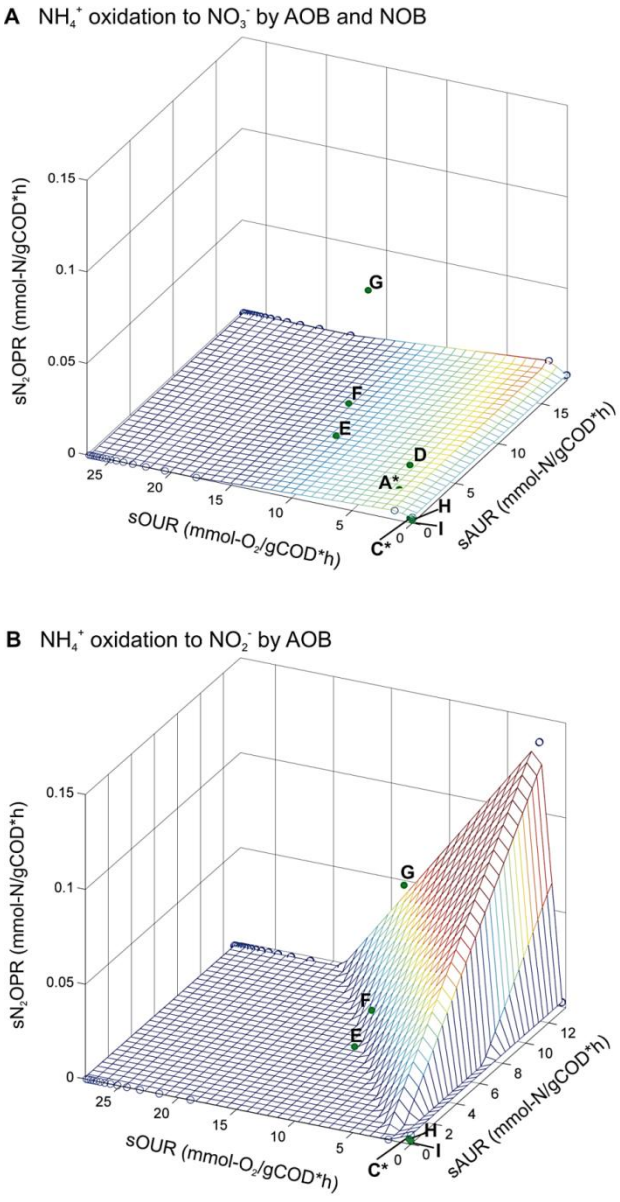


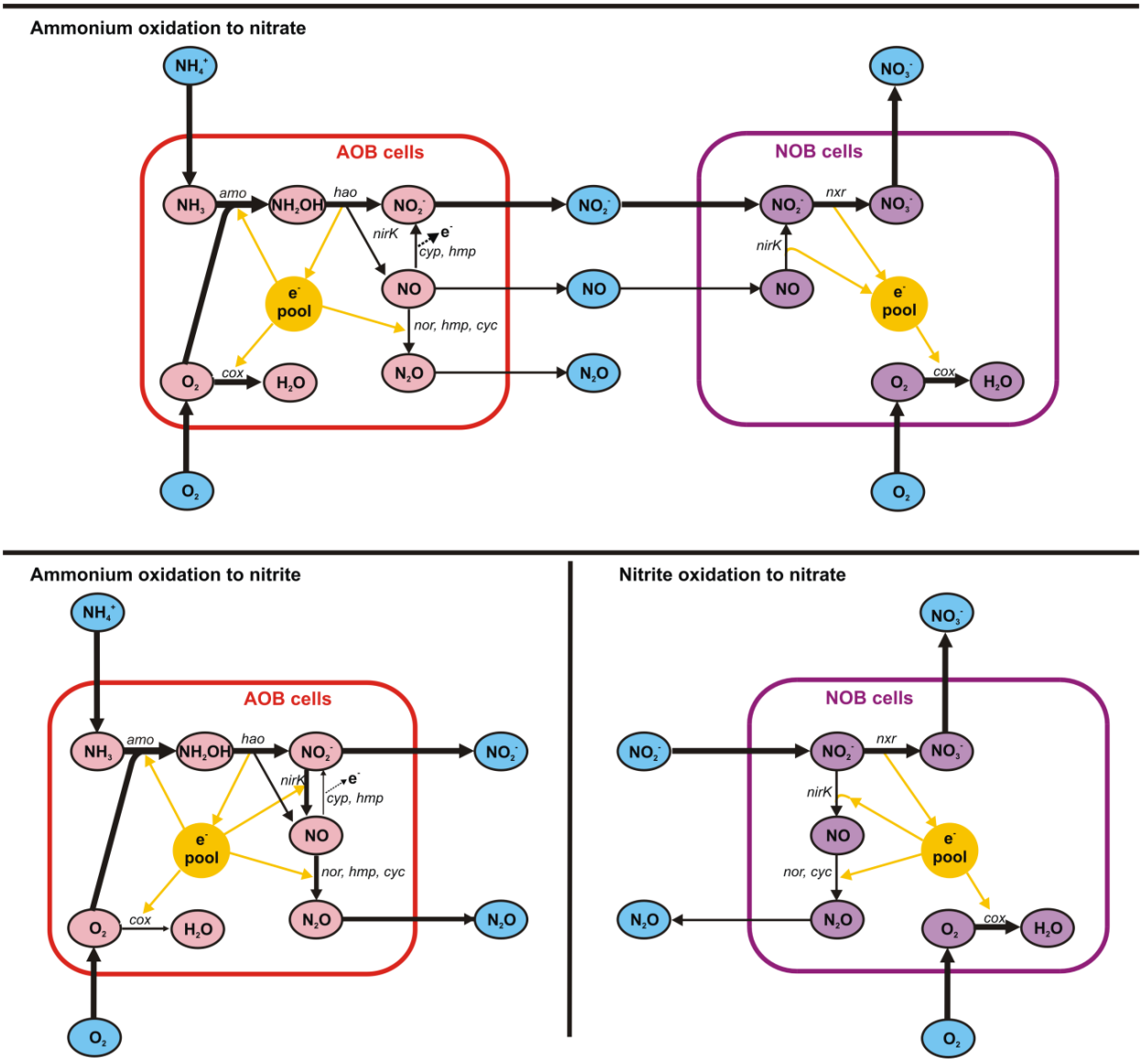
Figure 5. Fitness between experimental and estimated sAUR, sOUR and sN_2OPR



The model was used to define expected sN_2OPR with respect to electron donor and acceptor uptake rates (*Fig. 5*). The range of N_2O production rates estimated for ammonia oxidation to nitrate processes spams from 0 to 0.02 mmol-N/gCOD*h, maximum value being expected only at NH_4^+ concentrations above 35 mg-N/L. In contrast, the range of N_2O production rate estimated for ammonia oxidation to nitrite processes was from 0 to 0.13 mmol-N/gCOD*h, with expected maximum value only at NH_4^+ concentrations above 30 mg-N/L (*Fig.5B*). The N_2O production rate correlates in positive linear fashion to the cell's ammonium uptake rate, in line with the correlations found by (Law et al., 2012). However, the intercept to y axis value of the line formed with this correlation is inversely related to oxygen uptake rates. This means that the oxygen uptake rate affects this correlation by modifying the minimum value of the ammonium uptake rate required before N_2O production begins to be observed. For example, at an oxygen uptake rate of 2 mmol-O_2/gCOD*h, N_2O production starts at an ammonium uptake rate of 0.5 mmol-N/gCOD*h. Whereas, at an oxygen uptake rate of 15 mmol-O_2/gCOD*h, N_2O production starts at an ammonium uptake rate of 10 mmol-N/gCOD*h.

Figure 6 depicts the metabolic mechanism inferred from the results of the fitness analysis. During ammonium oxidation to nitrate, NOB cells have the ability to contribute to the reduction of N_2O formation in full nitrification processes by consuming NO_2^- and NO produced by AOB. In this case, both molecules would be used as electron donors in NOB metabolism. Independently of process operation conditions, AOB and NOB species richness and abundance profiles were found to affect the amount of N_2O produced in a given nitrification process. In other hand, in processes of ammonium oxidation to nitrite, NO oxidation to NO_2^- occurs only in AOB cells; however this reaction is not as relevant as in processes of full ammonium oxidation to nitrate.

Figure 6. Proposed role of reactions catalysed by Cytorchrome P460 (cyp), flavohemoglobins (hmp), and oxidative nitrite oxidoreductase (nitK) in nitrifying processes



4 N_2O ACCUMULATION IN DENITRIFYING MIXED MICROBIAL CULTURES

4.1 OVERVIEW

Microbial heterotrophic denitrification is commonly used during biological nitrogen removal (BNR) from wastewater, where soluble nitrate (NO_3^-) is converted to nitrogen gas (N_2), which results in the removal of nitrogen from wastewater. As an intermediate molecule in the denitrification process, N_2O can accumulate and then be emitted into the atmosphere (Kampschreur et al., 2009). This N_2O production pathway in denitrification is known as the "dissimilatory nitrite reduction pathway in denitrifiers" or "incomplete denitrification". Preliminary estimations suggest that the carbon footprint of a typical biological nitrogen removal wastewater plant would increase by approximately 30% when just 1% of denitrified nitrogen is emitted as N2O (de Hass and Hartley, 2004; Pan et al., 2013b).

The COD/N ratio of growth medium, i.e. the proportion of carbon substrate concentration measured as chemical oxygen demand (COD), to nitrate substrate concentration has been reported to significantly influence N_2O accumulation during denitrification (Hanaki et al., 1992; Kishida et al., 2004; Pan et al., 2013a). Pan, et al., (2013a) showed that low COD/N ratios in growth medium enhance competition for electron equivalents between the four denitrification reductase enzymes, consequently limiting the proportion of electrons distributed to the Nos enzyme, thus promoting N_2O accumulation. Differences in enzyme affinity constants for electrons would be the determining factor of electron distribution (Pan et al., 2013b).

Previous studies that implicate COD/N limitation in N_2O emission inherently assume that the electron affinity of the upstream nitrogen reduction steps is higher than the affinity of N_2O reductase (Nos). In such a case, the upstream nitrogen reductases would be more competitive to accepting the electrons from a given electron donor. However electron competition occurs not only under carbon-limiting but also in carbon-abundant conditions (Lu and Chandran, 2010; Pan et al., 2013a), meaning that the upstream electron supply reactions forming the carbon oxidation and electron transport chain pathways also influence the distribution of electron delivery to the four main denitrification reactions. Intracellular carbon storage compounds (i.e. poly-β-hydroxybutyrate (PHB) and long chain fatty acids) with relatively low biodegradability can be used as electron donors for denitrification (Kampschreur et al., 2009; Schalk-Otte et al., 2000). This indicates that not only the activity of the nitrogen reduction pathways but also the activity of carbon oxidation pathways influences electron distribution and N_2O production during denitrification. For example, Schalk-Otte et al, (2000) observed that as soon as PHB became the growth substrate due to COD limitation, N_2O started to accumulate. Moreover, electron equivalents in cells of common species of denitrifier organisms, like *Paracoccus denitrificans* and *Pseudomonas aeruginosa*, can be supplied to the electron transport chain through different metabolic compounds, i.e. NADH, lactate, succinate, $FADH_2$, glycerol-P and glycolate (Ferguson, 1998; Richardson et al., 2009). These metabolites are an indication as to which carbon oxidation pathways could potentially affect the rate of electron supply into denitrification reactions.

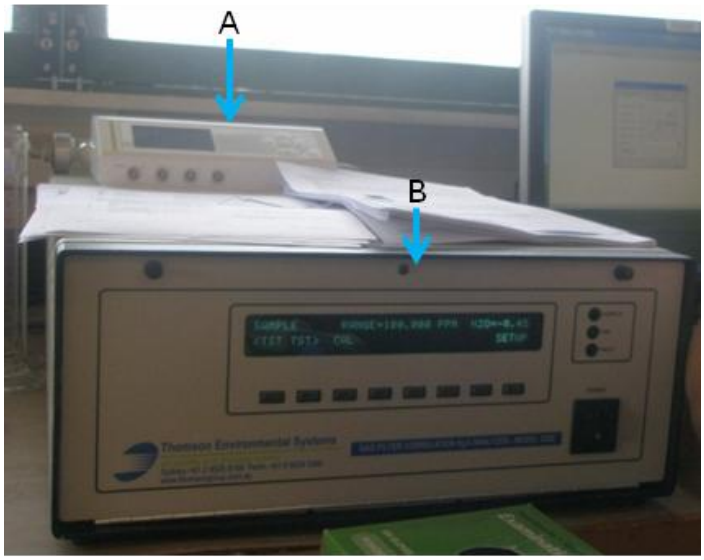
The goal of the present study is to identify the carbon oxidation pathways of denitrifying cultures producing N_2O as a consequence of exposure to the COD/N ratio of different growth media by performing metabolite profiling and metabolic network modeling of biomass in denitrifying batch cultures

4.2 METHODS

4.2.1 DENITRIFICATION BATCH EXPERIMENTS

Denitrifying batch cultures in medium with COD/N ratio of 4 and 11 were grown in two lab-scale bioreactors with a working volume of 3 litres seeded with activated sludge from a domestic wastewater treatment plant in Auckland, New Zealand. The *Photograph 1* shows the denitrifying reactors used for this research; the bioreactor on the left is completely mixed, while the one on the right is sediment sludge. The experimental setup consisted of two identical and independent reactors connected to an influent-effluent hydraulic line and interconnected through a recirculation hydraulic line Reactors were equipped with a tubing system to provide aeration and drag gaseous emissions to the 320E Teledyne N_2O analyser. Mixed liquor NO_2^--N and N_2O-N concentrations were respectively measured in real time with biosensors and Clark-type microsensors connected to a multimeter (Unisense, Aarhus, Denmark). These instruments were connected to a computer for online data recording. Photograph 2 shows the equipment used to online measure liquid (A) and gaseous (in bioreactors' headspace) (B) N_2O concentrations in bioreactors. Each experimental conditions (i.e. cultures with COD/N ratio of 11 or 4) was tested in triplicate by running three identical batch cultures.

Photograph 1. Denitrifying cultures in bioreactors of three litters of working volume.



Photograph 2. NO and N_2O detection equipment: A) Unisense Microsensor Multimeter used to process signal from NO_2^- and N_2O microsensors; B) The 320E Teledyne Infra-Red gas filter N_2O analyser.

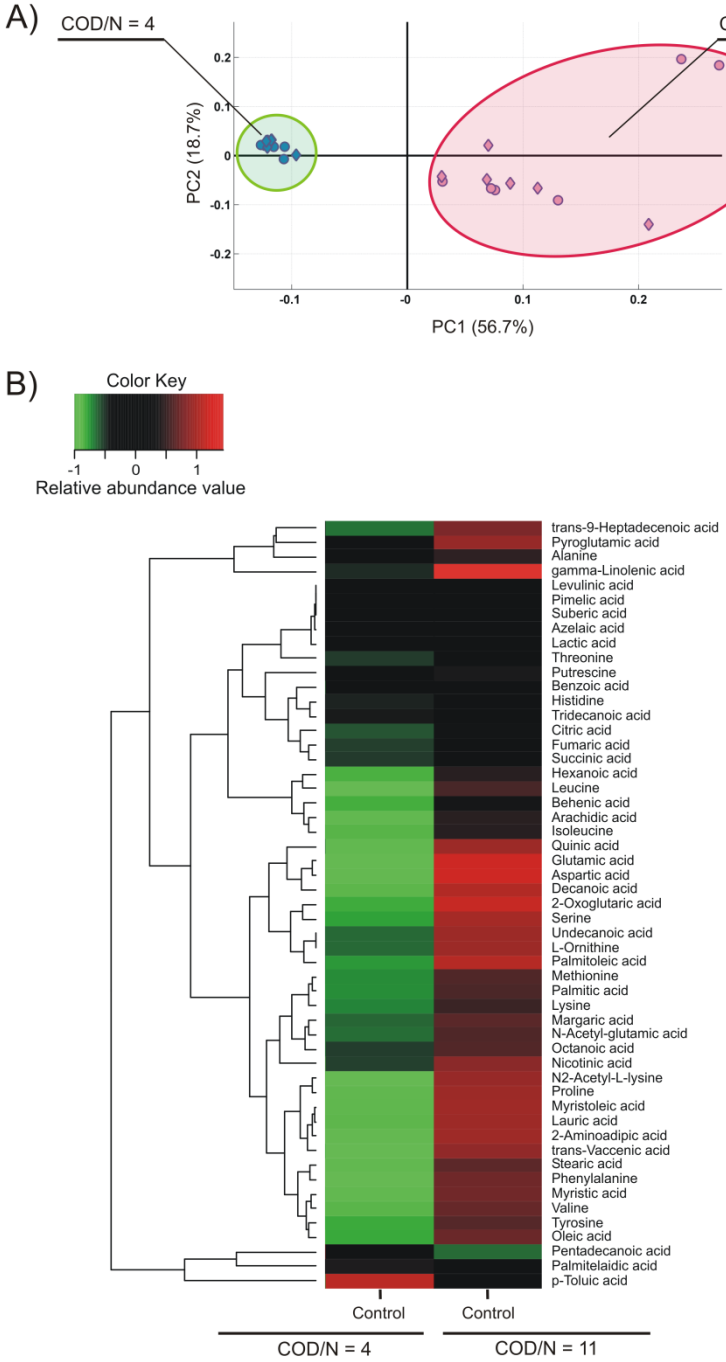
4.2.2 METABOLITE PROFILE

Relative abundance of the intracellular metabolites in the cultures was measured following the protocol described in Smart et al (2010), developed in the Metabolomics Laboratory of The University of Auckland and adapted to process mixed liquor samples. During the NO_3^--N oxidation phase, two 25mL samples were taken from the bioreactor and each filtered immediately using a vacuum filtration system with 0.45 µm pore-size cellulose acetate filter. Biomass quenching was done by rapidly washing the pellet on the filter with 10 mL of saline solution (0.9 % (wt/vol) NaCl) at 4 ºC. Thirty milligrams of pelletized biomass were recovered and placed in a 50 mL centrifuge tube containing 2.5 mL of cold methanol-water solution (1:1 (vol/vol)) at -30 ºC. The internal standard 2,3,3,3-d4-alanine (0.5 µmol/sample) was added to each sample and vigorously mixed with vortex for 30 seconds. Intracellular extracts were stored at -80 ºC for two to four weeks until further processing. Intracellular metabolite extraction was done by subjecting samples to freeze-thaw cycles as described in Smart et al (2010). Cell debris from this metabolite extraction step was used to quantify biomass (as a soluble protein) by using the Bradford method. Sample extracts were concentrated by adding 10 mL of cold (4 ºC) bidistilled water, freezing the diluted extracts to -80 ºC and then freeze-drying them. Freeze-dried samples were re-suspended in 200 µL of NaOH (1 N) and then derivatized using the methyl chloroformate (MCF) protocol (Smart et al., 2010). The MCF derivatives were analysed by GC-MS (Agilnet GC7890 coupled with MSD597) operated following the equipment setup specified in Smart et al (2010). In total six samples were analysed for each experimental condition. In other words, each culture was sampled in duplicate and each of the conditions was reproduced in triplicate cultures.

4.3 RESULTS

Metabolites profiles from the two experimental conditions significantly different as shown in the principal component analysis shown in *Figure 7A*. A total of 53 intracellular metabolites were identified in biomass samples from denitrifying cultures (*Fig 7B*), although metabolite relative abundance was affected by culture medium COD/N ratio. In general metabolite relative abundances of COD/N=4 cultures were lower than that of COD/N=11 cultures indicating that cells in COD/N=4 cultures were limited of carbon substrate presented low metabolic activity (*Fig 7B*).

Figure 7. Metabolite profiles obtained from biomass in denitrifying cultures in COD/N ratios of 4 and 11



Denitrifying biomass consuming carbon substrates at low a COD/N ratio tends to produce more N_2O than biomass with a high carbon substrate consumption rate. Not only do these denitrifying cultures produce more N_2O, they also produce it for longer periods of time than cultures operated at high COD/N ratios. This is due to a chronic lack of electron equivalents to completely reduce NO_3^- to N_2. However, at high COD/N ratios, sN_2OPR is higher than at low COD/N ratios. Metabolite profiles revealed that relative abundances of TCA intermediates i.e. citric, fumaric and succinic acids, remained at similar levels in all tested conditions. Moreover, posterior PAPi analysis highlighted that central carbon and glycolysis pathways remained active in all these conditions. The metabolite profiles also revealed that low COD/N conditions minimise amino acid abundance in denitrifying cells. Under these conditions cells are almost depleted of carbon-rich metabolites. However, long-chain fatty acids (palmitoleic, undecanoic, margaric, octanoic, palmitic and gamma-linolenic) are detected at higher relative abundances than amino acids, indicating the presence of storage compounds and, therefore, of potential electron donors. It can be concluded from this study that electron competition between denitrifying enzymes and subsequent N_2O accumulation is enhanced by the utilization of slow, degradable, carbon sources, such as long-chain fatty acids. In deed storage compounds such as these long-chain fatty acids and PHB's are known to have a low metabolic oxidation rate (Marshall et al., 2013)

5 CONCLUSIONS

From the simulation and experimental studies of this research, it can be concluded that different operational conditions of BNR processes that lead to changes in environmental availability of electron donors or acceptors cause an imbalance between production and consumption of electron equivalents in microbial nitrifying and denitrifying cells, and promotes N_2O production or accumulation as a consequent by-product of the metabolic adjustment to those imbalances (*Fig. 8*). In nitrification processes, an excess of electron equivalent production leads to N_2O formation and subsequent emission. In contrast, for denitrification processes, insufficient electron equivalent production results in N2O accumulation and subsequent emission. *Figure 8* and *Table 2* summarise the conclusions from this research on the relationship between BNR operational conditions and dominant N_2O production pathway.

Figure 8. Effect of operational parameters on the activation of microbial N_2O production pathways

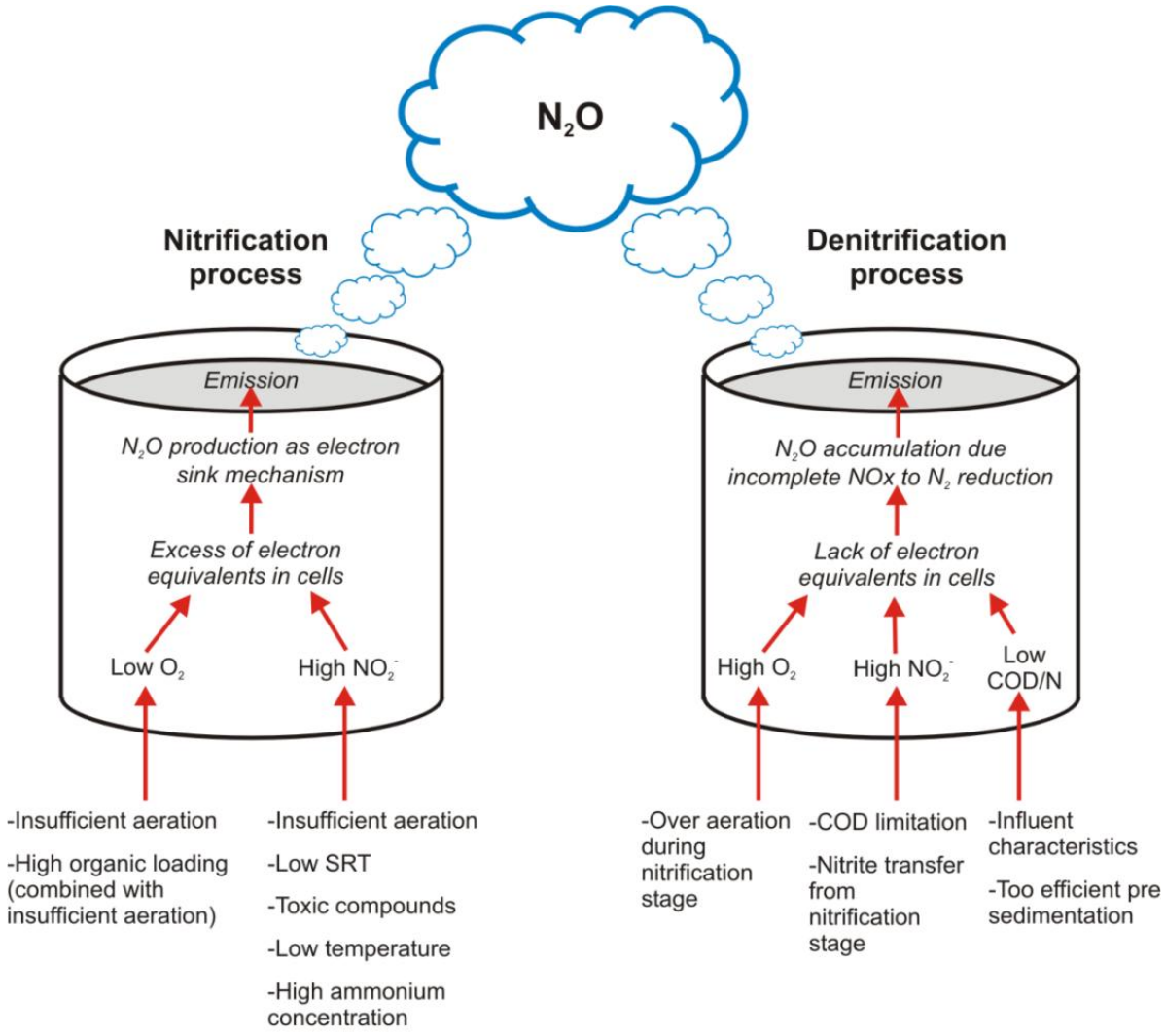


Table 2. Summary of operational conditions, metabolic mechanisms and pathways associated with N_2O emission

Process	Operational parameter/condition	Metabolic mechanism	Dominant N_2O production pathway
Nitrification	Low dissolved oxygen	NO leaks from NO reaction due low rate of terminal oxidize with respect to AMO. Not enough electron equivalent for NirK	HAO mediated NO production pathway
	Anoxic-oxic transition	AMO rate is high with respect to terminal oxidize rate Cyt_{aa3}	Nitrifier denitrification pathway, HAO mediated NO production, and NO detoxification
	High ammonium	High availability of electron equivalents. N_2O production as electron sink mechanism	Nitrifier denitrification pathway HAO mediated NO production, and NO detoxification
	High nitrite	NO_3^- and NirK competes with O_2 and Cyt_{aa3} for electron equivalents	Nitrifier denitrification pathway
	Presence of NOB	NOB consumes NO_{2-} and NO as electron donors	HAO mediated NO production and NO detoxification
Denitrification	High dissolved oxygen	Electron donor, incomplete NO_3^- reduction to N_2	Incomplete denitrification
	High nitrite	Increase of electron acceptor leads to depletion of electron equivalents. Incomplete NO_3^- reduction to N_2	Incomplete denitrification
	Low COD/N ratio	Lack of electron donor. Incomplete NO_3^- reduction to N_2. Use of storage compounds as electron donor	Incomplete denitrification

This research introduces SMN modelling and metabolomics (metabolic profiling) into environmental engineering practice. The research approach is based on the complementary use of computational and analytical methods to investigate complex metabolic phenomena in biological processes. SMN modelling formalizes vast quantities of biological data into flexible mathematical models; while metabolomics provides analytical measurements of hundreds of metabolic compounds. Therefore, these powerful techniques provide meaningful and accurate information about true cell phenotype. The research approach allowed us to generate a mechanistic hypothesis of how N_2O is produced in relationship to changes in electron donor and acceptor availability. Such mechanistic hypotheses were systematically developed by fitting the SMN models to experimental data to quantify the metabolic reaction rates. Practical implications about reactor operation strategies found during this research are illustrated by following three examples:

- The production of N_2O triggered by NH_4^+ shock loading can be minimised by adjusting the O_2 set point in bioreactor according to the influent NH_4^+ and biomass concentrations.

- Various previously published experiments show that full nitrification processes produce less N_2O than partial nitrification process. From the results generated in this study we now have a clearer picture of how much more N_2O (from 250% to 500% more) could be produced during partial nitrification compared to full nitrification (Fig. 5).

- Limiting or low COD/N ratios do not necessary cause denitrifiers produce N_2O, but does put them in a susceptible metabolic state to do so. In this case, effective process operation would involve maintaining a balance between carbon and nitrogen substrates in reactor considering the biomass concentration.

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