QUANTIFICATION, UNDERSTANDING AND MITIGATION OF NITROUS OXIDE EMISSIONS FROM BIOLOGICAL NITROGEN REMOVAL PROCESSES

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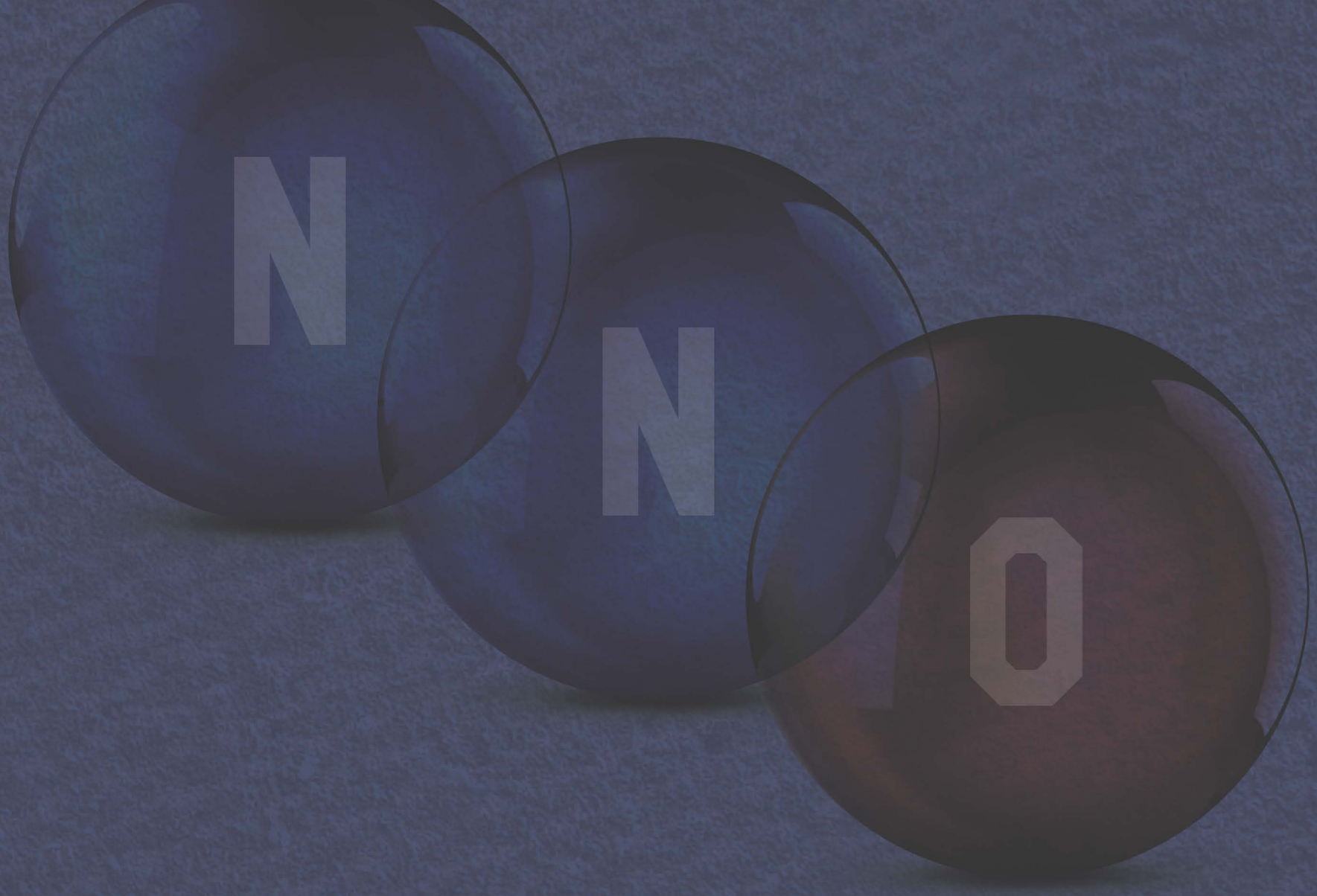

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"The great tragedy of science – the slaying of a nice hypothesis by an ugly fact"

Thomas Huxley, 1825 -1895

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Quantification, understanding and mitigation of nitrous oxide emissions from biological nitrogen removal processes

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Notation Index

AOB	ammonia-oxidising bacteria
AOR	ammonia oxidation rate
AnAOB	anaerobic ammonia-oxidising bacteria
bCOD	biodegradable fraction of the chemical oxygen demand
BES	bioelectrochemical system
BNR	biological nitrogen removal
BOD	biological oxygen demand
CC	closed circuit
CEM	cation exchange membrane
COD	chemical oxygen demand
DGGE	denaturating gradient gel eletrophoresis
DO	dissolved oxygen
ES	electrochemical system
FISH	fluorescent *in-situ* hybridization
GHG	greenhouse gas
GWP	global warming potential
HB	haber-bosch
HDN	heterotrophic denitrifiers
HRT	hydraulic residence time
IPCC	international panel for climate change
LCA	life cycle assessment
MBR	membrane bioreactor
MLVSS	mixed liquor suspended solids
MSW	municipal solid waste
NAC	net anodic compartment
NCC	net cathodic compartment
N/DN	nitrification/denitrification
NOB	nitrite-oxidising bacteria
OC	open circuit
ODP	ozone depleting potential
PCR	polemerase chain reaction
PN/A	partial nitritation/anammox

SBR	sequencing batch reactor
SHE	standard hydrogen electrode
SRT	sludge retention time
VSS	volatile suspended solids
WWTP	wastewater treatment plant

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Chapter 1 Introduction

Chapter redrafted after: *Desloover, J., Vlaeminck, S.E., Clauwaert, P., Verstraete, W. & Boon, N. (2011). Strategies to mitigate N_2O emissions from biological nutrient removal systems. Current Opinion in Biotechnology, 23(3), 1-9.*

1 Anthropogenic impact on the nitrogen cycle

Nitrogen (N) is, next to carbon (C), phosphorus (P), oxygen (O) and sulphur (S), an essential chemical element necessary for life. This compound has the greatest total abundance in Earth's atmosphere, hydrosphere and biosphere, however more than 99% of this nitrogen is not available to more than 99% of living organisms (Galloway et al., 2003). The reason for this is that almost all nitrogen is present in the form of unreactive dinitrogen gas (N_2). The latter constitutes 78% (v/v) of the Earth's atmosphere, but is not usable as such by most organisms as the two nitrogen atoms are held together by a strong triple bond.

Reactive forms of nitrogen can be formed and introduced into the biosphere by lightning, fossil fuel combustion and biological and anthropogenic nitrogen fixation.
The first two processes are able to oxidise N_2 to atmospheric nitric oxide (NO) and nitrogen dioxide (NO_2). These compounds can be further oxidised to nitric acid (HNO_3), which enters the biosphere by wet and dry deposition (Galloway et al., 2004). The contribution of lightning is estimated at 5.4 Tg N $year^{-1}$, whilst fossil fuel combustion is around 25 Tg N $year^{-1}$ (Galloway et al., 2008).
Biological and anthropogenic nitrogen fixation are processes able to reduce unreactive N_2 into bioavailable ammonia (NH_3) or ammonium (NH_4^+). Firstly, biological nitrogen fixation is a pathway exclusively performed by prokaryotes. These so-called diazotrophs have the ability to reduce N_2 into NH_4^+ by means of a nitrogenase, the key enzyme for this process (Martinez-Romero, 2006). On a global scale, continental nitrogen fixation amounts to 138.5 Tg N $year^{-1}$ (Galloway et al., 1995). Secondly, anthropogenic nitrogen fixation through the Haber-Bosch process catalytically combines hydrogen and dinitrogen gas into ammonia under high pressure (15-25 MPa) and temperature (300-350 ºC) (Chagas, 2007). This process is widely applied for fertilizer production, a powerful invention able to answer the food demand of the growing world population. In 2005, Haber-Bosch accounted for a nitrogen fixation of 120 Tg N $year^{-1}$ (Galloway et al., 2008), and is expected to increase to 165 Tg N $year^{-1}$ in 2050 (Galloway et al., 2004), thereby exceeding the natural creation rate.

Fig. 1.1 demonstrates the tremendous increase in fertiliser input since 1950 and the rapid growth of the world population and increase of meat production. Increased meat production increases nitrogen usage due to additional nitrogen required for animal feed production and the related inefficiencies of nitrogen use in meat-based diets compared to plant-based diets

(Erisman et al., 2008). Remarkably, without the Haber-Bosch process, only about 50% of the world population could be sustained. This increasing input of nitrogen however led to a large imbalance in the global nitrogen cycle and is causing accumulation of reactive nitrogen in many natural ecosystems, which is now a worldwide environmental problem. The latter is exemplified in Fig. 1.2, showing that next to biodiversity loss and climate change, the anthropic distortion of the nitrogen cycle has far exceeded the planetary boundary.

To date, the global population releases 20 Tg N $year^{-1}$ in wastewater of which more than 99% is not treated and thus released as such in the environment, mostly under the form of ammonium (Galloway et al., 2008). Increased nitrogen levels lead to eutrophication of lakes, rivers and marine ecosystems, causing oxygen depletion, loss of biodiversity and even extensive fish mortality. Furthermore, increased ammonium availability leads to enhanced microbial nitrification, resulting in the formation of toxic compounds such as nitrite and nitrate. These chemicals can contaminate groundwater and drinking water, thereby posing a severe threat to human health. Clearly, the nitrogen load to receiving water bodies through municipal and industrial wastewater disposal needs to be minimised in order to prevent aforementioned issues. Therefore, wastewater treatment plants all over the world are being upgraded by the implementation of a biological nitrogen removal step.

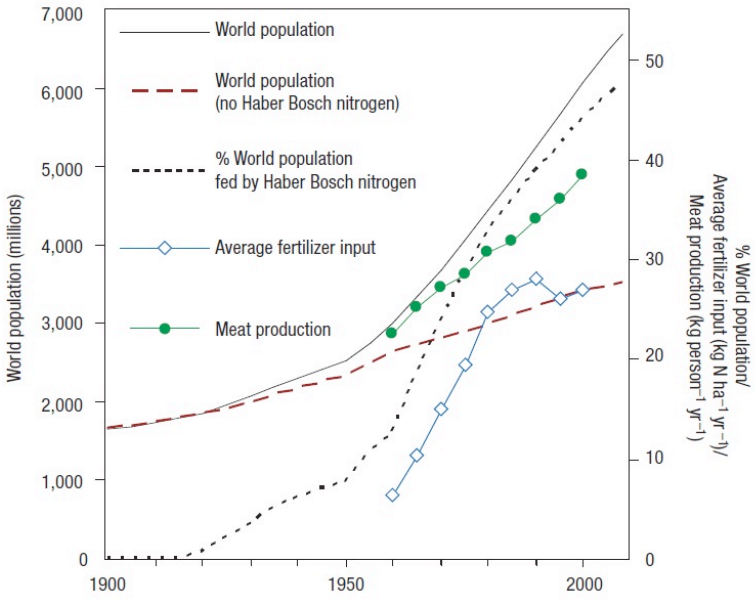

Figure 1.1 Trends in human population and nitrogen use throughout the twentieth century. Of the total world population, an estimate is made of the number of people that could be sustained without reactive nitrogen from the Haber-Bosch process, also expressed as a percentage of the global population. The recorded increase in average fertilizer use per hectare of agricultural land and the increase in per capita meat production is also shown (source: Erisman et al. (2008)).

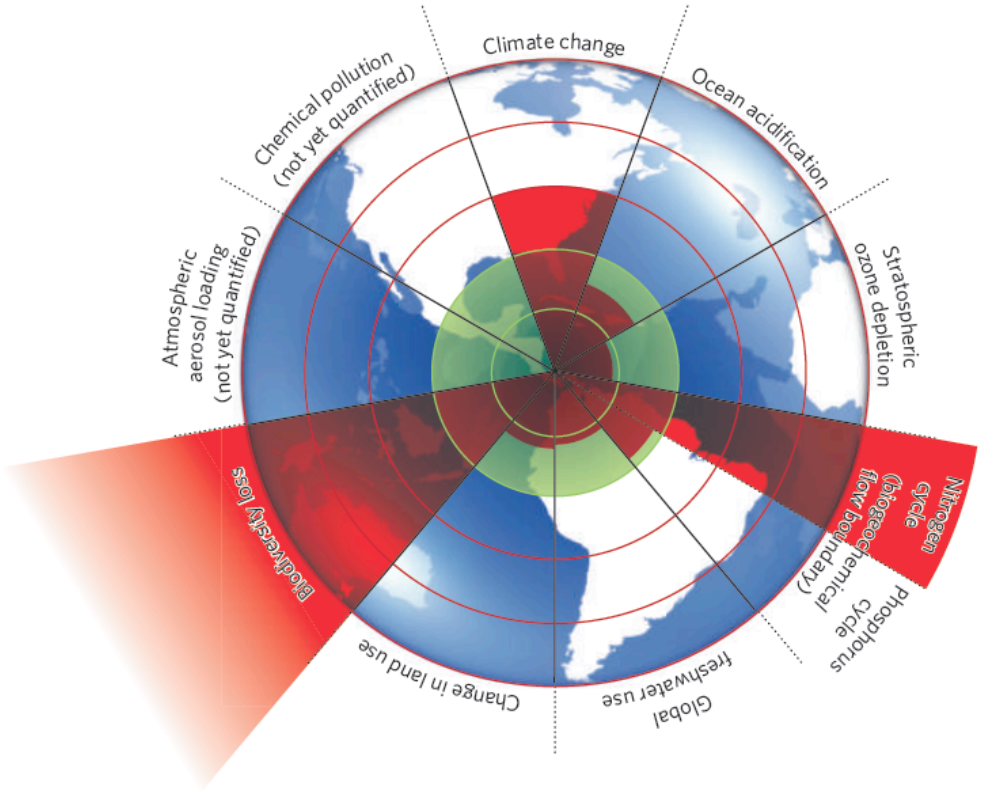

Figure 1.2 Beyond the boundary. The inner green shading represents the proposed safe operating space for nine planetary systems. The red wedges represent an estimate of the current position for each variable. The boundaries in three systems (rate of biodiversity loss, climate change and human interference with the nitrogen cycle), have already been exceeded (source: Rockstrom et al. (2009)).

2 Biological nitrogen removal (BNR)

Biological nitrogen removal (BNR) is a well-established technology and has become an important treatment step within a biological wastewater treatment plant (WWTP). At present, activated sludge is the most widely used process for the biological treatment of wastewater. The sludge consists of a microbial community able to metabolize organic and inorganic compounds present in the wastewater, rendering a dischargeable effluent. Multiple configurations of BNR systems have been developed, however they all rely on the same microbial processes.

2.1 Key microbial reactions

Four key microbial processes can be applied to remove nitrogen from wastewater: (1) nitritation by autotrophic aerobic ammonia oxidising bacteria (AOB), (2) nitratation by autotrophic nitrite oxidising bacteria (NOB), (3) denitrification by heterotrophic denitrifying bacteria (HDN) and (4) anammox by autotrophic anoxic ammonia oxidising bacteria (AnAOB). The combination of (1) and (2) is generally known as nitrification. Each catabolic microbial reaction is briefly discussed below. Furthermore, an overview of the overall stoichiometry of these microbial processes as well as combinations of these reactions commonly applied during BNR is given in Table 1.1.

2.1.1 Nitritation (AOB)

During nitritation, AOB catalyse the oxidation of ammonia to nitrite in two sequential steps (Hooper et al., 1997, Poughon et al., 2001, Wood, 1986):

$NH_3 + O_2 + 2\ H^+ + 2\ e^-$ → $NH_2OH + H_2O$

$NH_2OH + H_2O$ → $NO_2^- + 5\ H^+ + 4\ e^-$

$0.5\ O_2 + 2\ H^+ + 2\ e^-$ → H_2O

$NH_3 + 1.5\ O_2$ → $NO_2^- + H^+ + H_2O$ ($\Delta G^{o\prime} = -271\ kJ\ mol^{-1}$)

Firstly, ammonia (NH_3) is oxidised to hydroxylamine (NH_2OH) by the membrane bound enzyme ammonia monooxygenase (AMO), and is followed by the oxidation of hydroxylamine to nitrite (NO_2^-) by periplasmatic hydroxylamine oxidoreductase (HAO). The second reaction renders 4 electrons of which 2 are used for ammonia oxidation whereas the

remaining 2 electrons are used to reduce oxygen (O_2) by a terminal oxidase, thereby generating proton motive force.

2.1.2 Nitratation (NOB)

NOB further oxidise the nitrite produced by AOB to nitrate (NO_3^-) according to the following stoichiometry (Starkenburg et al., 2011):

$NO_2^- + H_2O \rightarrow NO_3^- + 2\ H^+ + 2\ e^-$

$0.5\ O_2 + 2\ H^+ + 2\ e^- \rightarrow H_2O$

$NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$ ($\Delta G^{o\prime} = -54$ kJ mol^{-1})

The oxidation of nitrite is catalysed by a nitrite oxidoreductase (NOR). This reaction renders 2 electrons which are used to generate proton motive force through oxygen reduction by a terminal oxidase.

2.1.3 Denitrification (HDN)

Denitrification, performed by HDN, comprises the stepwise reduction of nitrate (NO_3^-) and nitrite (NO_2^-) to nitric oxide (NO), nitrous oxide (N_2O), and dinitrogen gas (N_2) (Zumft, 1997):

$NO_3^- + 2\ e^- + 2\ H^+ \rightarrow NO_2^- + H_2O$ ($\Delta G^{o\prime} = -161$ kJ mol^{-1})

$NO_2^- + e^- + 2\ H^+ \rightarrow NO + H_2O$ ($\Delta G^{o\prime} = -76$ kJ mol^{-1})

$NO + e^- + H^+ \rightarrow ½\ N_2O + ½\ H_2O$ ($\Delta G^{o\prime} = -306$ kJ mol^{-1})

$½\ N_2O + e^- + H^+ \rightarrow ½\ N_2 + ½\ H_2O$ ($\Delta G^{o\prime} = -340$ kJ mol^{-1})

These stepwise reduction reactions are catalysed by nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (NOS), respectively. According to Zumft (1997) and Richardson (2000), each reaction in the denitrification pathway, catalysed by the abovementioned enzymes, is coupled to the generation of proton motive force.

2.1.4 Anammox (AnAOB)

The anammox reaction, executed by AnAOB, combines ammonium and nitrite to dinitrogen gas according to the following catabolic reactions (Strous et al., 1998, Strous et al., 2006):

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

$NH_4^+ + NO + 3e^- + 2\ H^+ \rightarrow N_2H_4 + H_2O$

$N_2H_4 \rightarrow N_2 + 4\ H^+ + 4\ e^-$

$NO_2^- + NH_4^+ \rightarrow N_2 + H_2O \qquad (\Delta G^{o\prime} = -358\ kJ\ mol^{-1})$

A nitrite reductase (NIR) reduces nitrite to nitric oxide, which is subsequently combined with ammonium to hydrazine (N_2H_4) by a hydrazine hydrolyse (HH). Finally, hydrazine is oxidised to dinitrogen gas by a hydroxylamine oxidoreductase (HAO) like enzyme.
To date, it was not yet possible to grow pure culture anammox bacteria. Hence, uncertainties exist regarding their metabolic capabilities. A model for the anabolism and catabolism of *Kuenenia stuttgartiensis* based on available genomic data was proposed recently (Strous et al., 2006).

Table 1.1 Overall stoichiometry of the 4 key microbial processes (nitritation, nitratation, denitrification and anammox) as well as combinations conventionally applied during BNR (nitrification/denitrification and partial nitritation/anammox) (Vlaeminck, 2009).

Process	**Number**	**Sub reaction**	**Stoichiometry**
Nitritation (AOB)	1	Substrates	$NH_4^+ + 1.382\ O_2 + 0.091\ HCO_3^-$
		Products	$0.982\ NO_2^- + 1.891\ H^+ + 0.091\ CH_{1.4}O_{0.4}N_{0.2} + 1.036\ H_2O$
Nitratation (NOB)	2	Substrates	$NO_2^- + 0.488\ O_2 + 0.003\ NH_4^+ + 0.01\ H^+\ 0.013\ HCO_3^-$
		Products	$NO_3^- + 0.013\ CH_{1.4}O_{0.4}N_{0.2} + 0.008\ H_2O$
Denitrification (HDN)	3	Substrates	$NO_3^- + 1.080\ CH_3OH$
		Products	$0.467\ N_2 + OH^- + 0.760\ CO_2 + 0.325\ CH_{1.4}O_{0.4}N_{0.2} + 1.440\ H_2O$
Anammox (AnAOB)	4	Substrates	$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+$
		Products	$1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O$
Nitrification/denitrification	1 + 2 + 3	Substrates	$NH_4^+ + 1.856\ O_2 + 1.058\ CH_3OH$
		Products	$0.457\ N_2 + 1.010\ H^+ + 0.641\ CO_2 + 0.421\ CH_{1.4}O_{0.4}N_{0.2} + 2.349\ H_2O$
Partial nitritation/anammox	1 + 4	Substrates	$NH_4^+ + 0.792\ O_2 + 0.080\ HCO_3^-$
		Products	$0.435\ N_2 + 1.029\ H^+ + 0.111\ NO_3^- + 0.052\ CH_{1.4}O_{0.4}N_{0.2} + 0.028\ CH_2O_{0.5}N_{0.15} + 1.460\ H_2O$

2.2 Conventional configurations

BNR plants are typically aerobic/anoxic processes based on nitrification/denitrification (N/DN) or partial nitritation/anammox (PN/A). A simplified process lay-out of these commonly applied BNR technologies is presented in Fig. 1.3.

2.2.1 Nitrification/denitrification systems

Nitrification/denitrification systems can be operated as a one- or two-stage process. The simplest method is a two-stage process with post-denitrification. In the first reactor, complete nitrification as well as oxidation of biodegradable organic material occurs. The effluent of the first reactor is subsequently treated in a second activated sludge reactor. In this denitrifying reactor, no aeration is provided but an extra source of reducing equivalents often needs to be added to promote complete denitrification, such as methanol or pre-settled sewage.

A more interesting method however is a two-stage system with pre-denitrification, also known as a modified Ludzack-Ettinger (MLE) system. In this system, a non-aerated anoxic section is installed in front of the nitrification reactor. By returning the nitrate-rich effluent of the nitrification reactor back to the anoxic section, the biodegradable organic material in the incoming wastewater can be used as a carbon and electron source for denitrification. In this way, dosing of external carbon source can be minimised.

In view of the fact that oxygen diffusion into the centre of an average sized sludge floc is fairly slow, simultaneous nitrification and denitrification can be promoted in a one-stage system, e.g. an oxidation ditch. Nitrifiers located on the outside of the floc scavenge the oxygen and the nitrate formed diffuses together with soluble organics inside the floc, thus supporting denitrification at the centre. Obviously, the aeration must be carefully controlled in this case.

Finally, next to separating different stages (anoxic/aerobic) in space as the systems described above, it is also possible to separate them in time in a so-called sequencing batch reactor (SBR). In this way, all the phases in continuous systems that are spatially separated are now provided in a single reactor.

2.2.2 Partial nitritation/anammox systems

Partial nitritation/anammox is a quite novel approach but has yet found the way to several full-scale applications. In anammox based processes, only half of the ammonium is oxidized to nitrite by AOB. After this, AnAOB combine the produced nitrite together with ammonium

into dinitrogen gas and some nitrate. This technology is particularly suitable for the treatment of wastewaters containing high ammonium levels, but a low amount of organics. Similar to N/DN systems, also PN/A systems can be operated as a one- or two- stage process.
In a two-reactor configuration, typically half of the ammonium is first oxidized to nitrite (partial nitritation) in an aerated reactor, after which the anammox process takes place in a second (anoxic) reactor (van der Star et al., 2007). The first process is also called SHARON (single reactor for high activity ammonia removal over nitrite), while the second step is called ANAMMOX (anaerobic ammonium oxidation).
Alternatively, in a one-reactor configuration, partial nitritation and anammox reactions take place in the same reactor. In this configuration, nitritation takes place at the outer side of a floc, biofilm or granule whereas the anammox process takes places in the deeper anoxic zones. Several terminologies exist for this one-reactor configuration. The most common names are CANON (completely autotrophic nitrogen removal over nitrite) (Third et al., 2001) and OLAND (oxygen-limited autotrophic nitrogen nitrification/denitrification) (Kuai and Verstraete, 1998).

The most important advantages of PN/A systems compared to N/DN systems are clear from the comparison of the different stoichometries (Table 1.1): (1) no external carbon source required due to the autotrophic nature of the involved microbial processes, (2) 60% lower aeration demand and (3) 75% lower sludge production. In this way, up to 85% of the operational costs can be saved compared to N/DN systems (Vlaeminck, 2009). The disadvantage however is the rather slow growth rate of AnAOB (doubling time of 1-2 weeks) (Strous et al., 1998), resulting in long start-up times of the reactors. Furthermore, according to the stoichiometries, only 89% of the nitrogen can be removed due the nitrate production by AnAOB (Table 1.1). This implicates that the effluent needs further polishing prior to discharge in the environment.

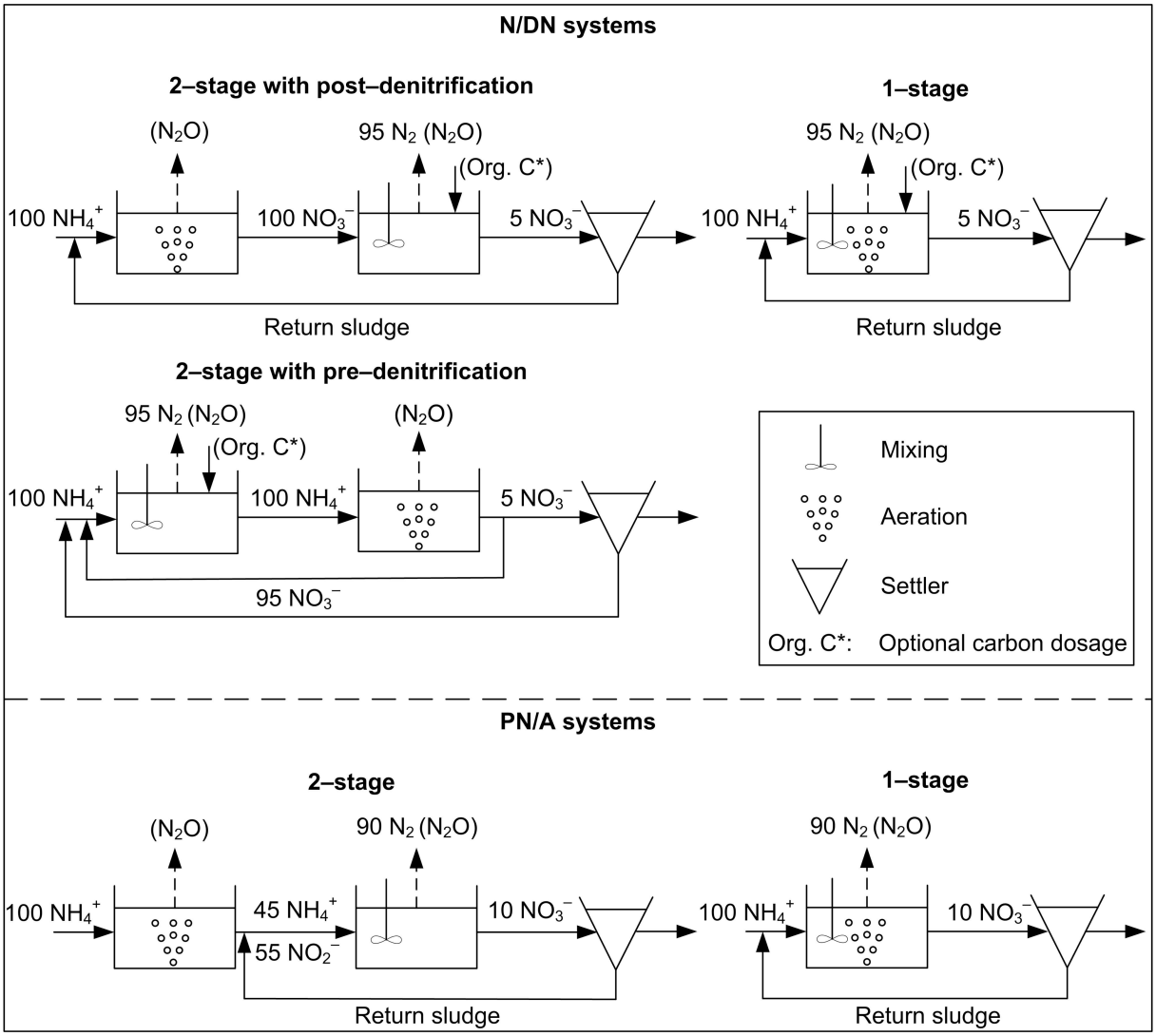

Figure 1.3 Simplified process layout of typical biological nitrogen removal (BNR) technologies for nitrification/denitrification (N/DN) and partial nitritation/anammox (PN/A), with approximate nitrogen mass conversion percentages.

2.3 N_2O emission from BNR systems

Nitrous oxide (N_2O) is a colourless non-flammable gas and applied in many fields, mainly as an oxidiser in rocket engines, aerosol propellant or anaesthetic. It is also commonly known as laughing gas due the euphoric effects caused by inhaling this compound. Nevertheless, N_2O is known to cause a severe adverse effect on the environment and the human health. It's an important greenhouse gas with a global warming potential of about 300 times CO_2 (considered over a 100 year time period) (Solomon et al., 2007). Additionally, N_2O has recently been assigned as the most important ozone-depleting substance in the 21st century (Ravishankara et al., 2009).

Nitrous oxide can be formed in the environment via several biological as well as chemical pathways. The intensification of the nitrogen cycle however, caused by the introduction of anthropogenic nitrogen fixation, led to a tremendous increase of N_2O emissions. By this, tropospheric N_2O levels increased from pre-industrial values of 270 ppb to a globally averaged value of 314 ppb in 1998. To date, about 40% of the total N_2O emission is attributed to human activities (calculated from Denman et al. (2007)). Next to agriculture, fossil fuel combustion and adipic and nitric acid production, treatment plants performing biological nitrogen removal are also considered as an important source.

2.3.1 Quantification

Global emission rates from sewage treatment were estimated at 0.22 Tg N_2O-N year^{-1} for 1990, accounting for about 3.2% of the anthropogenic N_2O emissions (Mosier et al., 1999). Furthermore, the contribution from BNR is likely significantly higher when including N_2O emission from manure (e.g. pig manure), landfill leachates and industrial nitrogenous effluents (no sufficient data for global estimation available yet).

An overview of intensive measurement campaigns to accurately quantify the N_2O emissions from full-scale BNR plants treating sewage and industrial streams is summarized in Table 1.2. A landfill leachate (non-intensive measurements) and manure treatment (pilot-scale) study was also included since sufficient accurate full-scale studies are still lacking. These studies confirmed that in general nitritation was the main responsible for N_2O emissions. The high variability of N_2O emissions among different BNR systems indicates the necessity for intensive on-site measurements, and accurate measurement protocols have been recently

provided (extended USEPA protocol by (Chandran, 2009); short easy-to-handle protocol (Chapter 2). Furthermore, Schneider et al. (2010) provided a comparison of different procedures used for the determination of N_2O emissions, and helps to evaluate the significance of available N_2O emission data.

At this moment, insufficient data are available to compare different BNR technologies on the basis of their N_2O emission potential. To our knowledge, until now only one in-depth study has been performed at long-term in which the N_2O emissions were compared from a lab-scale bioreactor operated sequentially in full-nitrification and partial-nitritation modes (Ahn et al., 2011). The N_2O emissions peaked during the transition period but stabilized afterwards, demonstrating the adaptive power of the involved microbial communities. Nevertheless, partial nitritation emissions were still statistically higher compared to full nitrification.
For nitrification-denitrification technologies, no clear difference in N_2O emissions between 1- and 2-stage systems has been observed until now. In contrast, regarding partial-nitritation/anammox technologies, 1-stage systems are expected to have a lower N_2O emission potential compared to 2-stage systems as nitrite (NO_2^-), an important precursor for N_2O production, is immediately turned over and renders a 10-50 times lower NO_2^- level (± 1 mg N L^{-1}) (Desloover et al., 2011, Jeanningros et al., 2010, Kampschreur et al., 2008b). Furthermore, bubble less aeration systems such as a membrane-aerated biofilm reactor (MABR) showed a 100-fold lower N_2O emission compared to other partial nitritation-anammox systems (Pellicer-Nacher et al., 2010), but awaits confirmation at full-scale. Hence, more extensive studies comparing conventional nitrification/denitrification and partial-nitritation/anammox technologies and suspended, floccular, granular and biofilm-based systems in general are required.

Table 1.2 Summary of intensive measurement campaigns (short period at high frequency or long period at low frequency) executed to accurately quantify N_2O emission rates from full-scale systems performing BNR by on-line or intensive grab sample measurement campaigns. Exceptions were made for plants treating landfill leachate (non-intensive study) and manure (pilot-scale study) due to lack of more accurate or full-scale data.

Wastewater type	**Type of processes**	**N_2O emission (% of N-load)**	**Sampling (frequency / duration) Measurements**	**Remarks**	**Reference**
Municipal	Activated sludge (no dedicated BNR)	0.035	Weekly grab / 15 weeks Off-line	N/A	(Czepiel et al., 1995)
Municipal	2–stage N/DN	0.001	2-weekly grab / 1 year Off-line	N_2O emission increased with increased NO_2^- and NO_3^- concentrations	(Sumer et al., 1995)
Municipal	2–stage N/DN	0.02	1 or 2-weekly grab / 1.5 year	N/A	(Sommer et al., 1998)
Municipal	1–stage N/DN	0.01 – 0.08	Cont. / 4 x 2h Off-line	N_2O emission decreased proportionally with shorter aerobic phase	(Kimochi et al., 1998)
Municipal	12 activated sludge plants with different N/DN configurations	1.8 (0.01 – 3.3 of N processed)	Cont. / 1 day On-line	Aerobic zones had higher contribution to N_2O emission compared to anoxic zones Positive correlation of NO_2^- accumulation, NH_4^+ and DO with N_2O emissions	(Ahn et al., 2010b)

Municipal	7 activated sludge plants with different N/DN configurations	0.06-25.3 (of N denitrified)	1-2 hourly grab / 2-4 h for 2 days Off-line	Lower N_2O production observed in plants with low total nitrogen effluent concentrations High NO_2^- concentration leads to high N_2O emissions	(Foley et al., 2010b)
Municipal	Plug flow N/DN	0.036	Cont. / 8 weeks On-line	Both anoxic and aerobic conditions in nitrifying lane may be behind the mechanistic triggers for N_2O formation	(Aboobakar et al., 2012)
Municipal	Plug flow N/DN	2.8	Cont. / 16 months On-line	N_2O emission represented 78% of the carbon footprint of the WWTP	(Daelman et al., 2013b)
Municipal digestate	2–stage PN/A	2.3	Cont. / 4 days Off-line	1.7% of the N_2O emissions originated from the nitritation stage N_2O emissions increased with decrease in DO (aerobic stage) and increase of NO_2^- concentration (anoxic stage)	(Kampschreur et al., 2008b)
Municipal digestate	1–stage PN/A	1.3	Grab every 15 min / 3 x 8 h Off-line	N_2O emission equalled 8 times the CO_2 emission on a CO_2 equivalent basis	(Weissenbacher et al., 2010)
Municipal digestate	Nitritation	3.8	Cont. / 12 x 6 h On-line	N_2O emission correlated positively with length of previous anoxic period (settling and decantation) and with ammonium oxidation rate	(Gustavsson and Jansen, 2011)
Industrial potato processing	1–stage PN/A	1.2	Cont. / 5 days Off-line	NO_2^- accumulation caused higher N_2O emission Higher aeration flow seemed to	(Kampschreur et al., 2009a)

factory digestate				increase N_2O emission	
Industrial potato processing factory digestate	2–stage PN/A	5.1 – 6.6	Cont. / 3 days On-line	Nitritation stage responsible for N_2O emission ± 50% decrease of N_2O emission would render CO_2 neutral operational footprint	(Chapter 2)
Manure piggery wastewater digestate	1–stage nitritation denitritation	0.07 – 0.15	Grab every 0.5 – 1 h / 2 x 12 h Off-line	Aerobic period was the main source of N_2O emission	(Rajagopal and Beline, 2011)
Landfill leachate	5 landfill sites with different N/DN configurations	0.0002 – 0.0531	Grab / not further specified Off-line	High N_2O production and emission after the leachate was aerated	(Lin et al., 2008)

2.3.2 N_2O production pathways

A state-of-the-art overview of the N_2O production and consumption pathways is presented in Fig. 1.4. Biological N_2O production can occur during autotrophic ammonia-oxidation by AOB and heterotrophic denitrification by heterotrophic denitrifiers (HDN). Next to these biological pathways, also chemical production of N_2O can take place during BNR. The key pathways are described below.

2.3.2.1 N_2O production by AOB

Two possible biological N_2O production pathways can occur during nitritation by AOB, i.e. nitrifier denitrification and hydroxylamine (NH_2OH) oxidation. Ammonia-oxidising archaea (AOA) have been suggested to be largely responsible for N_2O emission from oceans (Santoro et al., 2011). AOA can be significantly present in certain BNR systems treating low ammonium levels (Park et al., 2006), or operating at low dissolved oxygen (DO) levels and high sludge ages (Park et al., 2006). Nevertheless, their possible contribution to N_2O emissions from BNR has not yet been investigated.

Nitrifier denitrification

Nitrifier denitrification stands for dissimilatory reduction of NO_2^- to N_2O over NO by NO_2^- (NIR) and NO (NOR) reductase (Bock et al., 1995). Although N_2 production could be observed, no genes encoding N_2O (NOS) reductase have been found, suggesting that N_2O is the main end product of this pathway. Possible electron donors for this reduction reaction are hydroxylamine (NH_2OH) (Bock et al., 1995), hydrogen (H_2) and pyruvate (Bock et al., 1995), and ammonia (Poth and Focht, 1985). Nitrifier denitrification has been reported to especially play a role under anoxic to suboxic conditions (Goreau et al., 1980, Kampschreur et al., 2008a, Kampschreur et al., 2008b), whereby AOB can shift from O_2 to NO_2^- as their electron acceptor in case O_2 becomes limiting.

Hydroxylamine oxidation

Hydroxylamine oxidation is catalysed by a hydroxylamine oxidoreductase (HAO). A study by (Igarashi et al., 1997) suggests that the oxidation of NH_2OH leads to an intermediate called nitrosyl radical (NOH) which is subsequently converted to NO_2^-. However, this radical is very unstable which can break down into N_2O (Poughon et al., 2001). Furthermore, also NO can be formed due to enzymatic splitting of NOH (Andersson and Hooper, 1983) and

eventually end up as N_2O by enzymatic reduction (Stein, 2011b). This pathway seems to play an important role in case a high ammonia oxidation rate (AOR) is observed. A study by Law et al. (2012a) demonstrated that the N_2O production rate was exponentially correlated to the AOR, which could be represented by a metabolic model based on N_2O production through NOH degradation.

2.3.2.2 N_2O production by HDN

A second important biological source of N_2O during BNR is denitrification by heterotrophic denitrifiers (HDN). Nitrous oxide is the last intermediate in the denitrification pathway (Zumft, 1997) and can thus be released in case incomplete denitrification occurs. On the other hand, HDN can also serve as an important N_2O sink in case the N_2O reductase (NOS) is expressed and active. This pathway is not considered as a major contributor to the overall N_2O production during BNR. However, it can play a significant role in case of insufficient carbon source or N_2O reductase inhibition by a too high dissolved O_2 level in the reactor (Kampschreur et al., 2009b).

2.3.2.3 Chemical N_2O production

Next to biological production, also chemical production of N_2O can take place through reaction between NH_2OH and NO_2^-, or NO_2^- reduction with (in)organic compounds (Van Cleemput, 1998). Chemical N_2O production is generally considered to play a minor role. However, a study by Kampschreur et al. (2011) demonstrated that NO and N_2O production through ferric iron reduction could be of significance in case of iron-rich wastewaters.

Finally, other conversions applied during BNR such as nitratation by nitrite-oxidizing bacteria (NOB; (Lucker et al., 2010, Starkenburg et al., 2008)) and anammox by anoxic ammonia-oxidizing bacteria (AnAOB; (Kartal et al., 2011)) are not directly involved in N_2O production.

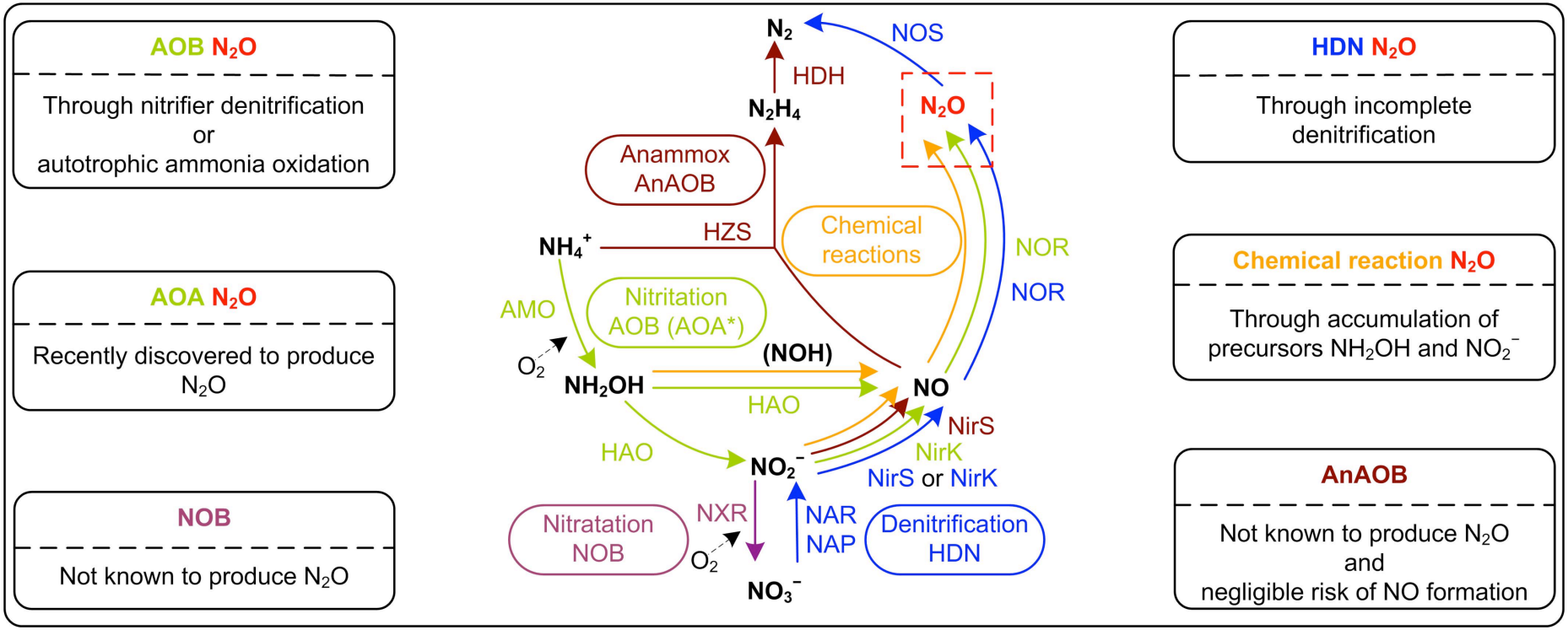

Figure 1.4 Conceptual overview of the N_2O production and consumption pathways during BNR, and the involved microbial communities and enzymes (N cycle redrafted after (Vlaeminck et al., 2011) and (Kartal et al., 2011). The key microbial communities are aerobic ammonia-oxidizing bacteria (AOB), anoxic ammonia-oxidizing bacteria (AnAOB), ammonia-oxidizing archaea (AOA), heterotrophic denitrifying bacteria (HDN) and nitrite-oxidizing bacteria (NOB). The related enzymes are ammonia monooxygenase (AMO), hydroxylamine oxidoreductase (HAO), hydrazine dehydrogenase (HDH), hydrazine synthase (HZS), periplasmatic nitrate reductase (NAP), membrane-bound nitrate reductase (NAR), Cu-containing nitrite reductase (NirK), cytochrome *cd1* nitrite reductase (NirS), nitric oxide reductase (NOR) and nitrous oxide reductase (NOS). *For archaeal nitritation, proposed intermediates are NH_2OH or HNO (Walker et al., 2010).

2.3.3 Key operational factors influencing N_2O production

The influencing parameters and operational conditions that lead to N_2O production are being increasingly understood. The most important operational factors influencing N_2O production are summarised and discussed below.

2.3.3.1 Dissolved oxygen regime

The dissolved oxygen (DO) concentration is considered as a very important parameter controlling N_2O production, both during nitrification and denitrification (Kampschreur et al., 2009b). During nitrification, sufficient oxygen supply is crucial for AOB. In case oxygen limitation occurs, AOB can shift to nitrite as their terminal electron acceptor and produce N_2O through the nitrifier denitrification pathway (Kampschreur et al., 2008a, Tallec et al., 2006). By this, AOB can save oxygen for the oxidation of ammonia to hydroxylamine.
Alternatively, over aeration of the nitrification reactor/zone might result into introduction of higher DO levels in the denitrification reactor/zone. As the N_2O reductase is more sensitive to oxygen compared to the other denitrification enzymes, N_2O production can be significant (Otte et al., 1996). Also, over-aeration leads to increased stripping of dissolved N_2O, a purely physico-chemical effect (Kampschreur et al., 2008b).

In practice, activated sludge is usually recirculated between anoxic and aerobic compartments/periods, thereby exposing the sludge to repeatedly changing conditions. Furthermore, such fluctuations can also take place in the reactor compartment itself depending on the feeding and aeration regime. To date, it has become clear that transient changes in the DO concentration are known to cause immediate production of N_2O, especially from AOB (Kampschreur et al., 2008a, Kester et al., 1997, Yu et al., 2010). More specific for AOB, imposition of anoxia and recovery from anoxia to aerobic conditions have been reported to be responsible for increased N_2O production.

Imposition of anoxia
Kampschreur et al. (2008a) demonstrated increased NO and N_2O production rates from a nitrifying culture upon the imposition of anoxia from fully aerobic conditions. It was suggested that nitrifier denitrification was the main N_2O production pathway as both the presence of nitrite and ammonium was required to observe this effect. This was investigated more into detail by Yu and Chandran (2010), who looked at the gene expression and

transcription level of an exponential phase *Nitrosomonas europaea* batch culture in response to low DO levels. Higher mRNA concentrations for *amoA* and *hao* were observed in cultures grown at lower DO. As a result, the authors postulated that *N. europaea* increases its efficiency to metabolize ammonia and hydroxylamine under oxygen-limiting conditions. However, this effect was not observed in stationary phase cells, suggesting that the response is dependent on the physiological growth state of the culture.

Recovery from anoxic to aerobic conditions

A study by Yu et al. (2010) demonstrated that the recovery from anoxic to aerobic conditions, rather than imposition of anoxia, causes the production of N_2O from AOB. The results from a *N. europaea* grown batch culture showed that NO accumulated under anoxic conditions, whereas N_2O was only produced upon recovery to aerobic conditions. Furthermore, the N_2O production correlated positively with the accumulation of ammonium during anoxia and the oxygen concentration upon recovery. Therefore, the authors concluded that the production of N_2O was mainly related to a shift in the metabolism from low to high specific AOB activity. The results of this pure culture study seem to be valid in practice as similar effects have been observed at several full-scale plants (Ahn et al., 2010a).

2.3.3.2 Nitrite concentration

Similar to oxygen, nitrite is an important factor in N_2O production from nitrification and denitrification. However, the effect of nitrite in N_2O production is much less understood as multiple contradictory observations have been reported.

Some nitrification studies demonstrated increased N_2O production rates in the presence of nitrite, and a correlation could be established between N_2O production by AOB and high nitrite concentrations in full-scale studies (Desloover et al., 2011, Foley et al., 2010b, Kampschreur et al., 2009b, Kampschreur et al., 2008b, Sumer et al., 1995). Furthermore, dosing nitrite pulses at 10 mg N L^{-1} to nitrifying cultures has been shown to increase N_2O production (Tallec et al., 2006). In contrast, Hynes and Knowles (1984) demonstrated that addition of exogenous nitrite to a *N. europaea* pure culture did not cause an increase in N_2O production.

For denitrification, the presence of nitrite has been reported to affect the activity of the N_2O reductase and consequently resulted into increased N_2O production (Itokawa et al., 2001). However, von Schulthess et al. (1995) suggested that NO produced from nitrite rather than nitrite itself is the true inhibitor of the enzyme.

2.3.3.3 pH

The pH is an important factor to take into account in studies concerning N_2O production. It not only has a direct effect on enzyme activity, it also determines the NH_3/NH_4^+ and NO_2^-/HNO_2 ratio. NH_3 and HNO_2 are considered as the true substrates during nitrification (NH_3 and HNO_2) and denitrification (HNO_2). Therefore, distinguishing between the effect of pH and NH_3 or HNO_2 is extremely difficult. Furthermore, both compounds can become toxic at certain concentrations and also influence enzyme activity.

Hynes and Knowles (1984) demonstrated that N_2O production by *N. europaea* was highest at a pH of 8.5 and lowest at a pH of 6. Law et al. (2011) observed similar results with an enriched AOB culture. For denitrification, Thorn and Sorensson (1996) only observed N_2O formation below a pH of 6.8. The latter is probably related to the high sensitivity of N_2O reductase to a low pH (< 6.5) (Ghosh et al., 2007).

Nevertheless, in general WWTPs operate at a rather stable pH of 7-8, suggesting that the effect of pH is probably playing a minor role.

2.3.3.4 Organic carbon source

The availability of organic carbon, usually represented as chemical oxygen demand (COD), is an important factor influencing N_2O production during denitrification. Insufficient carbon source is known to cause increased N_2O production (Chung and Chung, 2000, von Schulthess and Gujer, 1996). In order to promote complete denitrification, the COD/N ratio in the reactor should be above 4. In case this ratio is lower, competition for electrons between the denitrification enzymes comes into play. As the nitrate and nitrite reductase have a relatively higher affinity for electrons (Knowles, 1982), NO and N_2O is expected to accumulate.

Besides the amount of organic carbon available, also the type of carbon source has been demonstrated to play a role in N_2O production (Christensson et al., 1994, Hallin and Pell, 1998). Feeding different types of carbon sources leads to the enrichment of different denitrifying communities. These communities might in turn have a different susceptibility to operational parameters known to influence N_2O production, such as nitrite and oxygen. As such it was demonstrated that a methanol-fed community had a higher susceptibility to oxygen inhibition compared to an ethanol-fed community (Lu and Chandran, 2010).

2.3.3.5 Consumption of internal storage compounds

Biological phosphorus removal can be operated as anaerobic/aerobic or anaerobic/anoxic (Kuba et al., 1993). When operated as anaerobic/anoxic, denitrification can take place during the anoxic phase. Some studies reported that denitrification by glycogen accumulating organisms (GAOs) leads to increased N_2O production (Lemaire et al., 2006, Zeng et al., 2003a, Zeng et al., 2003b). GAOs are, just as phosphate accumulating organisms (PAOs), able to take up organic carbon for storage during anaerobic periods and subsequently degrade the stored polyhydroxybutyrate (PHB) during aerobic/anoxic periods. As PHB consumption is reported to be the rate-limiting step (Murnleitner et al., 1997), N_2O production is possible resulting from the competition of the slowly released electrons between the denitrification enzymes. However, there is yet no conclusive evidence whether there is an intrinsic relation between storage compounds and N_2O production as it cannot be excluded that N_2O production was caused by nitrite accumulation, which could inhibit the N_2O reductase (Zhou et al., 2008).

3 Nitrogen removal versus nitrogen recovery

The choice between nitrogen removal and nitrogen recovery is highly dependent on the cost. To date, it is considered that biological nitrogen removal is the most cost-effective option for wastewaters containing up to 5 g N L^{-1}, a condition met by many domestic and industrial wastewaters (Mulder, 2003). Next to the economical aspect, also sustainability is emerging as a decisive tool. From an energy point of view, nitrogen recovery could be more sustainable if the energy input is lower than the sum of nitrogen removal (0.9-2.3 kWh kg^{-1} N) followed by re-fixation through the Haber-Bosch process (9.6-12.4 kWh kg^{-1} N) (Mulder, 2003).

At first sight, nitrogen recovery from waste streams seems pointless since air (78 v/v% N_2) can be considered as an unlimited resource. However, taking a closer look at the global nitrogen cycle shows that nitrogen recovery from manure, industrial and domestic wastewater could constitute about 50% of the reactive nitrogen produced through the HB process (calculated from Sutton et al. (2013)). This can be considered as a very important driving force, and a strong incentive to look deeper into more cost-effective nitrogen recovery techniques.
Moreover, treating nitrogen-rich waste streams first through direct nitrogen recovery prior to biological nitrogen removal for effluent polishing could mitigate a significant amount of N_2O. The latter follows from studies showing that BNR systems treating high nitrogen-loaded wastewater can emit N_2O up to 6.6% of the nitrogen load (Table 1.2).

Various techniques have been investigated to recover ammonium from nitrogen-rich waste streams such as digestates, manure and urine. At this stage mainly air or steam stripping has been applied (Lei et al., 2007, Siegrist, 1996). However, next to inefficient stripping and the need for chemicals, mainly the high energy requirement has impeded full-scale application. Next to this, nitrogen recovery by ion exchange through the use of for instance zeolites has been investigated intensively, but yet not widely applied (Hedstrom, 2001).
An emerging approach is precipitating nitrogen and phosphorus as struvite ($MgNH_4PO_4$) (Miles and Ellis, 2001). However, this technique is mainly considered for phosphorus recovery. The latter can be explained by the fact that the molar N/P ratio of wastewater is usually considerably higher than the molar N/P ratio of struvite (equal to one). This means that for efficient nitrogen recovery additional phosphate would need to be added, which is a very unsustainable approach.

At this moment, membrane-driven processes such as reverse-osmosis (Mondor et al., 2008), electrodialysis (Ippersiel et al., 2012) and (bio)electrolysis (Desloover et al., 2012, Kuntke et al., 2012) are gaining a lot of interest from researchers for nutrient recovery, mainly because of the fact that the prices of membranes have decreased considerably.

4 Objectives and outline of this research

At the start of this research, the magnitude of N_2O emissions derived from BNR was largely unknown as accurate measurements were lacking. Furthermore, there is much debate concerning the dominant microbial processes and the biochemical pathways responsible for the production of N_2O. Also, mitigation strategies aiming to lower the overall N_2O emission from BNR systems were scarce. Therefore, five research chapters were elaborated in this research aiming at accurate quantification and assessing the impact of N_2O emission (Chapter 2), improved understanding of the N_2O production processes and influencing parameters (Chapter 2 and 3), and the exploration of innovative mitigation strategies (Chapter 4, 5 and 6). In Chapter 7, the obtained results are discussed in the framework of the research objectives: the impact of N_2O emission from BNR is considered at a global versus local scale; a proposal for N_2O mitigation through process optimisation based on literature (Chapter 1) and own research findings (Chapter 2 and 3); and innovative mitigation strategies (Chapter 4, 5 and 6) are discussed in terms of practical applicability. Finally, conclusions are drawn and perspectives for further research are presented. A graphical overview of the different research chapters is presented in Fig. 1.5.

- **Chapter 2**: There is a lack of accurate N_2O measurements from full-scale BNR systems. Furthermore, the influence of operational parameters and the actual impact on the CO_2 footprint of a BNR plant needs further understanding. The goal of this chapter was to accurately quantify the N_2O emission from a full-scale BNR system, and to unravel which reactor zone and related microbial process was responsible for N_2O production. Also, the impact of N_2O on the operational CO_2 footprint was assessed.

- **Chapter 3**: There is a general consensus that ammonia-oxidising bacteria (AOB) are the most important contributors to N_2O production during BNR. However, AOB possess several pathways that can lead to N_2O, and there is strong debate concerning the mechanisms involved and the dominant pathways. Hydroxylamine (NH_2OH) can be considered as the key intermediate that leads to N_2O production, and the nitrite and dissolved oxygen (DO) concentration as the most influencing parameters. Therefore, the objective of this chapter was to investigate the effect of NH_2OH loading on N_2O

and NO_2^- production by an enriched AOB culture in absence and presence of ammonium NH_4^+, and its relation to the DO and NO_2^- concentration.

- **Chapter 4:** To date, no dedicated biological N_2O removal technology has been developed for curative mitigation of N_2O emissions. In this chapter, a bioelectrochemical system (BES) was investigated for its potential as a biological N_2O mitigation technology. The BES was initially developed with a denitrifying biocathode with nitrate as the electron acceptor. Next, the system was operated with N_2O as the sole electron acceptor in the cathode. The performance of the N_2O reducing biocathode was fully characterised in terms of maximum removal rate, current efficiency and optimal cathode potential.

- **Chapter 5:** N_2O reduction to N_2 is the final step of the denitrification process and thus an important N_2O sink during BNR. However, the presence of oxygen has a strong inhibitory effect on the enzyme (NOS) catalysing this reaction. Furthermore, there is debate concerning the existence of an alternative N_2O sink, that is, assimilatory N_2O reduction to microbial biomass nitrogen. This chapter was devoted to the enrichment and isolation of the dominant N_2O reducers from activated sludge under anoxic and oxic conditions. Also, the existence of an assimilatory N_2O consumption pathway was investigated by isotope tracing. The practical application could be situated in the field of bio-augmentation or curative treatment in a bioreactor.

- **Chapter 6:** A close look at the global nitrogen cycle shows that about half of the current reactive nitrogen demand, delivered by the Haber-Bosch process, could potentially be provided by the direct recovery of reactive nitrogen present in manure, industrial and domestic wastewater. This approach would have the advantage that considerably less nitrogen has to pass through a BNR system, thereby significantly lowering the overall N_2O emission from BNR. In this chapter, an electrochemical cell was investigated for direct nitrogen recovery. Firstly, important operational parameters were investigated with a synthetic medium. Secondly, the performance of this technology was validated with a nitrogen-rich digestate originating from a municipal solid waste digester.

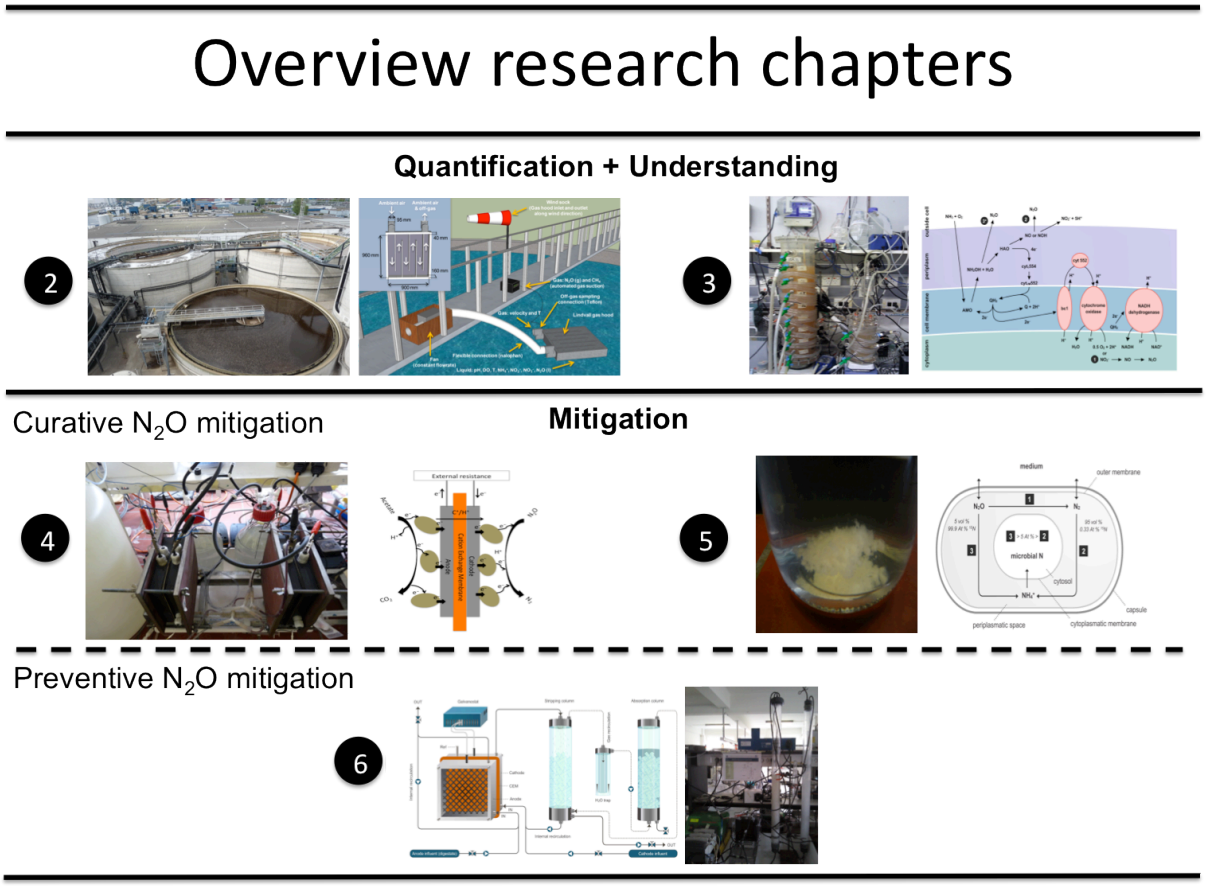

Figure 1.5. Overview of the different research chapters. The numbers refer to the respective chapters in this thesis.

Chapter 2 Performance and N_2O emission from a full-scale BNR system

Abstract

New Activated Sludge (NAS®) is a hybrid, floc-based nitrogen removal process without carbon addition, based on the control of solids retention times (SRT) and dissolved oxygen (DO) levels. The aim of this study was to examine the performance of a retrofitted four-stage NAS® plant, including on-line measurements of greenhouse gas emissions (N_2O and CH_4). The plant treated anaerobically digested industrial wastewater, containing 264 mg N L^{-1}, 1154 mg chemical oxygen demand (COD) L^{-1} and an inorganic carbon alkalinity of 34 meq L^{-1}. The batch-fed partial nitritation step received an overall nitrogen loading rate of 0.18-0.22 kg N m^{-3} d^{-1}, thereby oxidized ammonium to nitrite (45-47%) and some nitrate (13-15%), but also to N_2O (5.1-6.6%). This was achieved at a sludge retention time (SRT) of 1.7 d and DO around 1.0 mg O_2 L^{-1}. Subsequently, anammox, denitrification and nitrification compartments were followed by a final settler, at an overall SRT of 46 d. None of the latter three reactors emitted N_2O. In the anammox step, 0.26 kg N m^{-3} d^{-1} was removed, with an estimated contribution of 71% by the genus *Kuenenia,* which constituted 3.1% of the biomass. Overall, a nitrogen removal efficiency of 95% was obtained, yielding a dischargeable effluent. Retrofitting floc-based nitrification/denitrification with carbon addition to NAS® allowed to save 40% of the operational wastewater treatment costs. Yet, a decrease of the N_2O emissions by about 50% is necessary in order to obtain a CO_2 neutral footprint. The impact of emitted CH_4 was 20 times lower.

Chapter redrafted after: *Desloover, J., De Clippeleir, H., Boeckx, P., Du Laing, G., Colsen, J., Verstraete, W. & Vlaeminck, S.E. (2011). Floc-based sequential partial nitritation and anammox at full scale with contrasting N_2O emissions. Water Research, 45(9), 2811-2821.*

1 Introduction

Biological nitrogen removal is economically preferred above physicochemical nitrogen recovery for wastewaters containing less than 5 g N L^{-1} (Mulder, 2003). If the ratio of biodegradable chemical oxygen demand (bCOD) to nitrogen is relatively low (typically ≤ 3), nitrogen removal with partial nitritation/anammox is possible, decreasing 30-40% of the overall nitrogen removal costs compared to nitrification/denitrification (Fux and Siegrist, 2004). Partial nitritation oxidizes about half of the ammonium with oxygen to nitrite, and subsequent anammox oxidizes the residual ammonium with the formed nitrite to nitrogen gas and some nitrate. Depending on the wastewater characteristics and reactor operation, additional nitrogen conversions can take place, including aerobic nitrite oxidation to nitrate (nitratation) and reduction of nitrate or nitrite with organic carbon to nitrogen gas (heterotrophic denitrification). The latter requires at least 4.1 g bCOD g^{-1} NO_3^--N and 2.7 g bCOD g^{-1} NO_2^--N using wastewater organics (Mateju et al., 1992).

Partial nitritation and anammox can be executed in one reactor stage (Jeanningros et al., 2010, Joss et al., 2009, Vlaeminck et al., 2010, Wett, 2006), or in two sequential stages (van der Star et al., 2007, van Dongen et al., 2001). Although separate conversion stages entail higher investment costs related to the construction of the different reactors, such configuration allows to attune and optimize the conversion stages individually. Furthermore, in case of higher bCOD levels in the influent, the anammox bacteria will experience less bCOD in case of separate stages, leading to a lower chance that denitrifiers overgrow the anammox bacteria (Lackner et al., 2008). For an anammox stage, biomass retention is crucial given the high doubling time of the anammox bacteria (7-14 d; (Strous et al., 1998)). All application reports so far relied on the growth of anammox bacteria in biofilms or granules to obtain a sufficiently high SRT (Fernández et al., 2008, López et al., 2008, van der Star et al., 2007). Given the fact that some floccular applications exist for one-stage partial nitritation and anammox in sequential batch reactors (Joss et al., 2009, Wett, 2006), the feasibility of a separate floccular anammox step could be expected, yet remained to be demonstrated with realistic operational parameters.

Next to energy- and cost-efficiency, sustainability is evolving as a benchmark in wastewater treatment industry. An important sustainability aspect is the CO_2 footprint of a wastewater treatment plant (WWTP). Since 1 kg CH_4 and 1 kg N_2O have the global warming potential of

25 and 298 kg CO_2 on a 100-yr time horizon, respectively (Solomon et al., 2007), follow-up of these emissions is particularly warranted. The formation of methane through methanogenesis is well understood and the sustainability aspect of direct CH_4 emissions has been taken into account for quite some time (e.g. (Keller and Hartley, 2003). In contrast, N_2O emissions concern a more complex matter, as the interplay of many parameters determines N_2O production from nitritation, denitrification and chemical reactions (Ahn et al., 2010b, Kampschreur et al., 2009b). Furthermore, given the highly dynamic nature of N_2O emissions, accurate quantifications can only be obtained from grab samples taken at high frequency (e.g. 5-15 min) or from continuous on-line measurements (e.g. 0.5-5 min).

The aim of this study was to examine the performance of a novel, floc-based partial nitritation and anammox process, including quantification of the emissions of the greenhouse gases CH_4 and N_2O with a continuous on-line measurement set-up. The characterized full-scale nitrogen removal process discharges effluent to surface water and is preceded by anaerobic digestion and struvite precipitation (Anphos®), jointly representing the WWTP of a potato-processing factory. Previously, the nitrogen removal plant was operated as a floccular nitrification/denitrification system comprising a first nitrification stage followed by two subsequent denitrification stages and a final nitrification stage. As the COD/N ratio of the wastewater entering the denitrification stage was around 2, additional carbon was added by deviating 10% of the anaerobic digester influent, hence lowering the biogas production. However, by choosing appropriate DO setpoints and SRT, the system was retrofitted to a hybrid nitrogen removal process, consisting of partial nitritation, anammox, denitrification and nitrification (Fig. 2.1). This novel process was designated New Activated Sludge (NAS®), removing nitrogen without external carbon addition nor pH or temperature control.

2 Material and methods

2.1 Plant operation and sampling strategy

The operation of the industrial WWTP (Bergen op Zoom, the Netherlands) follows the production cycle of the potato company, i.e. in cycles of 2 weeks consisting of 12 days of factory effluent treatment and 2 idle days. In the idle days, the nitrogen plant receives no influent, but DO setpoints and recirculation flow rates are maintained. In weeks 10-17 (2010), reactor operation parameters and wastewater characteristics of each nitrogen removal compartment were monitored on a daily basis. In week 16 (2010), on day 1 of the operation

cycle, exploratory measurements of the gaseous emissions of the partial nitritation compartment were performed over a 3-h period. In week 17 (2010), at days 8-11 of the operation cycle, the snapshot characterization of each of the four reactor compartments comprised on-line gas sampling of the reactor off-gas, and liquid grab sampling of all streams entering and leaving a reactor compartment. The latter was executed every 30 min over a 3-h period, i.e. the cycle duration of the partial nitritation reactor. Since the exploratory test yielded relatively high N_2O emissions from the partial nitritation, this basin was monitored for three snapshot periods (batches 1, 2 and 3), whereas the other basins were only measured for one snapshot period. Also, automated on-line gas sampling was continued overnight for each reactor compartment. Table 2.1 presents an overview of the snapshot sampling schedule per day and night. Given the inherent variability of factory wastewater, the snapshot characterizations of each stream deviated slightly in composition and flow rate between subsequent snapshot periods.

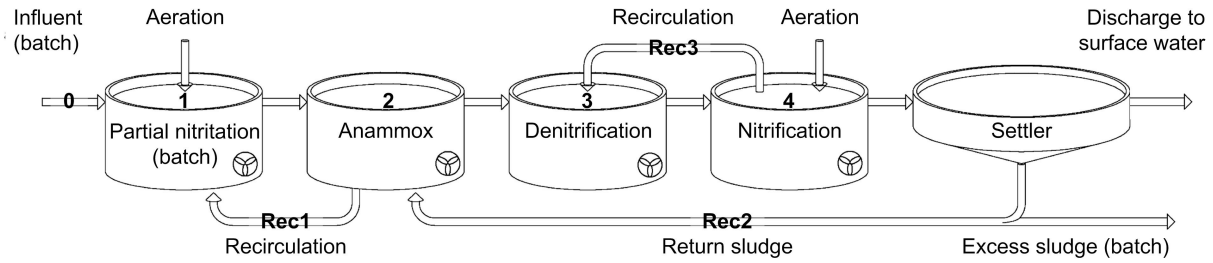

Figure 2.1 Schematic overview of the examined four-stage hybrid nitrogen removal process. The processes and flows were operated in a continuous mode, except for the feeding of the partial nitritation step and the wasting of excess sludge.

2.2 On-line gas sampling

The on-line gas sampling set-up of the snapshot sampling period (week 17) is presented in Fig. 2.2. The used Lindvall hood is an example of the wind tunnel approach to quantify gaseous emissions, whereas Ahn et al. (2010b) previously quantified N_2O emissions with the dynamic flux chamber approach. Both techniques provide sensitive fluxes, yet the former allows to control the sweep velocity of the mimicked wind more precisely (Downing and Nerenberg, 2007). A floating, aluminium Lindvall gas hood was used to capture the gaseous emissions from 0.864 m^2 of covered reactor surface. A fan with air intake opposite to the wind direction was used to blow ambient air through the internal snake pattern of the hood (Fig. 2.2 inset), mimicking relatively low wind conditions at the water surface under the hood, i.e. 0.4-0.5 m s^{-1} over a section of 20800 mm^2. The gas velocities and temperature were measured in triplicate every 30 min with a Testo hot-bulb probe (Ternat, Belgium) at the

outlet of the hood (7090 mm^2), and were 1.8±0.3 m s^{-1} at 30±2 °C (partial nitritation), 1.2±0.4 m s^{-1} at 30±1°C (anammox), 1.4±0.2 m s^{-1} at 28±1°C (denitrification), and 1.8±0.2 m s^{-1} at 25±1°C (nitrification). Hence, the aeration in the partial nitritation and nitrification compartments contributed only 22-33% of the measured gas velocity, and superficial gas velocities in these reactors were 0.0033-0.0049 m^3 m^{-2} s^{-1}. A Teflon tube at the hood outlet was connected to a photo-acoustic infrared Brüel & Kjær Multi-Gas Monitor 1302 (Nærum, Denmark), measuring and storing the N_2O and CH_4 level every 3 min. Calibration was done with 250 ppmv N_2O and 50 ppmv CH_4. Measured off-gas concentrations were corrected by subtraction of background levels, which were recorded for about 30 min before and after the 3-h intensive sampling period. Average background concentrations of N_2O and CH_4 were 1.63±1.26 and 6.73±1.01 ppmv, 1.13±0.38 and 9.37±2.51 ppmv, 0.54±0.27 and 7.03±1.24 ppmv, 0.58±0.22 and 5.76±0.11 ppmv during the intensive sampling periods of the partial nitritation, anammox, denitrification and nitrification reactor, respectively. Calculation of the gas emissions (kg d^{-1}) was based on the concentration corrected for background levels, converted to molar concentrations with the ideal gas law at atmospheric pressure and at the measured gas temperature. The measured gas velocity and cross-sectional area of the gas hood outlet, with a diameter of 95 mm (Fig. 2.2), yielded the off-gas flow rate. The overall emissions were obtained by extrapolating the flux from the covered surface (0.864 m^2) to the overall surface area of the relevant reactor compartment.

2.3 Grab liquid sampling

During the long-term monitoring (weeks 10-17), the pH, DO, inorganic nitrogen species, COD and sludge characteristics were monitored for every reactor compartment, and the total inorganic carbon (TIC) and phosphorus were measured in the influent and effluent. During the snapshot characterization (week 17), DO, pH and water temperature were measured in triplicate close to the gas hood every 30 min (Fig. 2.2). For the inorganic nitrogen species and dissolved N_2O, grab samples were taken every 30 min from every stream entering or leaving the reactor, except for the influent samples of the batch-fed nitritation where only one sample per batch was taken. The latter samples were additionally examined for Kjeldahl nitrogen.

A Consort C532 meter with probe was used for pH measurements (Turnhout, Belgium), and DO concentration and water temperature were measured with a Hach-Lange LDO meter (Düsseldorf, Germany). Hach-Lange cuvette tests (Düsseldorf, Germany) were used for ammonium (LCK302, 303 or 304), nitrite (LCK341 or 342) and nitrate analysis (LCK339).

Free ammonia and free nitrous acid levels were calculated based on the reactor ammonium and nitrite concentration, pH and water temperature (Anthonisen et al., 1976). Kjeldahl nitrogen was analysed according to standard methods (Greenberg, 1992). For dissolved N_2O measurement, a 1-mL filtered (0.45 µm) sample was brought into a 7 mL vacutainer (−900 hPa) and measured afterwards by pressure adjustment with He and immediate injection at 21°C in a Shimadzu GC-14B gas chromatograph equipped with an electron capture detector (Columbia, Maryland). In a control experiment, dissolved N_2O concentrations with and without prior filtration were on average 6.06±0.16 and 6.13±0.05 mg N L^{-1}, respectively, showing no significant influence of the filtration step (t-test p-value > 0.05). A Shimadzu TOC-VCPN analyser and ASI-V autosampler (Columbia, Maryland) were used for TIC determination, and the IC alkalinity was calculated from the TIC concentrations by taking into account pH and temperature (Crittenden et al., 2005). Total and volatile suspended solids (TSS and VSS) content, sludge volume index (SVI), COD and total phosphorus were determined according to standard methods (Greenberg, 1992).

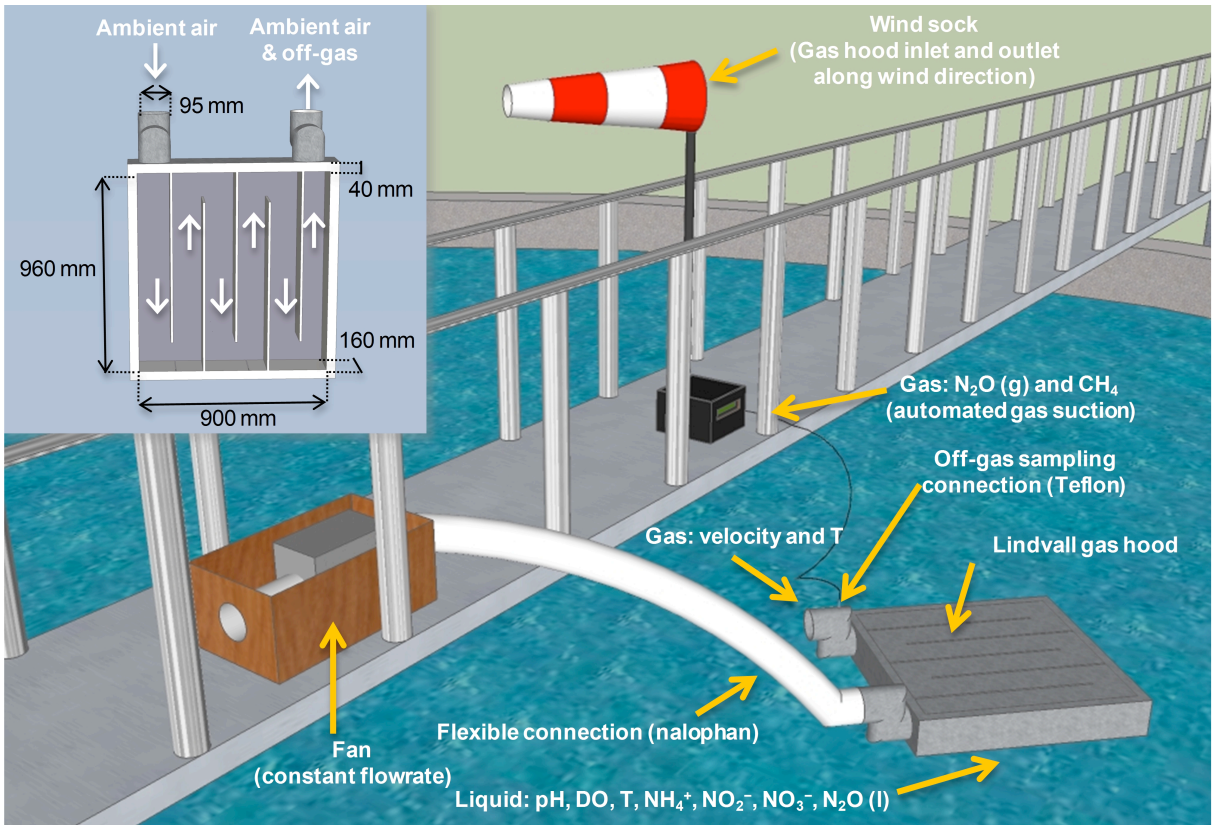

Figure 2.2 Set-up for the on-line measurement of gaseous N_2O and CH_4 emissions, with position of the liquid grab sampling, and detail of the Lindvall gas hood (inset). Q: flow rate; T: temperature; DO: dissolved oxygen; N_2O (l) and N_2O (g): dissolved and gaseous N_2O, respectively. For each reactor compartment, the gas hood was placed at one randomly chosen position.

2.4 Anammox batch tests

One grab sample was harvested from the anammox and from the denitrification compartment to determine the specific anammox activity rates. Prior to the batch activity tests, the biomass was washed with a phosphate buffer (100 mg P L^{-1}, pH 8) to remove residual dissolved reactor compounds. Anoxic ammonium conversion tests were previously described in detail (Vlaeminck et al., 2007), and were performed in triplicate.

2.5 Fluorescent in-situ hybridization

Fluorescent *in-situ* hybridization (FISH) was performed to identify the anammox bacteria, and to quantify nitritation bacteria and anammox bacteria in each reactor compartment. One mixed liquor sample of each compartment was fixed in a 4% paraformaldehyde solution and FISH was performed according to Amann et al. (1990). An equimolar probe mixture of Nso1225 and Nso190 for the β-proteobacterial nitritation bacteria genera *Nitrosomonas* and *Nitrosospira*, and probes Kst157, Amx1240, Sca1309 and Amx820 for the anammox bacterium *"Candidatus* Kuenenia stuttgartiensis", *"Candidatus* Brocadia anammoxidans", *"Candidatus* Scalindua" and *"Candidatus* Brocadia and Kuenenia", respectively. Probe sequences and formamide concentrations were applied according to probeBase (Loy et al., 2003), unless for the equimolar mixture of Nso1225 and Nso190, 35% formamide was applied (Pynaert et al., 2003). The target group was quantified by dividing the signal of the specific probe to the signal of the DNA stain 4',6-diamidino-2-phenylindole (DAPI). Images from ten random fields of view were acquired on a Carl Zeiss Axioskop 2 Plus epifluorescence microscope (Jena, Germany), which were subsequently analysed with ImageJ freeware.

3 Results

3.1 Partial nitritation reactor

Reactor operation parameters and wastewater characteristics during the long-term monitoring period are presented in Tables 2.1 and 2.2, respectively. The incoming streams of the partial nitritation reactor included (i) batches of effluent from the struvite precipitation reactor, containing mainly ammonium, and (ii) a continuous recirculation stream from the anammox reactor (Fig. 2.1).

The partial nitritation reactor received a new influent batch every 3 h during a feeding phase of 0.5 h, exchanging around 10% of the reactor volume per cycle (Fig. 2.3.A). Although the partial nitritation influent was fed in batches, the reactor's effluent was pumped continuously to the following anammox step. During regular factory operation, the nitrogen streams of the partial nitritation reactor were closely monitored for three influent batches. For batches 1 and 2, the reactor was operated at the normal DO setpoint (0.9-1.0 mg O_2 L^{-1}), while from the start of batch 3, the DO was set at 0.4 mg O_2 L^{-1}, to test the effect of a lower aeration rate on the emission of N_2O. The batch-feeding process had a clear influence on the reactor pH and DO. The influent was characterized by a relatively high pH (9.0 ± 0.1) and oxygen demand due to the ammonium (223-243 mg N L^{-1}; Table 2.3) and COD (1154 ± 110 mg COD L^{-1}; Table 2.2) content, resulting in pH peaks and DO valleys upon the addition of fresh influent (Fig. 2.3.B; 2.3.C). The partial nitritation reactor was not heated and was at a constant temperature of 36 ± 0°C.

On average, the snapshot reactor total nitrogen loading rates were 0.18-0.22 kg N m^{-3} d^{-1}, also taking into account the organic nitrogen loads of 36, 72 and 25 kg N d^{-1} for the batches 1, 2 and 3, respectively (Table 2.3). The incoming nitrogen was mainly oxidized to nitrite (45-47%) and nitrate (13-15%). Effluent nitrite to ammonium ratios were 1.37-1.53, which is in the vicinity of the required ratio of 1.32 for the subsequent anammox step (Strous et al., 1998). In congruence herewith, β-proteobacterial nitritation bacteria represented 30 ± 10% of the biomass, as determined by FISH analyses.

Table 2.1 Overview of the reactor parameters over the long-term monitoring period and during the snapshot characterizations (average ± standard deviation). For the latter, the sampling strategy was clarified with day and night numbers indicating the relative position in the 14-d production cycle. Numbers between brackets refer to the numbers in the schematic process overview (Fig. 2.1). Flow rates (Q) are the sum of the different streams entering a compartment. HRT: hydraulic residence time; DO: dissolved oxygen; TSS and VSS: total and volatile suspended solids concentration, respectively; SRT: sludge residence time; SVI: sludge volume index; am: morning; pm: afternoon; T: temperature.

		Partial nitritation (1)			Anammox (2)	Denitrification (3)	Nitrification (4)
Volume (m^3)		2370			1650	1600	2300
Long-term (weeks 10-17)	**Q (m^3 d^{-1})**	1851 ± 298			5931 ± 298	10731 ± 298	10731 ± 298
	HRT (d)	1.3 ± 0.2			0.28 ± 0.01	0.15 ± 0.00	0.21 ± 0.00
	pH (-)	7.5 ± 0.1			7.9 ± 0.1	7.7 ± 0.1	7.6 ± 0.1
	DO (mg O_2 L^{-1})	0.75 ± 0.05			0.0 ± 0.0	0.0 ± 0.0	5.7 ± 0.7
	VSS (g VSS L^{-1})	0.25 ± 0.03			3.25 ± 0.23		
	SRT (d)	1.7 ± 0.5			46 ± 41*		
	SVI (mL g^{-1} TSS)	100 ± 23			167 ± 34		
Snapshot (week 17)	**Night monitoring**		Night 9		Night 10	Night 11	Night 8
		Batch 1	Batch 2	Batch 3			
	Day monitoring	Day 9, am	Day 9, pm	Day 10, am	Day 10, pm	Day 11, am	Day 8, pm
	pH (-)	7.6 ± 0.1	7.6 ± 0.1	7.8 ± 0.1	8.0 ± 0.1	8.0 ± 0.1	7.6 ± 0.1
	DO (mg O_2 L^{-1})	1.0 ± 0.1	0.9 ± 0.2	0.4 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	3.0 ± 0.2
	T (°C)	36 ± 0	36 ± 0	36 ± 0	32 ± 0	31 ± 0	30 ± 0

* Average and standard deviations were calculated on weekly wasted sludge amounts, and since no sludge was wasted in weeks 12 and 13, this yielded a high standard deviation

Table 2.2 Overview of long-term wastewater characteristics (weeks 10-17) of the influent and different reactor compartments (average ± standard deviation). Numbers between brackets refer to the numbers in the schematic process overview (Fig. 2.1). Inorganic carbon (IC) alkalinity was calculated from total inorganic carbon (TIC), pH and temperature values. COD: chemical oxygen demand, respectively; Kj-N: Kjeldahl nitrogen; P_{tot}: total phosphorus; <LOD: below limit of detection.

(Weeks 10-17)	**Influent (0)**	**Partial nitritation (1)**	**Anammox (2)**	**Denitrification (3)**	**Nitrification (4)**
pH (-)	9.0 ± 0.1	7.5 ± 0.1	7.9 ± 0.1	7.7 ± 0.1	7.6 ± 0.1
COD (mg L^{-1})	1154 ± 110	442 ± 69	123 ± 49	95 ± 34	48 ± 7
Kj-N (mg N L^{-1})	264 ± 39				<LOD
NH_4^+ (mg N L^{-1})	193 ± 39	48 ± 13	4.7 ± 5.2	2.4 ± 0.8	<LOD
NO_2^- (mg N L^{-1})	<LOD	115 ± 25	12 ± 6	1.6 ± 1.2	<LOD
NO_3^- (mg N L^{-1})	<LOD	39 ± 8	7.4 ± 2.7	6.5 ± 4.1	9.1 ± 3.8
P_{tot} (mg P L^{-1})	23 ± 2				17 ± 1
TIC (mg C L^{-1})	391 ± 27				211 ± 41
IC Alkalinity (meq L^{-1})	34 ± 2				17 ± 3

Table 2.3 Water and nitrogen flows from the snapshot characterization (week 17) of the partial nitritation reactor (averages ± standard deviations). The number or label of each flow refers to the number in the schematic process overview (Fig. 2.1). The gaseous and dissolved N_2O streams, respectively labelled N_2O (g) and N_2O (l), were also expressed as percentages of the incoming nitrogen load, which further consisted of 36, 72 and 25 kg organic N d^{-1} for the batches 1, 2 and 3, respectively. Q: flow rate; <LOD: below limit of detection.

(Week 17)	**Partial nitritation (1)**								
	Batch 1			**Batch 2**			**Batch 3**		
Flow	0	+ Rec1	→ 1	0	+ Rec1	→ 1	0	+ Rec1	→ 1
Q (m^3 d^{-1})	1740	36	1776	1756	36	1792	2087	36	2123
NH_4^+ (kg N d^{-1})	388	0.3±0.0	133±7	420	0.3±0.0	136±7	507	0.2±0.0	174±13
NO_2^- (kg N d^{-1})	1.5	0.3±0.0	202±7	0.8	0.3±0.0	209±15	<LOD	0.4±0.0	238±6
NO_3^- (kg N d^{-1})	1.0	0.1±0.0	65±7	0.1	0.8±0.0	65±2	0.8	0.1±0.0	71±6
N_2O (l) (kg N d^{-1})		<LOD	0.7±0.1 (0.17%)		<LOD	0.8±0.1 (0.16%)		<LOD	1.1±0.2 (0.21%)
N_2O (g) (kg N d^{-1})			28±1 (6.6±0.2%)			27±1 (5.5±0.2%)			27±0 (5.1±0.0%)

Concentrations of ammonium, nitrite and nitrate were relatively stable along the batch cycle. After the feeding phase, ammonium decreased slightly whereas nitrite accumulated near the end of the batch (Fig. 2.3.E). However, the free ammonia (NH_3) and free nitrous acid (HNO_2) changes were more pronounced, because of the pH decrease due to the proton production associated with nitritation. At high pH values, i.e. at the beginning of a new batch, free ammonia reached up to 2.7-3.8 mg N L^{-1}, whereas free nitrous acid obtained a maximum of 5.6-9.3 µg N L^{-1} at the end of the batch cycle (Fig. 2.3.H).

At the unsteady state conditions of the onset of a two-weekly operation cycle, 9.0 ± 1.0% of the nitrogen load, i.e. the sum of Kjeldahl nitrogen, NO_2^- and NO_3^-, was emitted as N_2O. The gaseous N_2O emissions during normal operation were lower, amounting to 5.1-6.6% of the overall nitrogen load (Table 2.3). In comparison, the dissolved N_2O stream in the effluent of the partial nitritation reactor, was 25-40 times lower, representing 0.16-0.21% of the nitrogen load. In batch 3, the DO setpoint was lowered from 0.9 to 0.4 mg O_2 L^{-1} (Fig. 2.3.C). Despite the lower aeration rate of batch 3 (Fig. 2.3.F), the emitted N_2O flow was not lower in batch 3, which might have been caused by the higher dissolved N_2O concentrations (Fig. 2.3.D). Dissolved N_2O concentrations showed increasing trends in batches 1 and 3, and reached the highest levels in batch 3 with the lower DO setpoint and slightly higher nitrite levels (Fig. 2.3.B; D). In agreement with the latter, gaseous N_2O concentrations showed an increasing trend in batch 3, but were not higher compared to batches 1 and 2 (Fig. 2.3.D). The latter could be due to the higher dilution of the aeration flow with the wind flow in batch 3, as the gaseous concentrations were determined on this flow mixture. The overnight trends of gaseous N_2O levels were slightly decreasing per cycle, similar to the decrease in aeration rate (Fig. 2.4). Overall, no clear or uniform N_2O formation or emission trend could be derived.

It should be noted that it is in practice difficult to obtain fully closed mass balances from snapshot measurements, given the impact of a number of previous batches on the actual reactor concentrations. Nevertheless, besides the significant loss as gaseous N_2O, the balances indicate that no considerable quantities of nitrogen gas were removed from the liquid phase (Table 2.3).

Next to N_2O, some CH_4 was emitted during the partial nitritation step. The average emission from the three batches corresponded to 0.9±0.0 g CH_4 m^{-3} d^{-1} (2.1±0.0 kg CH_4 d^{-1}), and the CH_4 peaks at the beginning of a new batch (Fig. 2.3.G and Fig. 2.4) indicated that these

emissions were mainly due to stripping of residual dissolved CH_4 from the anaerobic digestion step.

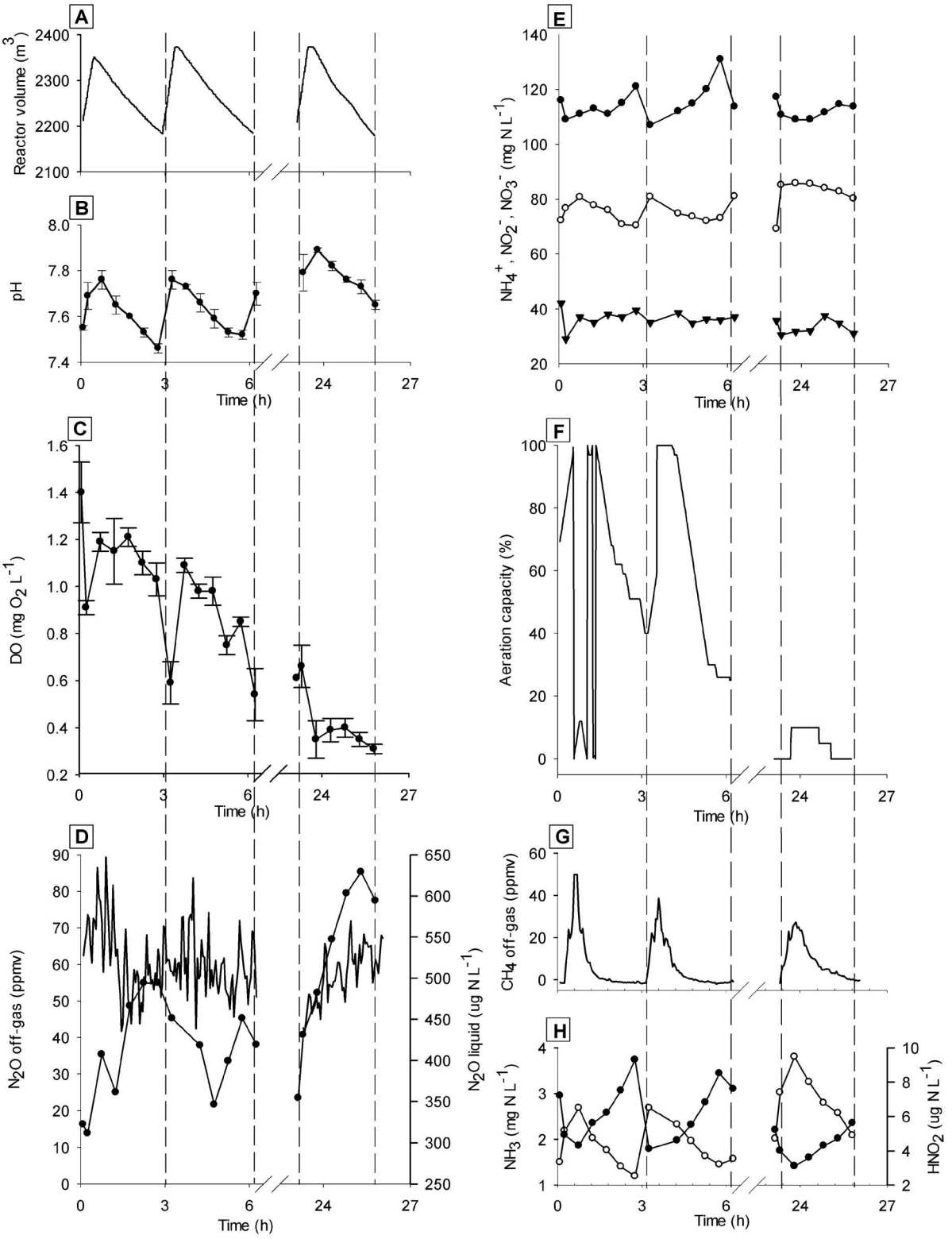

Figure 2.3 Profiles of reactor parameters and concentrations during three monitoring batches on the partial nitritation reactor. Dashed lines indicate beginning or end of a sampling period. An additional liquid grab sample was taken 10 minutes prior to the start of batch 1 and 3. Panel D: Concentrations of dissolved N2O (circles) and gaseous N2O (solid line); panel E: Concentrations of nitrite (full circles), ammonium (empty circles) and nitrate (triangles); panel H: concentrations of free ammonia (empty circles) and free nitrous acid (full circles).

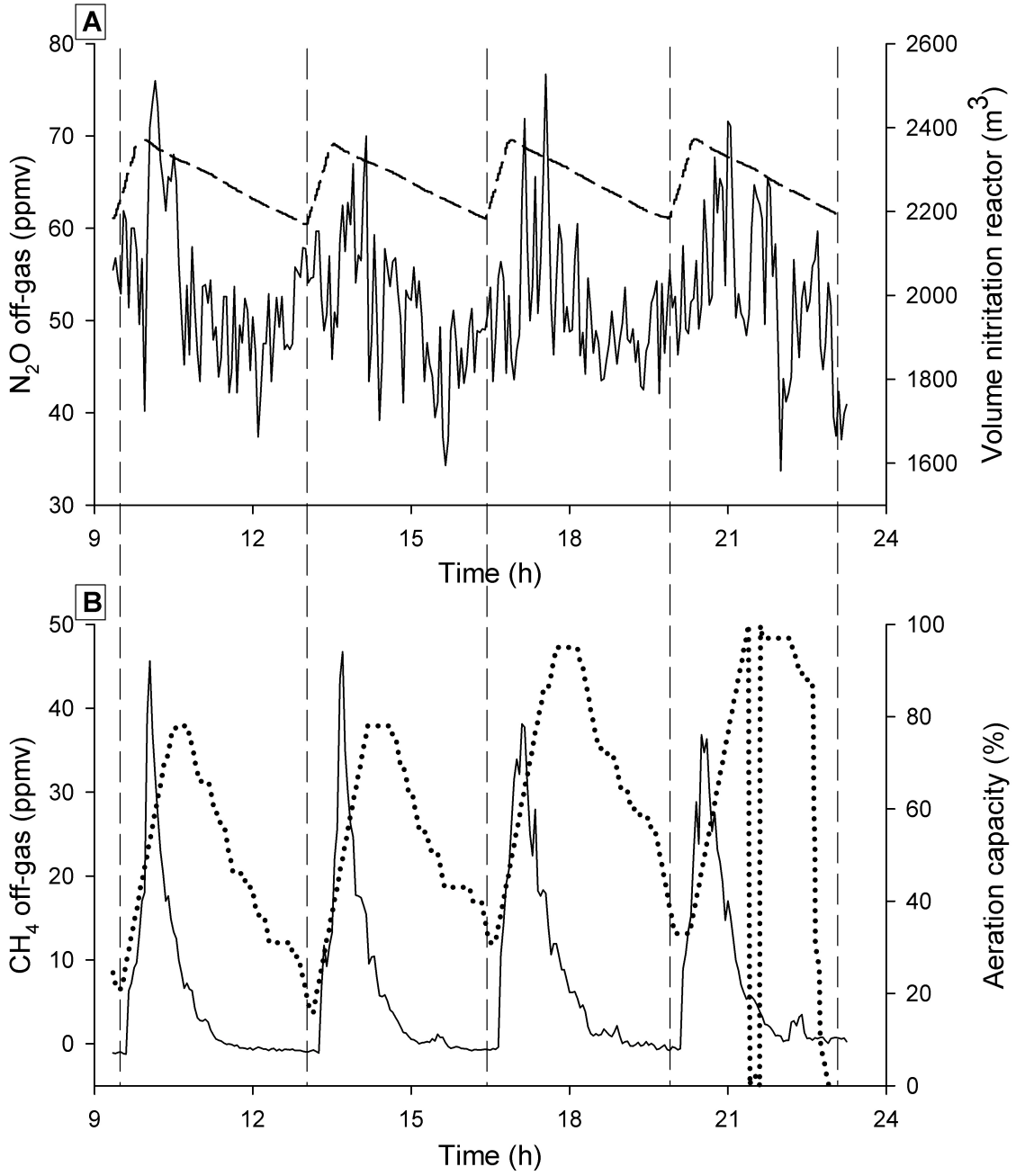

Figure 2.4 Overnight profiles of the partial nitritation reactor, with vertical dashed lines indicate the beginning of a new batch. A: Gaseous N_2O concentrations (solid lines) and reactor volume (medium dashed lines). B: Gaseous CH_4 concentrations (solid line) and use of aeration capacity (dotted line).

3.2 Anammox reactor

The anammox step was fed with partial nitritation effluent and with return sludge (Fig. 2.1). A hydraulic minor fraction of the anammox effluent was recycled to the partial nitritation stage, whereas the majority was delivered to the denitrification stage. Over weeks 10-17 (2010), the combined anammox, denitrification and nitrification stage was operated at a SRT of 46 d and a floccular sludge was obtained with a fair settleability (Table 2.1).

During the snapshot sampling period, the anammox stage was loaded with 0.33 kg N m^{-3} d^{-1} of ammonium, nitrite and nitrate and removed 77% of this nitrogen load, at a nitrite to ammonium nitrogen consumption ratio of 1.45/1 (Table 2.4). Assuming that only anammox bacteria were responsible for the anoxic ammonium removal (0.090 kg N m^{-3} d^{-1}), this would result in a concurrent nitrite removal rate of 0.118 kg N m^{-3} d^{-1} and a nitrate production rate of 0.023 kg N m^{-3} d^{-1}, as calculated from the anammox stoichiometry (Strous et al., 1998). Hence, anammox bacteria actually removed 0.19 kg N m^{-3} d^{-1}. The biomass from the anammox stage exerted a specific ammonium oxidation rate of 25 ± 2 mg NH_4^+-N g^{-1} VSS d^{-1}, as determined in a batch activity test, and consisted for 3.1 ± 2.0% out of the anammox genus *Kuenenia*. The anammox genera *Brocadia* and *Scalindua* could not be detected with FISH.

Besides the anammox activity, concurrent nitrite and nitrate denitrification was supported by the results. Firstly, expected nitrite removal by the anammox bacteria was 0.012 kg N m^{-3} d^{-1} lower than the actual nitrite consumption, indicating nitrite denitrification. Secondly, nitrate was consumed at 0.041 kg N m^{-3} d^{-1} (Table 2.4) and also the expected nitrate production from anammox (0.024 kg N m^{-3} d^{-1}) could not be detected, indicating *in-situ* consumption by nitrate denitrification. Overall, the denitrification rate estimate amounts to 0.076 kg (NO_2^-+ NO_3^-)-N m^{-3} d^{-1}, or 29% of the overall nitrogen removal.

The anammox reactor pH (8.0), DO level (0.0 mg O_2 L^{-1}) and temperature (32°C) were constant and not controlled (Table 2.1). In comparison with the preceding partial nitritation, the pH was higher, probably due to concurrent denitrification, and the temperature was lower, given the absence of heating and the mixing with the colder recirculation stream.

No gaseous N_2O emissions could be detected during the 3-h sampling period or the overnight gas measurement period and moreover, the incoming dissolved N_2O from the partial nitritation reactor was consumed in the anammox stage (Table 2.4). However, some CH_4 was detected in the off-gas, corresponding to an average emission of 0.8±0.1 g CH_4 m^{-3} d^{-1} (1.3±0.2 kg CH_4 d^{-1}). The emitted CH_4 levels were constantly around 11±3 ppmv, and since no concentration trends were observed in 3-h cycles (Fig. 2.5), CH_4 was likely formed *in-situ,* in contrast to stripping of dissolved CH_4 entering the partial nitritation reactor. Given the

presence of low nitrite and nitrate levels (3-8 mg N L^{-1}; Table 2.4), methanogenesis might have occurred in anaerobic microniches inside the flocs.

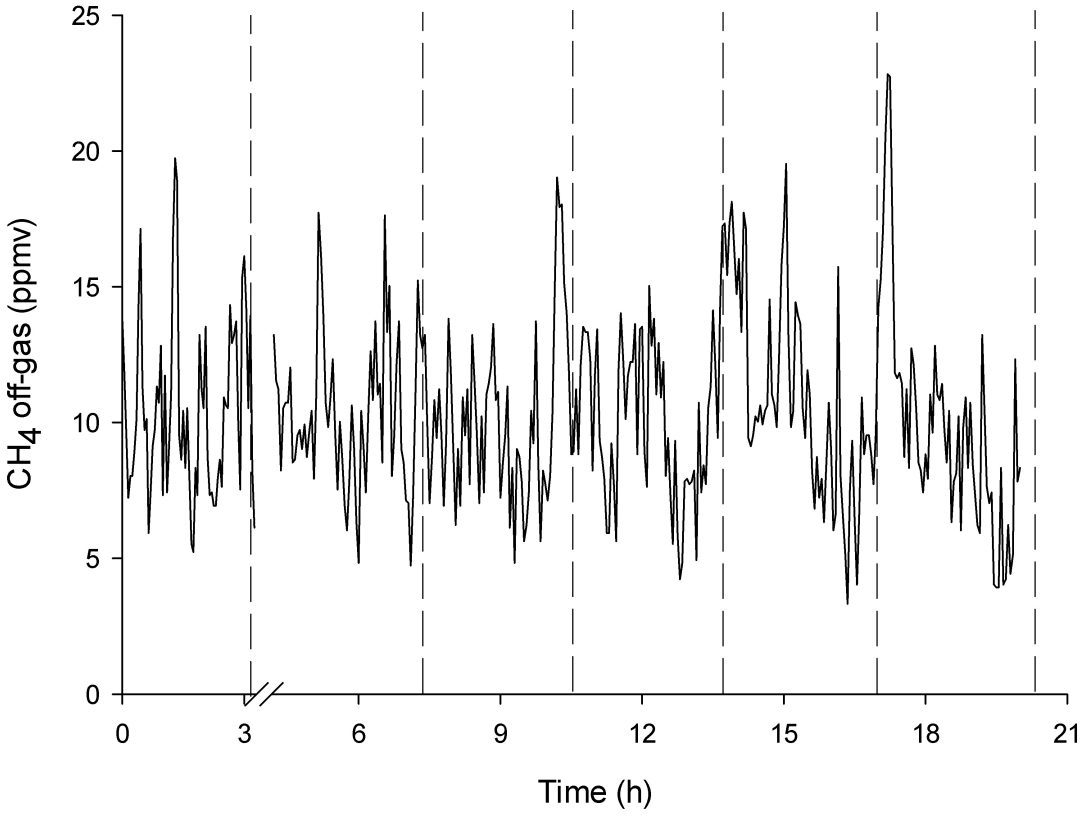

Figure 2.5 Profile of the gaseous CH_4 concentrations in the off-gas from the anammox reactor, recorded during the intensive monitoring period (first 3h) and overnight. Time gap represents omitted ambient air measurements. Vertical dashed lines indicate the beginning of a new batch in the partial nitritation reactor, from which the anammox step was fed continuously.

3.3 Denitrification and nitrification reactors

The final denitrification and nitrification steps provided effluent polishing. The denitrification received influent from the anammox reactor and a recirculation stream from the nitrification reactor (Fig. 2.1). During weeks 10-17 (2010), the nitrification effluent contained on average 9.1 ± 3.8 mg NO_3^--N L^{-1}, and no other nitrogen species (Table 2.2). Hence, over this period the four-stage nitrogen removal plant yielded consistently a dischargeable effluent (< 10 mg N L^{-1}), and an overall nitrogen removal efficiency 95 ± 2%.

In the denitrification reactor, pH (8.0) and anoxic conditions were identical to the preceding anammox reactor (Table 2.1). In the following nitrification stage, a DO setpoint of 5.7 mg O_2

L^{-1} was applied, yielding a significantly lower pH (7.6), probably due to proton production associated with nitritation and the stripping of some CO_2 formed in the previous stages.

During the snapshot sampling, the denitrification stage obtained a combined nitrite and nitrate removal rate of 0.058 kg N m^{-3} d^{-1}, i.e. 70% of the loading rate. The specific anammox activity of the denitrification stage biomass was 29 ± 6 mg NH_4^+-N g^{-1} VSS d^{-1}, as determined in a batch activity test, and consisted for 2.6 ± 2.0% out of the anammox genus *Kuenenia.* As the same sludge is cycled over the anammox and denitrification compartments, the detection of anammox activity and bacteria in the denitrification compartment was expected. Following denitrification, the nitrification stage oxidized all residual ammonium and nitrite to nitrate, without removing any nitrogen.

Besides the once-only measured presence of some dissolved N_2O in the influent of the nitrification stage, no N_2O flows were observed during the monitoring periods of both denitrification and nitrification compartments (Table 2.4). Also, these stages did not emit any CH_4.

Table 2.4 Water and nitrogen flows from the snapshot characterization (week 17) of the anammox, denitrification and nitrification reactors (averages ± standard deviations). The number or label of each flow refers to the number presented in the schematic overview of the nitrogen removal process (Fig. 2.1). Gaseous and dissolved N_2O streams are labelled N_2O (g) and N_2O (l), respectively. Q: flow rate; <LOD: below limit of detection.

(Week 17)	**Anammox (2)**				**Denitrification (3)**			**Nitrification (4)**		
Flow	1	+ Rec2	→ Rec1	+ 2	2	+ Rec3	→ 3	3	→ Rec3	+ 4
Q (m^3 d^{-1})	2366	4080	36	6410	5601	4800	10401	10997	4800	6197
NH_4^+ (kg N d^{-1})	203±15	<LOD	0.3±0.0	55±1	<LOD	<LOD	<LOD	74±3	<LOD	<LOD
NO_2^- (kg N d^{-1})	266±9	1.0±1.0	0.3±0.0	52±3	89±9	<LOD	27±3	1.0±0.0	<LOD	<LOD
NO_3^- (kg N d^{-1})	79±8	10±3	0.1±0.0	21±4	20±4	24±2	13±3	27±1	45±3	58±4
N_2O (l) (kg N d^{-1})	0.6±0.1	<LOD	<LOD	0.0±0.1	<LOD	<LOD	<LOD	0.3±0.2	<LOD	<LOD
N_2O (g) (kg N d^{-1})				<LOD			<LOD			<LOD

4 Discussion

4.1 NAS® operation and technology

The NAS® process is one of the first examples applying anammox in a separate floccular stage under realistic operational conditions. As such, a dischargeable effluent (<10 mg N L^{-1}) was obtained through a hybrid nitrogen treatment train without external carbon addition, treating an industrial digestate with medium nitrogen concentrations (264 mg N L^{-1}), a relatively low COD/N ratio (4.4) and a relatively high alkalinity/N (0.13 meq mg N^{-1}).

The effluent nitrite to ammonium nitrogen ratios from partial nitritation were above 1.32 and hence suitable for ammonium removal through anammox. For the partial nitritation stage, the inorganic carbon content of the wastewater played an important buffering role. Given the proton production during nitritation, a buffering capacity of 0.13 meq mg^{-1} N oxidized is required (Barnes and Bliss, 1983). Equilibrating this with nitratation (Barnes and Bliss, 1983), anammox (Strous et al., 1998) and/or denitrification stoichiometries (Mateju et al., 1992) shows that a similar alkalinity is required for partial nitritation/anammox and nitrification/denitrification, i.e. 0.073 and 0.065 meq mg^{-1} N removed, respectively. The industrial wastewater in this study contained around 0.13 meq mg^{-1} N (Table 2.2), which is hence more than sufficient for any biological removal process.

Besides nitrite, some nitrate was produced in the first reactor, i.e. 13-15% of the oxidized ammonium nitrogen. The minimal nitrate production obtained in the partial nitritation reactor were likely the combined result of the chosen DO setpoint (0.9-1.0 mg O_2 L^{-1}) and SRT (1.7 d), as well as the prevailing free ammonia concentrations (2.7-3.8 mg N L^{-1}). Firstly, although the applied DO levels were relatively low, often levels of around 0.3 mg O_2 L^{-1} are, in practice, required for longer term suppression of nitratation bacteria (Joss et al., 2009, Vlaeminck et al., 2009, Wett, 2006). Secondly, at higher temperatures the lower doubling time of nitratation bacteria compared to nitritation bacteria can be exploited by choosing an intermediate SRT which does not largely exceed 1 d at 35°C (Hellinga et al., 1998). Finally, in addition to the growth rate differences, also the pH strongly influences the required SRT, through its effect on the NH_4^+/NH_3 and NO_2^-/HNO_2 equilibria. Anthonisen et al. (1976) reported inhibition of nitratation bacteria by 0.08-0.82 mg NH_3-N L^{-1} and 0.06-0.83 mg HNO_2-N L^{-1}, although some studies reported only inhibition of nitratation bacteria at higher

ammonia levels (6.0 mg N L^{-1}) and lower free nitrous acid levels (0.02 mg N L^{-1}) (Vadivelu et al., 2007). It should be noted that nitratation suppression through high free ammonia levels and a controlled SRT is an advantage of a separated partial nitritation reactor, although the concomitant high nitrite levels likely play a role in N_2O formation (Kampschreur et al., 2009b).

In comparison with highly loaded anammox systems (10 kg N m^{-3} d^{-1}; (van der Star et al., 2007)), the anammox step in the current study had an intermediate loading rate (0.33 kg N m^{-3} d^{-1}), requiring a larger reactor volume. Since retrofitting of the studied plant used the existing reactor compartments and external settler, space requirement or construction costs were no issue. In case of a limited area availability to construct a new plant, the NAS® technology can be executed in a more compact way by applying higher nitrogen loading rates and by operating the final process stage (nitrification stage) as a membrane bioreactor. In the latter configuration, a 2200 m^3 NAS® plant was built in Bergen (the Netherlands), which treated in 2009 a high-strength digestate (3350 mg N L^{-1}) at an overall nitrogen loading rate of 0.5 kg N m^{-3} d^{-1} and a nitrogen removal efficiency of 99.5%.

The anammox stage removed 77% of its loading rate, with an estimated contribution of 71% by anammox and 29% by denitrification. As a major part of the influent COD was removed in the preceding nitritation step, the wastewater entered the anammox reactor at a COD/N ratio of 2.2 (Table 2.2), which was sufficiently low to prevent heterotrophic denitrifiers from overgrowing anammox bacteria at a SRT of 46 d. The co-occurrence of anammox and denitrification was even advantageous for the nitrogen removal efficiency, allowing for net nitrate consumption, whereas another full-scale anammox reactor produced on average 0.25 kg NO_3^--N kg^{-1} NH_4^+-N oxidized (van der Star et al., 2007). Although anammox was the dominant nitrogen removing process (71%) in the anammox reactor, anammox bacteria only represented 3.1% of the bacterial community. Furthermore, the enduring anammox activity was remarkable taking into account the periodical exposure of the biomass to oxygen. Indeed, the settler HRT was 2.3 h, and the HRT over the anammox, denitrification and nitrification stages were 6.7, 3.6 and 5.1 h, respectively (Table 2.1), exposing the sludge at least 29% of its cycle time to high DO levels (5.7 mg O_2 L^{-1}; Table 2.1) in the nitrification compartment, which apparently caused no irreversible inhibition in contrast to previous observations (Egli et al., 2001, Strous et al., 1998). In line with the results of Jeanningros et al. (2010), no inoculum enriched in anammox bacteria was required for the plant retrofitting, in contrast to

the start-up of most other plants comprising anammox (e.g. Joss et al. (2009), van der Star et al. (2007) and Wett (2006)).

On the long term, retrofitting the plant from nitrification/denitrification with carbon addition to NAS® had economical advantages. Since no more carbon was deviated from the anaerobic digester, biogas production increased with 10% (450 m^3 d^{-1}), recovering an additional 302 m^3 CH_4 d^{-1} or 52 EUR d^{-1}. Furthermore, electricity consumption for aeration decreased with 33% (859 kWh_{el} d^{-1}), saving 69 EUR d^{-1}, sludge production decreased with 50% (2.7 ton dewatered sludge d^{-1}), saving 110 EUR d^{-1}. Overall, these savings amount to 230 EUR d^{-1} or 40% of the operational costs for the wastewater treatment plant, not taking into account the value of the sold struvite.

4.2 Greenhouse gas emissions

During partial nitritation, 5.1 to 6.6% of the total nitrogen load was emitted as N_2O during normal reactor operation, and even 9.0% after the two-weekly non-feeding period. An average N_2O emission load of 27 kg N d^{-1} (Table 2.3) corresponds to an equivalent of 12644 kg CO_2 d^{-1}. In contrast, no N_2O emissions were detected from the anammox, denitrification and nitrification stages. In comparison, reported N_2O emissions from full-scale WWTPs range from 0.01 to 3.3% of the nitrogen load, as determined with an intensive gas sampling methodology (Ahn et al., 2010b, Joss et al., 2009, Kampschreur et al., 2009a, Kampschreur et al., 2009b, Weissenbacher et al., 2010).

The critical parameters for the production of N_2O during nitritation include high nitrite and ammonium values, DO setpoint around 1.0 mg O_2 L^{-1}, a DO increase after anoxia, as well as rapidly changing operational conditions (Ahn et al., 2010b, Kampschreur et al., 2009b, Yu et al., 2010). Moreover, Yoshinari (1990) reported that chemical production of N_2O can take place if nitrite concentrations exceed 14 mg N L^{-1}, and known mechanisms for chemical N_2O formation require the presence of the nitritation intermediate hydroxylamine (NH_2OH) (Kampschreur et al., 2009b). For the final nitrification stage, none of the aforementioned triggers for N_2O emission were fulfilled, in agreement with the absence of N_2O emissions from this stage. In contrast, the conditions in a partial nitritation reactor are inherently more likely to induce some N_2O production, though the measured emissions in the current study were substantially higher than the 1.7% from another full-scale separated partial nitritation step (Kampschreur et al., 2008). Firstly, nitrite and ammonium in the current study were

simultaneously high (resp. 114-117 and 75-82 mg N L^{-1}; Fig. 2.3.E), although still considerably lower than the 600-700 mg N L^{-1} levels reported in the study of Kampschreur et al. (2008b). Secondly, DO levels in our study (≤ 1 mg O_2 L^{-1}) were below the setpoint of 2.5 mg O_2 L^{-1} in the reactor of Kampschreur et al. (2008b), and might have been closer to the 'optimum' for high N_2O emissions (Tallec et al., 2006). During batch 3, the significantly lower DO level of 0.4±0.1 mg O_2 L^{-1} resulted in a build-up of dissolved N_2O towards the end of the batch, which was most likely caused but by decreased N_2O stripping, given the lower aeration rate at low DO levels. Finally, the reactor studied by Kampschreur et al. (2008b) was fed continuously, in contrast to the relatively more variable process conditions of our study, inherent to a batch feeding operation mode, which might also have contributed to the high nitritation emissions in the current study. The effect of variable process conditions was most apparent from the higher emissions from the introduction of fresh influent after two days of non-feeding. Although it awaits quantification of the N_2O emissions from more partial nitritation reactors, it may be that higher N_2O emissions are partly inherent to configurations with a separate nitritation step, mainly due to the higher nitrite concentrations.

The production of N_2O during denitrification is generally enhanced by rapidly changing process conditions, high DO and nitrite levels and low COD/N ratios (Ahn et al., 2010b, Kampschreur et al., 2009b). The absence of residual DO in the anammox and denitrification stages (Table 2.1) and the relatively low nitrite levels in the anammox and denitrification compartments (8 and 3 mg N L^{-1}, respectively; Table 2.4) as well as the high COD/N ratio of 5.1 in the denitrification stage (Table 2.2) were in agreement with the absence of N_2O emissions. Apparently, the low COD/N ratio of 2.2 in the anammox compartment (Table 2.2) was not a trigger for anoxic N_2O emissions. The only other study on a separate full-scale anammox step reported N_2O emissions of 0.6% of the nitrogen load, attributed to the activity of washed-out nitritation bacteria (Kampschreur et al., 2008b). Interestingly, this was not the case in our study. Possibly the intermediate anammox loading rate, as well the absence of a stripping gas led to the *in-situ* consumption of any dissolved N_2O, either derived from the influent (0.6-1.1 kg N d^{-1}; Table 2.3) or locally formed by nitritation bacteria or denitrifiers.

Methane emissions were observed from the partial nitritation and anammox stages. Emissions from the first stage (2.1 kg CH_4 d^{-1}) likely derived from the stripping of residual dissolved CH_4 from the anaerobic digester. At 67% CH_4 atmosphere and 35°C, the CH_4 solubility is around 12 mg CH_4 L^{-1} (Perry et al., 1997), so 22.4 kg CH_4 d^{-1} was expected to leave the

digester (flow rate of 1867 m^3 d^{-1}) dissolved in the effluent, of which around 10% was stripped in the partial nitritation step, and presumably 90% in the preceding struvite precipitation step. Given the stripping of residual dissolved CH_4 in the partial nitritation reactor and the relatively constant CH_4 concentrations in the off-gas of the anammox reactor, emissions from the latter reactor (1.3 kg CH_4 d^{-1}) likely derived from *in-situ* production through methanogenesis. Hence, the overall WWTP CH_4 emissions were estimated at 23.8 kg CH_4 d^{-1}, equivalent to 594 kg CO_2 d^{-1}.

The importance of the N_2O emissions follows from the calculation of the simplified operational CO_2 footprint of the WWTP, comprising energy consumption and recovery, and the emission of CH_4 and N_2O. It should be noted that CO_2 emissions from the degradation of organic carbon present in the wastewater are not taken into account, since this carbon is of biogenic origin (Metz et al., 2007). Over weeks 10-17, the anaerobic digester produced 4163 m^3 biogas d^{-1}, on average. With a CH_4 content of 67% and an energy content of 10 kWh m^{-3} CH_4, this is equivalent to 11160 kWh_{el} d^{-1} and 12550 kWh_{th} d^{-1}, given electrical and thermal conversion efficiencies of 40 and 45%, respectively, in the combined heat and power generation unit. Note that the factory effluent is already at 40°C and that the recovered heat from biogas is fully used to produce steam. Given the average omitted fossil-fuel sources of 0.45 kg CO_2 kWh_{el}^{-1} and 0.28 kg CO_2 kWh_{th}^{-1} in the EU15 (Fruergaard et al., 2009), this sums up to a sequestration of 8535 kg CO_2 d^{-1}. On the other side, WWTP energy consumption (4475 kWh_{el} d^{-1}, equivalent to 2014 kg CO_2 d^{-1}), estimated CH_4 emissions (594 kg CO_2 d^{-1}) and measured N_2O emissions (12644 kg CO_2 d^{-1}) give rise to an emitted equivalent of 15251 kg CO_2 d^{-1}. From the latter it is clear that the N_2O emission represented about 80% of the operational CO_2 footprint, and that a decrease of about 50% of the N_2O emissions from partial nitritation would render the carbon footprint neutral. The impact of direct CH_4 emissions was a factor 20 lower. However, reducing the N_2O emissions in the given process configuration is not straightforward, given the difficulty to elucidate the most critical parameter influencing N_2O production. However, the results indicated the production cycle variability as an important trigger for N_2O emission. Inserting a volumetric buffer tank prior to the partial nitritation reactor would make it possible to operate the partial nitritation step with a lower variability by a continuous influent flow. This would eliminate the possible effect of idle days without fresh influent provision with continued recirculation, and the batch feeding operation mode.

5 Conclusions

The main findings of the NAS® plant characterization can be listed as follows:

Advantages:

- Industrial wastewater with a relatively low COD/N ratio (4.5) was treated without carbon addition at high removal percentages (95%) yielding dischargeable effluent qualities (<10 mg N L^{-1}).
- A floccular anammox stage was achieved at a COD/N ratio of 2.2, which was sufficiently low to prevent heterotrophic denitrifiers from overgrowing anammox bacteria at a SRT of 46 d, and which allowed for concurrent denitrification and hence higher nitrogen removal efficiencies.
- Retrofitting from nitrification/denitrification to NAS® operation allowed to save 40% of the operational costs, due to 10% higher biogas production, 33% lower aeration and 50% lower sludge production. Inoculation with anammox bacteria was not required.

Challenges:

- Nitrous oxide emissions from partial nitritation constituted 5.1-6.6% of the nitrogen load. These emissions should be decreased by about 50% to render the operational CO_2 footprint of the industrial wastewater treatment plant neutral. High N_2O emissions may be partly inherent to a separate nitritation step.
- Intermediate nitrogen loading rates and an external settler represented a considerable areal footprint for the nitrogen removal plant. New plants with higher loading rates and a membrane bioreactor can be made more compact.

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Chapter 3 Effect of externally supplied NH_2OH on the N_2O and NO_2^- production by an enriched AOB mixed culture

Abstract

The influence of externally supplied hydroxylamine (NH_2OH), an important metabolic intermediate of ammonia (NH_3) oxidation, on N_2O production by an enriched ammonia oxidising culture in the presence and absence of ammonium (NH_4^+) and under various dissolved oxygen (DO) and nitrite (NO_2^-) conditions was investigated. At low DO conditions (DO = 0.55 mg O_2 L^{-1}), the supply of different NH_2OH loading rates (from 0.0 – 0.4 mg N min^{-1}) in the presence of NH_4^+ dramatically slowed down the NO_2^- production rate and resulted in higher N_2O emissions factors (up to 100-fold increase). At a higher DO concentration of 2.0 mg O_2 L^{-1}, the NH_2OH loading rate did not have an obvious effect on the NO_2^- production rate. While the N_2O emission factor also increased with the NH_2OH loading rate (up to 10-fold increase), the emission factor was lower than in the DO = 0.55 mg O_2 L^{-1} case. We suggest that the addition of NH_2OH in the presence of NH_4^+ resulted in electron overloading and hence likely stimulated the nitrifier denitrification pathway, particularly under low DO conditions. When NH_2OH was provided as the sole electron donor, the NO_2^- and N_2O production rates were 10-fold and 7-fold lower, respectively, compared to the case where NH_4^+ was also present, and the increase in DO did not promote the NO_2^- production rate or lower the N_2O production rate. The results suggest that, during NH_2OH supply in the absence of NH_4^+, the nitrifier denitrification pathway may not be active, and the produced N_2O is likely from NH_2OH oxidation. Finally, N_2O production by the culture was also influenced by the NO_2^- concentration and again the presence or absence of NH_4^+ resulted in a completely different response in the N_2O production dynamics.This study provided valuable data for further elucidation of the N_2O production pathways by AOB, and their dependency on environmental factors.

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1 Introduction

Nitrous oxide (N_2O) emissions from wastewater treatment systems have gained a lot of interest given the powerful greenhouse gas effect (IPCC, 2007) and ozone-depleting potential (Ravishankara et al., 2009) of this compound. Multiple recent studies as well as Chapter 2 have indicated that ammonia (NH_3) oxidation to nitrite (NO_2^-) by aerobic ammonia-oxidising bacteria (AOB) is often the most important source of N_2O in wastewater treatment plants (Ahn et al., 2010a, Law et al., 2012b, Ni et al., 2013a).

NH_3 oxidation by AOB is a two-step process by which NH_3 is firstly oxidised to hydroxylamine (NH_2OH) by a membrane-bound ammonia mono-oxygenase (AMO), with molecular oxygen (O_2) as the electron acceptor. Secondly, NH_2OH is further oxidised to NO_2^- by a periplasmic hydroxylamine oxidoreductase (HAO). The latter renders four electrons, of which two are used to sustain NH_3 oxidation and the remaining two for O_2 reduction by a terminal oxidase and generation of proton motive force (Fig. 3.1). Additionally, AOB produce N_2O as a by-product through three hypothesized pathways (Fig. 3.1). One pathway is generally known as 'nitrifier denitrification' during which AOB use NO_2^- and NO as their terminal electron acceptors, with electrons indirectly derived from NH_2OH oxidation as electron donor (Ritchie and Nicholas, 1972, Stuven and Bock, 2001). This pathway involves the sequential reduction of NO_2^- to NO and N_2O by a copper-containing NO_2^- reductase (NIRk) and a heam-copper NO reductase (NOR), respectively. A second pathway can take place during biological NH_2OH oxidation by which a nitroxyl radical (NOH) or NO could be formed as intermediates. NOH is a very unstable compound that can easily breakdown chemically to form N_2O and NO (Anderson, 1964, Hooper, 1968), and NO can be enzymatically reduced to N_2O by NOR or certain types of cytochromes (Stein, 2011b). Finally, a third pathway which is indirectly related to AOB activity, has been described by which N_2O is formed through the chemical oxidation of NH_2OH with O_2 or NO_2^- (Ritchie and Nicholas, 1972, Stuven et al., 1992).

The oxidation of NH_2OH to NO_2^- is the sole electron donating step in the metabolism of AOB. Under aerobic conditions, O_2 reduction during the NH_3 oxidation to NH_2OH step and at the terminal oxidase are the two main electron consuming pathways in AOB (Fig. 3.1). Nitrifier denitrification is generally promoted under oxygen-limiting conditions (Goreau et al., 1980, Kampschreur et al., 2008a, Zheng et al., 1994). When O_2 availability decreases, it is proposed that *Nitrosomonas europaea* increase the nitrifier denitrification activity, utilising NO_2^- and NO as alternative electron acceptors to facilitate electron flow, leading to increased

N_2O production (Stein, 2011a). The nitrifier denitrification pathway relieves the electron bottleneck between HAO and the quinone pool (Fig. 3.1), therefore accelerates the NH_2OH oxidation activity and overall electron flow. Furthermore, in an *N. europaea* pure culture, the nitrifier denitrification pathway has been shown to support the oxidation of NH_3 to NO_2^- via NH_2OH (Jason et al., 2007).

Significantly more N_2O production was also observed in NH_2OH fed activated sludge in the absence of ammonium (NH_4^+) compared to when NH_4^+ was supplied under aerobic conditions (Kim et al., 2010, Wunderlin et al., 2012). Kim et al. (2010) postulated that electrons that would otherwise be channelled to AMO are directed to the nitrifier denitrification pathway, resulting in increased N_2O production. However, using isotopic analysis, Wunderlin et al. (2012) showed that N_2O produced from NH_2OH fed activated sludge was predominantly from biological NH_2OH oxidation rather than nitrifier denitrification. Nitrifier denitrification increased subsequently only when NH_2OH was gradually depleted (Wunderlin et al., 2012). In addition, the overall NO_2^- production rate of the sludge was slower than that when NH_3 was supplied (Wunderlin et al., 2012).

While an electron bottleneck from the decrease in O_2 availability generally lead to increase in N_2O production by nitrifier denitrification (Stein, 2011a), it is unclear whether electron overloading through the external supply of NH_2OH will trigger the same response in AOB. Under electron overloaded conditions, the presence of electron donors (NH_3 and NH_2OH) and electron acceptors (O_2 and NO_2^-) are expected to alter the N_2O and NO_2^- production of AOB. Therefore, this study aims to characterise the N_2O and NO_2^- production by an enriched AOB culture under continuous supply of NH_2OH, with NH_3, NO_2^- and DO concentration as the most influencing parameters. The AOB culture used was enriched in a partial nitritation system with synthetic digester liquor and therefore has constant exposure to high NH_4^+ and NO_2^- concentration of 500 mg N L^{-1}. Under such condition, the effect of increased NH_2OH loading was investigated at different DO levels (0.55 and 2.0 mg O_2 L^{-1}). The effect of O_2 (0.55 and 1.8 mg O_2 L^{-1}) and NO_2^- (5-1000 mg N L^{-1}) on the N_2O production were further examined in the absence and presence of NH_4^+ (500 mg N L^{-1}). We believe that NH_2OH dosing experiments at different operational conditions on an enriched AOB culture can significantly contribute to the understanding of AOB dependant N_2O production.

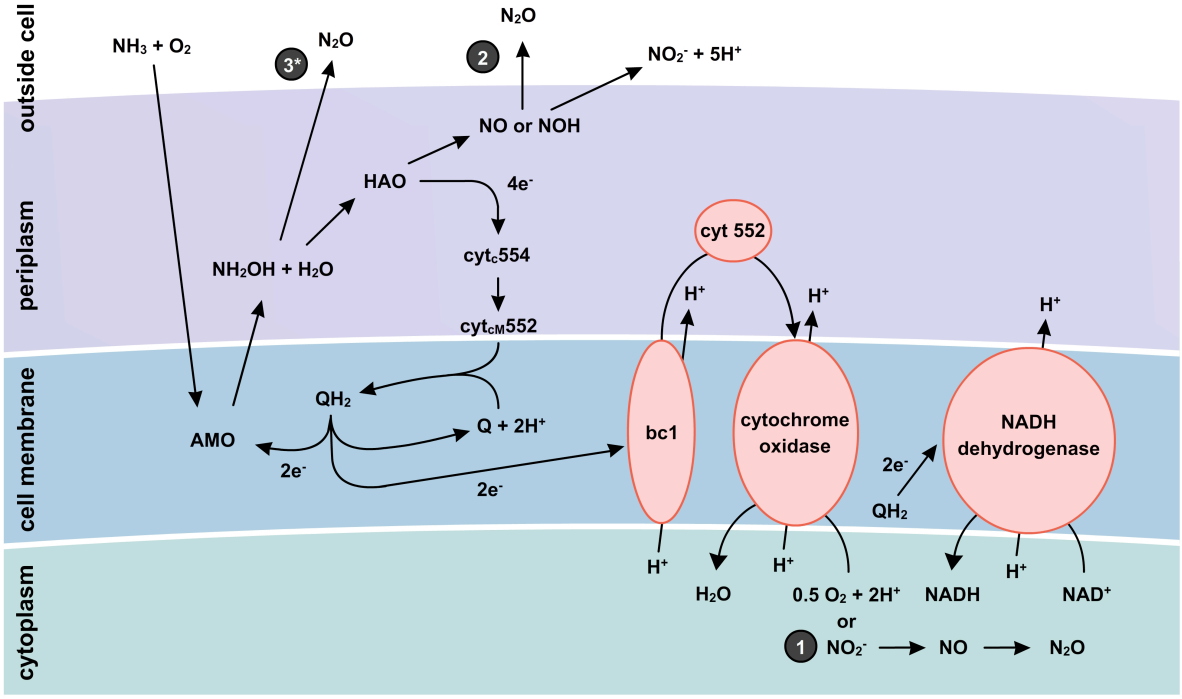

Figure 3.1 Model for the oxidation of ammonia to nitrite by AOB (modified from Arp and Stein (2003), Hooper et al. (1997) and Sayavedro-Soto and Arp (2011)) and known N_2O producing pathways (1-3). 3*: this pathway can be influenced by the concentration of O_2 and NO_2^-. AMO: ammonia monooxygenase; HAO: hydroxylamine oxidoreductase.

2 Material and methods

2.1 Enriched AOB culture

An enriched AOB culture was obtained from an 8 L sequencing batch reactor (SBR) performing nitritation. The SBR was seeded with activated sludge from a domestic wastewater treatment plant in Brisbane, Australia. The mixed liquor temperature was controlled at 33 ± 1 ºC using a water jacket. The SBR was operated in 6h cycles and consisted of 30 min settling, 10 min decanting, 2.5 min feeding I (aeration on), 65 min aerobic reaction I, 92 min idle I, 2.5 min feeding II (aeration on), 65 min aerobic reaction II, 92 min idle II and 1 min wasting (aeration on). The mixed liquor volatile suspended solid (MLVSS) concentration of the SBR was maintained at approximately 0.75 ± 0.05 g L^{-1} with a sludge retention time of 20 days. 2L of synthetic wastewater (composition described below) was fed every cycle giving a hydraulic retention time (HRT) of 1 day. The SBR was fed with synthetic wastewater mimicking characteristics of anaerobic digester liquor. The daily nitrogen load was 1 kg N m^{-3} d^{-1}. The composition of the wastewater (modified from Kuai and Verstraete (1998)) was: 5.63 g L^{-1} of NH_4HCO_3 (1 g L^{-1} NH_4^+-N), 0.064 g L^{-1} of each of KH_2PO_4 and K_2HPO_4 and 2 mL of a trace element stock solution. The trace element stock solution contained: 1.25 g L^{-1} EDTA, 0.55 g L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.40 g L^{-1} $CoCl_2 \cdot 6H_2O$, 1.275 g L^{-1} $MnCl_2 \cdot 4H_2O$, 0.40 g L^{-1} $CuSO_4 \cdot 5H_2O$, 0.05 g L^{-1} $Na_2MoO_4 \cdot 2H_2O$, 1.375 g L^{-1} $CaCl_2 \cdot 2H_2O$, 1.25 g L^{-1} $FeCl_3 \cdot 6H_2O$ and 44.4 g L^{-1} $MgSO_4 \cdot 7H_2O$. The feed had a pH of 8 ± 0.1 and a molar ratio of ammonium to bicarbonate of 1:1. 1 M $NaHCO_3$ was used for pH adjustment at the end of each aerobic phase to maintain pH above 6.4. Details of the reactor design, operation and performance can be found in Law et al. (2011).

At the time the experiments reported below were performed, the SBR had been operated for approximately 30 months and was displaying stable performance. Approximately 55% of the 1 g NH_4^+-N L^{-1} in the feed was converted to NO_2^- at the end of a cycle. Minimal NO_2^- oxidising bacterial (NOB) activity was detected with NO_3^- concentration lower than 20 mg NO_3^- -N L^{-1} at all times, and the N_2O emission factor was about 0.5% of the nitrogen load. Characterisation of the biomass composition using *Fluorescence in situ Hybridization* (FISH) revealed that approximately 80% of the bacterial populations are ammonia-oxidising *beta-proteobacteria,* which was dominated by *Nitrosomonas sp.* (~70%) (Law et al., 2011). The probes used included NEU, NSO1225, NSV443 (Mobarry et al., 1996), NIT3 (Wagner et al., 1996); Ntspa662 (Daims et al., 2001) and EUB-mix.

2.2 Batch experiments

Four series of batch tests were conducted, as summarised in Table 3.1.

Series I- NH_2OH was continuously loaded for 1.5h to a sample of the AOB culture at four different loading rates (0, 0.1, 0.2 and 0.4 mg N min^{-1}) and two DO concentration conditions (0.55 and 2.0 mg O_2 L^{-1}), all in the presence of NH_4^+ and NO_2^- and each at approximately 500 mg N L^{-1}. Each batch test was conducted for 1.5h and triplicated.

Series II- In a single experiment, the NH_2OH loading rate was stepwise increased every 0.5h from 0.1 mg N min^{-1} to 0.2 mg N min^{-1}, 0.4 mg N min^{-1} and finally to 0.8 mg N min^{-1}, in the absence of NH_4^+ and NO_2^-. This experiment was also repeated with biomass-free synthetic medium.

Series III- In the first part (1.6h) of this single experiment, the DO concentration was varied (0.55 to 1.8 to 0.55 mg O_2 L^{-1}) at 0.1 mg N min^{-1} NH_2OH loading in the absence of NH_4^+. In a second part (remaining 1.6h), the DO concentration was varied (0.55 to 1.0 to 1.8 mg O_2 L^{-1}) at 0.1 mg N min^{-1} NH_2OH loading in the presence of 500 mg N L^{-1} NH_4^+.

Series IV- In this single experiment, the NO_2^- concentration was stepwise increased from 0 to finally 1000 mg N L^{-1} at 0.1 mg N min^{-1} NH_2OH loading, both in the absence and presence (500 mg N L^{-1}) of NH_4^+. The experiment in the absence of NH_4^+ was also repeated with biomass-free synthetic medium.

For Test Series I, 0.4 L mixed liquor was withdrawn from the SBR and diluted with 0.6 L of pre-warmed (33^oC) synthetic medium supplemented with 500 mg N L^{-1} of NH_4^+ and NO_2^-. Experiments were then carried out on the 1.0 L diluted mixed sludge. For all batch experiments in Test Series II-IV with biomass, 1.0 L sludge was withdrawn from the SBR and settled to a volume of 0.1 L. The settled sludge was then centrifuged at 4000 rpm for 3 mins and washed with NH_4^+ free synthetic medium (otherwise identical to the feed). The washed sludge was centrifuged and re-inoculated into NH_4^+ and NO_2^- free synthetic medium. All batch experiments were carried out in a completely sealed 1.3 L batch reactor with a gas outlet on the lid. Unless otherwise stated, the pH and DO were maintained at 7.0 ± 0.5 and 0.55 ± 0.05 mg O_2/L, respectively, similar to the conditions applied to the SBR. DO in the

batch reactor was manually controlled using a gas mixture of N_2 and air. The N_2 flow and air flow were adjusted using two mass flow controllers (Smart- Trak 50 series- 1 L min^{-1} and 5 L/min, Sierra). The total gas flow rate in all batch tests was maintained constantly at 1 L min^{-1}. pH was controlled automatically using a programmable logic controller (PLC) system. In test series I, pH was controlled with solely $NaHCO_3$ instead of a mixture with NH_4CO_3 in order to follow up NH_3 oxidation. During experimental periods in Test Series II-IV whereby NH_4^+ was supplemented, a mixture of ammonium bicarbonate (NH_4HCO_3) and $NaHCO_3$ solution, with the NH_4^+ to HCO_3^- molar ratio adjusted to 0.5:1 per L, was used to control the pH and to replenish NH_4^+. Since 2 moles of HCO_3^- are required for the complete conversion of 1 mole of NH_4^+ to NO_2^-, using the pH control system, NH_4^+ could be maintained relatively constant at 500 ± 20 mg N L^{-1} in all experiments. Similar to the SBR, temperature was controlled at 33 ± 1 ^oC using a water jacket in all batch tests.

2.3 Data collection

During all experiments, DO and pH were continuously monitored online using a miniCHEM-DO_2 and pH meter. The gas phase N_2O concentration was also measured online and analysed with a URAS 26 infrared photometer (Advance Optima Continuous Gas Analyser AO2020 series, ABB). A measuring range of 0-100 ppmv was used with a detection limit of 1.0 ppmv. A water trap was installed at the gas inlet of the analyser to prevent moisture from entering into the analyser. A T-shaped tubing joint was fitted to the gas sampling tube connecting the gas outlet of the reactor in order to allow excess gas flow from the reactor to escape from the reactor. By this, the reactor was maintained at atmospheric pressure. The flow of the sampling pump was adjusted to be lower than the total gas flow rate in the reactor at all times.

Mixed liquor samples were taken periodically for NH_4^+, NO_2^-, NO_3^- and NH_2OH analyses. Samples were collected using a syringe and immediately filtered through disposable Milipore filters (0.22 μm pore size). The NH_4^+, NO_2^- and NO_3^- concentrations were analysed using a Lachat QuikChem8000 Flow Injection Analyser (Lachat Instrument, Milwaukee). NH_2OH concentration was measured using a standard colorimetric assay (Frear and Burrell, 1955). At the end of each test, a mixed liquor sample was taken to determine the mixed liquor suspended solids (MLSS) concentration and its volatile fraction (MLVSS). Each sample was analysed in triplicate according to the standard methods (Greenberg, 1992).

2.4 Calculations

For Test Series I, the NH_4^+, NO_2^- and NH_2OH transformation activity was each calculated based on the change in the N concentration from the initial to the end of each batch experiment normalised with the MLVSS concentration (g VSS L^{-1}). The % N converted to N_2O was calculated as the total amount of N_2O produced relative to the amount of NO_2^- produced. For Test Series II, III and IV, the biomass specific NH_2OH or combined NH_4^+ and NH_2OH (in cases where NH_4^+ was supplied) oxidation activity are reported as the specific NO_2^- production rate. This was calculated by normalising the NO_2^- production rate with the MLVSS concentration (g VSS L^{-1}). The N_2O production was either presented as N_2O production rate (mg N_2O-N h^{-1}) which was calculated by multiplying the measured gas phase N_2O concentration (mg N_2O-N L^{-1}) and the known gas flow rate (L h^{-1}) or as specific N_2O production rate (mg N_2O-N h^{-1} g VSS^{-1}) which was calculated by normalising the N_2O production rate with the MLVSS concentration (g VSS L^{-1}). The N_2O production rate was presented in Test Series II and IV to enable comparison with cell-free negative control batch experiments.

Table 3.1 Experimental conditions applied during batch Test Series I-IV.

Experiment	Biomass	pH (-)	NH_2OH (mg N min^{-1})	NH_4^+ (mg N L^{-1})	NO_2^- (mg N L^{-1})	DO (mg O_2 L^{-1})
Test Series I (Fig. 3.2)						
A1	Yes	7.0	0.1	500±50	500±50	0.55
A2	Yes	7.0	0.2	500±50	500±50	0.55
A3	Yes	7.0	0.3	500±50	500±50	0.55
A4	Yes	7.0	0.4	500±50	500±50	0.55
B1	Yes	7.0	0.1	500±50	500±50	2.0
B2	Yes	7.0	0.2	500±50	500±50	2.0
B3	Yes	7.0	0.3	500±50	500±50	2.0
B4	Yes	7.0	0.4	500±50	500±50	2.0
Test Series II (Fig. 3.3)						
(a)	Yes	7.0	0.1-0.2-0.4-0.8	0	0 at start	0.55
(b)	No	7.0	0.1-0.2-0.4-0.8	0	0 at start	0.55
Test Series III (Fig. 3.4)						
Part 1	Yes	7.0	0.1	0	0 at start	0.55-1.8-0.55
Part 2	Yes	7.0	0.1	500±50		0.55-1.0-1.8
Test Series IV (Fig. 3.5)						
(a)	Yes	7.0	0.1	0	0-100-200-400-600-800-1000	0.55
(b)	Yes	7.0	0.1	500±50	0-100-200-400-600-800-1000	0.55
(c)	No	7.0	0.1	0	0-200-400-600-800-1000	0.55

3 Results

3.1 Test Series I

N transformation at different NH_2OH loading rates in the presence of NH_4^+ and NO_2^- and the influence of DO

Fig. 3.2 shows the effect of (A) low (0.55 mg O_2 L^{-1}) and (B) high (2.0 mg O_2 L^{-1}) DO concentration on the N transformation activity at different NH_2OH loading rates. In all cases, the higher DO concentration resulted in a higher NH_4^+ oxidation and NO_2^- production activity. The total NH_4^+ oxidised and NO_2^- produced varied between 250-400 mg N g VSS^{-1} at a DO concentration of 2.0 mg O_2 L^{-1} as compared to 30-250 mg N g VSS^{-1} at a DO concentration of 0.55 mg O_2 L^{-1}. In addition, the increasing NH_2OH loading rate did not have a significant effect on the NH_4^+ oxidation and NO_2^- production activity at DO concentration of 2.0 mg O_2 L^{-1}. However, at a lower DO concentration, an overall decreasing trend was observed on the average NH_4^+ oxidised and NO_2^- produced with increasing NH_2OH loading rate. At the highest applied NH_2OH loading rate of 0.4 mg N min^{-1}, the ammonia oxidation activity significantly ($p < 0.05$) decreased by over 90% compared to that in the absence of external NH_2OH supply (Fig 3.2, A1 & A4). Similarly, the NH_2OH loading rate of 0.4 mg N min^{-1} only significantly decreased the oxidation activity of externally supplied NH_2OH at low DO concentration while the NH_2OH oxidation activity remained high at higher DO concentration of 2.0 mg O_2 L^{-1}.

Both the DO concentration and the externally supplied NH_2OH also had a clear effect on the N_2O emission factor. Firstly, in both DO cases, the N_2O emission factor increased with increasing NH_2OH loading rate. Secondly, the increase of the N_2O emission factor was 3-6 fold higher in the case of DO concentration of 0.55 mg O_2 L^{-1} compared to the case of 2.0 mg O_2 L^{-1}. Thirdly, In the absence of external NH_2OH supply, the N_2O emission factor was higher at DO of 2.0 mg O_2 L^{-1} (~0.51% N converted to N_2O) compared to that at DO of 0.55 mg O_2 L^{-1} (~0.21% N converted to N_2O), but were still 10-100 fold lower compared to the cases were NH_2OH was supplied.

In case of NH_2OH loading, the nitrite production was generally higher than the amount of NH_4^+ oxidised as a major part of the supplied NH_2OH was oxidised to NO_2^-. On average, nitrogen mass balances (consumed/produced) could be closed for 109±6 %.

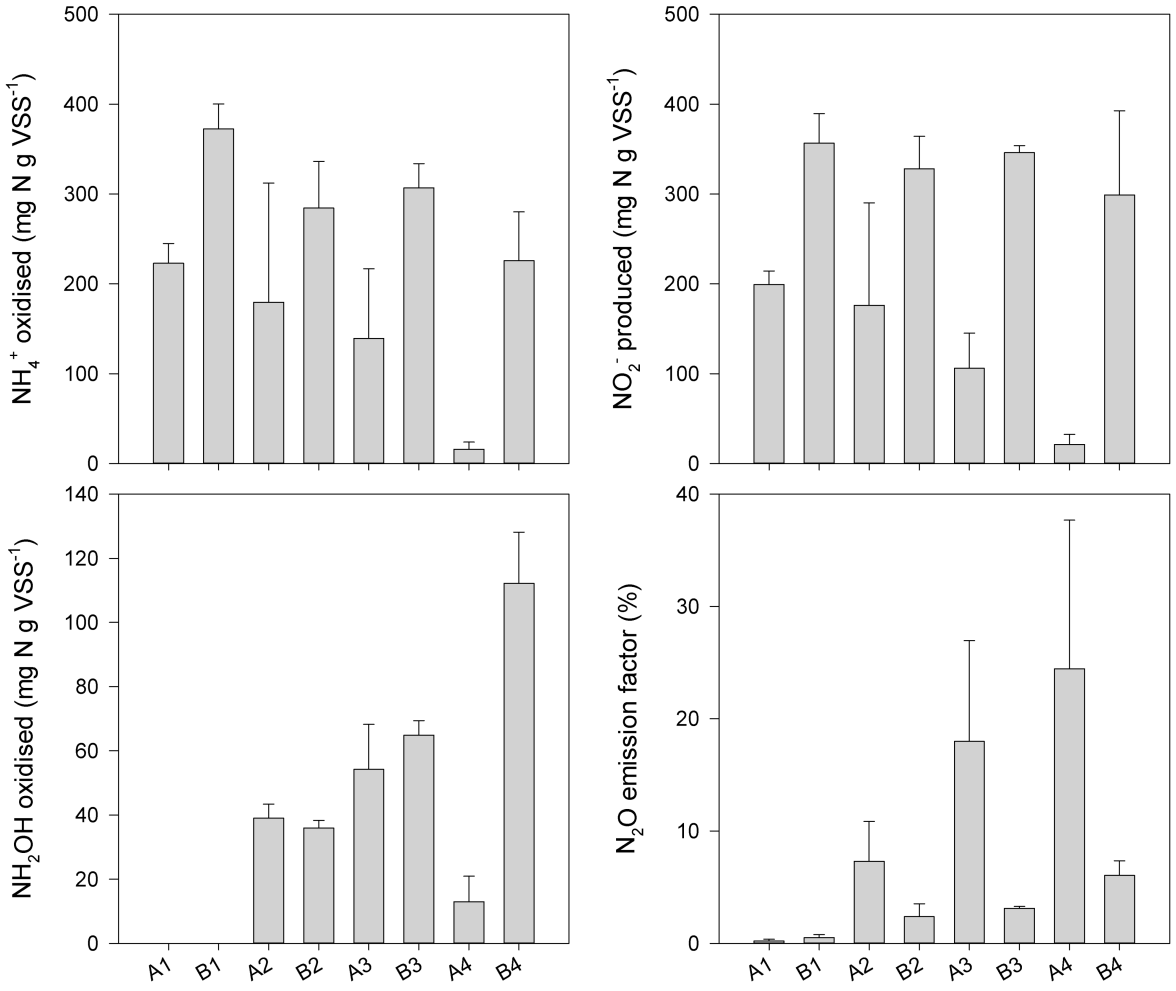

Figure 3.2 Effect of DO (0.55 versus 2 mg O_2 L^{-1}) on N conversion at different hydroxylamine loading rates in presence of ammonium and nitrite (starting concentration of 500 ± 50 mg N L^{-1}). Each batch test was performed for 1.5h. A: 0.55 mg O_2 L^{-1}, B: 2 mg O_2 L^{-1}. 1 to 4 means no NH_2OH, 0.1 mg N min^{-1}, 0.2 mg N min^{-1} and 0.4 mg N min^{-1}, respectively.

3.2 Test Series II

The influence of increasing NH_2OH loading on the N_2O and NO_2^- production rates in the absence of NH_4^+

In the absence of NH_4^+, the N_2O production rate also increased with the stepwise increase in the NH_2OH loading rate. With each step increase, a new pseudo-steady state N_2O production rate and NH_2OH concentration was achieved (Fig. 3.3a). The N_2O production rate increased from 0.2 up to 0.8 mg N_2O-N h^{-1} (corresponding to a biomass specific rate of 0.4-1.6 mg N_2O-N h^{-1} g VSS^{-1}) at the highest NH_2OH loading rate of 0.8 mg N min^{-1}. However, no clear effect could be observed on the NO_2^- production rate. The specific NO_2^- production rate remained relatively low (10-14 mg NO_2^--N h^{-1} g VSS^{-1}) despite an increase in NH_2OH

loading from 0.1-0.8 mg N min^{-1}. The relatively constant NO_2^- production rate resulted in the accumulation of NH_2OH up to 25 mg N L^{-1}. Therefore, an NH_2OH loading rate of 0.1 mg N min^{-1} was chosen for the remaining test series. The control experiment (Fig. 3.3b) confirmed that the N_2O produced was predominantly from biological activity as the N_2O production rate of <0.1 mg N_2O-N h^{-1} was 4-16 times lower compared to the biological experiment and did not increase with increased NH_2OH loading.

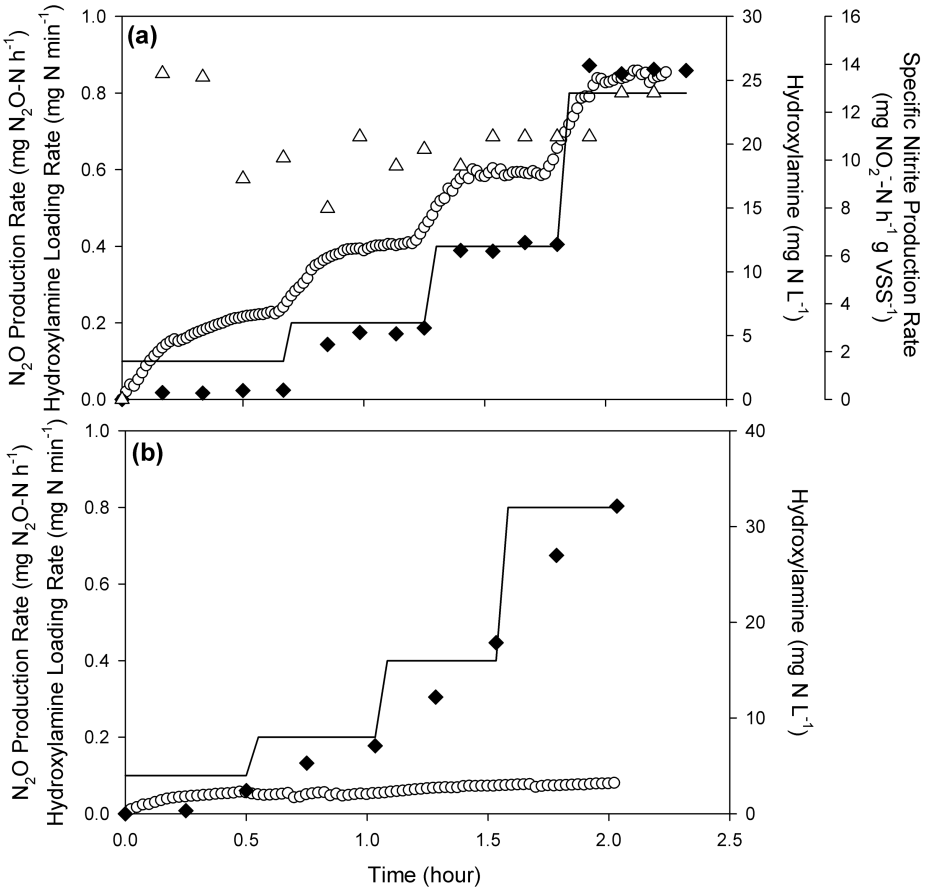

Figure 3.3 The effect of increasing hydroxylamine loading rate on N_2O production in (a) the enriched AOB culture and (b) synthetic medium. Nitrite from the production by AOB was less than 20 mg N L^{-1} in (a). In (a) and (b): specific nitrite production rate (triangles), gas phase N_2O (circles), hydroxylamine (diamonds) and hydroxylamine loading rate (solid line). DO concentration was controlled at 0.55 mg O_2 L^{-1}.

3.3 Test Series III

The influence of DO concentration on the N_2O and NO_2^- production rates with continuous NH_2OH loading in the presence or absence of NH_4^+

The increase in the DO concentration had an effect on the N_2O production rate in the presence of NH_4^+ but not the in the absence of NH_4^+ (Fig. 3.4), with a continuous supply of NH_2OH at 0.1 mg N min^{-1}. When NH_4^+ was absent, the specific N_2O production rate stayed relatively constant at 0.3 mg N_2O-N h^{-1} g VSS^{-1} when DO concentration was increased from 0.55 to 1.8 and decreased back to 0.55 mg O_2 L^{-1} (Fig. 3.4a). When 500 mg N L^{-1} of NH_4^+ was supplied, the N_2O production rate increased immediately 10-fold to 2.7 mg N_2O-N h^{-1} g VSS^{-1}. When the DO concentration was increased step-wise from 0.55 to 1.0 and 1.8 mg O_2 L^{-1}, the specific N_2O production rate decreased progressively down to 1.9 and 1.4 mg N_2O-N h^{-1} g VSS^{-1}, respectively.

The presence or absence of NH_4^+ also had a clear effect on the NO_2^- production rate. Upon the addition of NH_4^+, an apparent increase in the NO_2^- production rate could be observed (Fig. 3.4b). The specific NO_2^- production rate increased from 7.0 to 75 mg NO_2^--N h^{-1} g VSS^{-1} and further increased to 160 mg NO_2^--N h^{-1} g VSS^{-1} with the increase in DO concentration.

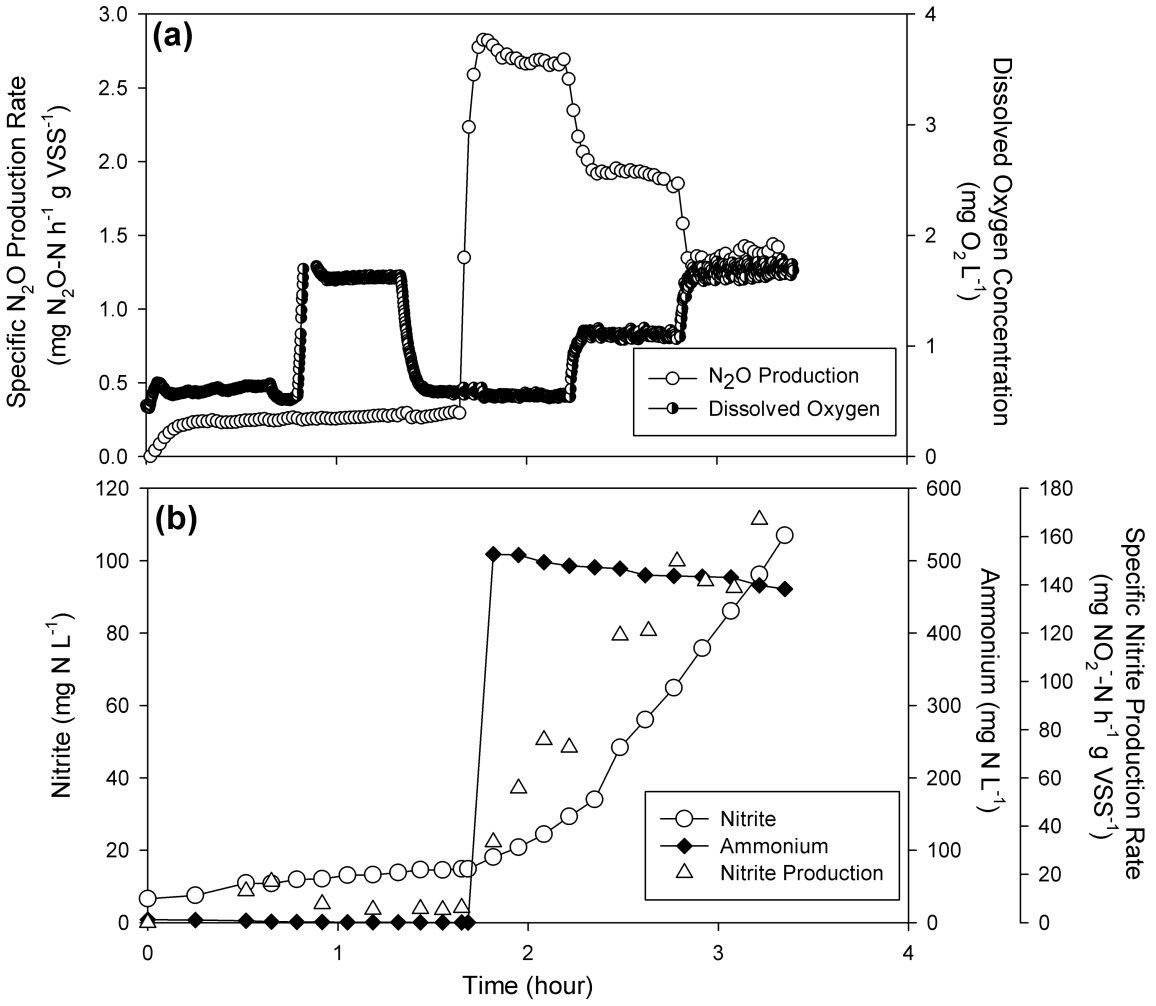

Figure 3.4 The effect of dissolved oxygen concentration on the N_2O production by enriched AOB culture in the absence of ammonium from 0 to 1.6 hour and after ammonium addition. Hydroxylamine was continuously loaded at 0.1 mg N min^{-1} and maintained at concentration of less than 1.0 mg N L^{-1} throughout the test.

3.4 Test Series IV

The Influence of NO_2^- concentration on the N_2O and NO_2^- production rate with continuous NH_2OH loading in the presence or absence of NH_4^+

Similar to Test Series III, NO_2^- also had different effects on N_2O and NO_2^- production when NH_4^+ and NH_2OH were both present (Fig. 3.5b) compared to the case where only NH_2OH was supplied (Fig. 3.5a). In the absence of NH_4^+, a pseudo-steady state N_2O production rate of 0.25 mg N_2O-N h^{-1} (specific rate of 0.5 mg N_2O-N h^{-1} g VSS^{-1}) was reached without external NO_2^- dosing. An increase in the N_2O production rate up to 0.55 mg N_2O-N h^{-1} (specific rate of 1.2 mg N_2O-N h^{-1} g VSS^{-1}) was observed when NO_2^- concentration was

increased to 1000 mg N L^{-1} (Fig. 3.5a). In contrast, when NH_2OH and NH_4^+ were co-supplied, a higher pseudo-steady state N_2O production rate of 1.5 mg N_2O-N h^{-1} (specific rate of 3.6 mg N_2O-N h^{-1} g VSS^{-1}) was achieved prior to any NO_2^- dosing (Fig. 3.5b). Furthermore, the step-wise increase in NO_2^- concentration above 200 mg N L^{-1} resulted in a decrease in N_2O production rate down to 1.1 mg N_2O-N h^{-1}. However, further increase in NO_2^- concentration between 600-1000 mg N L^{-1} caused a slight increase in the N_2O production rate up to 1.3 mg N_2O-N h^{-1}.

Similarly, the specific NO_2^- production rate was clearly dependent on the presence of NH_4^+. In the absence of NH_4^+, the specific NO_2^- production rate varied between 13-20 mg NO_2^--N h^{-1} g VSS^{-1} throughout the tested NO_2^- concentration range. Conversely, in the presence of NH_4^+, the specific NO_2^- production rate was significantly higher and increased to approximately 135 mg NO_2^--N h^{-1} g VSS^{-1} with increasing NO_2^- levels up to 500 mg N L^{-1}. A plateau was reached and no further increase was observed with increasing NO_2^- concentration above 500 mg N L^{-1}.

In the cell-free control experiment (Fig. 3.5c), the N_2O production rate did not have a clear step-wise increase or decrease trend with the increasing NO_2^- concentration. However the N_2O production rate increased gradually from 0.2 to 0.5 mg N_2O-N h^{-1} when NO_2^- concentration increased to 1000 mg N L^{-1}.

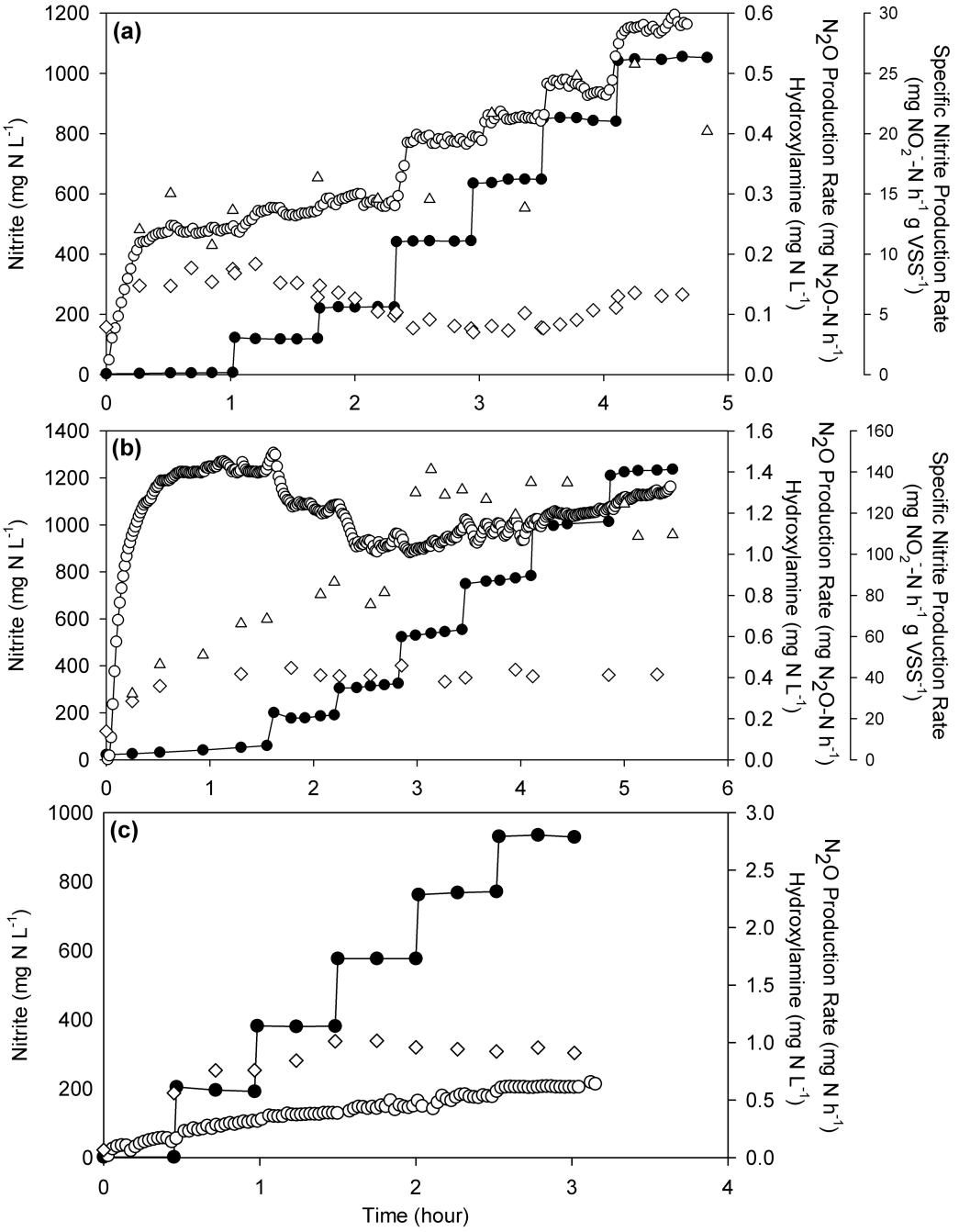

Figure 3.5 The effect of nitrite dosing on (a) enriched AOB culture with continuous hydroxylamine addition (0.1 mg N min^{-1}); (b) enriched AOB culture with continuous hydroxylamine addition (0.1 mg N min^{-1}) and 500 ± 50 mg NH_4^+-N L^{-1} maintained from the start; and (c) synthetic medium only with hydroxylamine manually maintained at concentration of < 1 mgN L^{-1}. Ammonium was not present in (a) and (c). In (a), (b) and (c): N_2O production rate (white circles), nitrite (black circles), hydroxylamine (diamonds) and specific nitrite production rate (triangles). DO concentration was controlled at 0.55 mg O_2 L^{-1}.

4 Discussion

4.1 Effect of NH_2OH overloading on the NO_2^- and N_2O production

Under conditions with ample availability of NH_4^+ and NO_2^- (500 ± 50 mg N L^{-1}), the external supply of NH_2OH to the AOB culture had a strong negative effect on the NH_4^+ oxidation/ NO_2^- production activity when operated at a DO concentration of 0.55 mg O_2 L^{-1} (Fig. 3.2). This could not be explained by NH_2OH toxicity as AOB have been reported to be tolerant to NH_2OH (Stein, 2011b). Recent studies have applied NH_2OH concentrations of 10-70 mg N L^{-1} to activated sludge without reporting on toxicity effects or inhibition on the ammonia oxidation activity (Harper et al., 2009, Kim et al., 2010, Wunderlin et al., 2012). For comparison, the average level accumulated at the end of the 1.5h batch experiments with highest NH_2OH loading of 0.4 mg N min^{-1} was 23±8 mg N L^{-1}. At a higher DO concentration of 2.0 mg O_2 L^{-1}, the NH_4^+ oxidation/ NO_2^- production activity did not show such a dramatic decrease (Fig. 3.2). The average NH_2OH level achieved (14±3 mg N L^{-1}) was not significantly different compared to the NH_2OH level during the low DO experiment ($p > 0.05$). Therefore, we believe that toxicity was not playing a major role. More likely, NH_2OH overloading resulted in an electron bottleneck in the electron transport chain, thereby slowing down the overall NH_4^+ conversion rates. As such, the AOB culture was thermodynamically constrained as all the intermediate electron transport components were likely fully reduced. Oxygen can to a certain extent alleviate this electron jam by acting as a terminal electron acceptor. However, a DO concentration of 0.55 mg O_2 L^{-1} was clearly too low. Indeed, a higher DO of 2.0 mg O_2 L^{-1} allowed to maintain a significantly higher NH_4^+ oxidation/ NO_2^- production activity (Fig. 3.2).

The overloading of NH_2OH also resulted in interesting dynamics of N_2O production. While the data reported cannot deliver conclusive results regarding the exact contribution of each N_2O production pathway, the results suggest that both the nitrifier denitrification and NH_2OH oxidation-based pathways were operational. At the DO concentration of 0.55 mg O_2 L^{-1}, the increasing NH_2OH load evidently promoted the N_2O production (Fig. 3.2 and Fig. 3.3). This is in agreement with Stein (2011a) who proposed that *N. europaea* utilise NO_2^- and NO as alternative electron acceptors to facilitate electron flow under conditions of electron congestion. In addition, the observed N_2O production was clearly oxygen sensitive, which points to the likely involvement of nitrifier denitrification in the presence of NH_4^+. The nitrifier denitrification pathway has been reported to decrease in activity with increasing DO

concentration (Anderson et al., 1993, Sutka et al., 2006). At increased DO concentration, the decrease in N_2O production coincided with an overall increase in NH_4^+ oxidation/ NO_2^- production activity (Fig. 3.2). This highlights once again that O_2 is able to alleviate the electron jam, and NO_2^- acts as a supplementary electron acceptor only if O_2 becomes limiting. The significant reduction of 2-6 fold in the N_2O emission factor at elevated DO concentration also suggests that the majority of N_2O was produced through nitrifier denitrification under NH_2OH overloaded conditions.

However, in case where no NH_2OH was supplied, the N_2O emission factor was higher at a higher DO concentration (Fig. 3.2). Using the same AOB culture, Law (2012a) observed that a higher DO concentration resulted in a higher ammonia oxidation rate which in turn exponentially increased the N_2O production rate through biological NH_2OH oxidation (pathway 2, Fig. 3.1). Stein (2011b) also reported that N_2O production from biological NH_2OH oxidation is favoured under increased DO concentration. Wunderlin et al. (2013) clearly demonstrated with isotopic analysis that N_2O production from biological NH_2OH oxidation is also active when 10 mg L^{-1} of NH_2OH is supplied to an activated sludge in the presence of NH_4^+. Therefore when NH_2OH was co-supplied, N_2O production from biological NH_2OH oxidation was also likely active though nitrifier denitrification was likely the dominant N_2O production pathway.

4.2 The importance of NH_4^+ in regulating the NO_2^- and N_2O production

While the co-supply of NH_2OH and NH_4^+ magnifies in particular nitrifier denitrification, this pathway did not appear to be functional when NH_2OH was solely supplied. This resulted in contradicting outcomes on the responses of the N_2O production activity with changes to key process conditions such as DO and NO_2^- concentration. When NH_2OH was co-supplied with NH_4^+, the N_2O production was susceptible not only to elevated DO concentration (Fig. 3.4) but also to high NO_2^- concentration (Fig. 3.5b). Using the same AOB culture, the activity of the main N_2O production pathway was also shown to decrease with increasing DO and NO_2^- concentration when NH_4^+ was provided as the sole electron donor (Law et al., 2013). Due to the sensitivity of the N_2O production pathway to O_2, it was postulated to be nitrifier denitrification. However, the N_2O production was not affected by DO concentration when NH_2OH was provided as the sole electron donor (Fig. 3.4). Also, the effect of NO_2^- on the N_2O production was quite different (Fig. 3.5 a and b) and most likely due to chemical NH_2OH oxidation in case NH_4^+ was absent (Fig. 3.5a)., The overall N_2O production rate was 7-8 folds lower than that when NH_4^+ is co-present and pinpoints the absence of a significant N_2O

production pathway, nitrifier denitrification. Since nitrifier denitrification activity in this study was shown to be active mainly under electron overloading condition, the lack of NH_2OH consumption resulted in the redundancy of the pathway and therefore was not functional when NH_2OH was present as the sole electron donor. The observed N_2O produced was mainly from biological NH_2OH oxidation with a relatively small contribution from chemical NH_2OH oxidation (Fig. 3.3).

The relatively low N_2O production rate also coincided with a low NO_2^- production activity. The NO_2^- production rate increased by approximately 10 fold when NH_4^+ was added to the AOB culture continuously fed with NH_2OH (Fig. 3.4). Although the oxidation of NH_2OH to NO_2^- is the sole electron donating step in the metabolism of AOB, the increased supply of NH_2OH did not stimulate the NO_2^- production rate, and the increased amount of N converted to N_2O is postulated to be derived from incomplete NH_2OH oxidation (Fig. 3.3a). It is possible that the lack of electron consumption by AMO, in the absence of NH_4^+ resulted in an electron bottleneck between HAO and the internal electron shuttles, thus significantly slowed down the overall NO_2^- and N_2O production rate. However, unlike in the batch test with the presence of NH_4^+ (Fig. 3.2), an increase in DO supply was still unable to speed up the NO_2^- production rate (Fig. 3.4). The activity of the AOB is at bear minimal with low levels of NH_2OH consumption activity as detected by the NO_2^- production rate, and neither the presence of more electron donor in the form of NH_2OH nor electron accepter in the form of O_2 or NO_2^- could increase its activity. According to the Michaelis-Menten equation for enzyme kinetics, the maximum reaction rate (Vmax) is low and the controlling factor as an increase of substrate (NH_2OH) concentration did not have an effect. The low overall reaction rate might therefore be a result of insufficient HAO turnover, and affects Vmax. The supply of NH_3 to a starved *N. europaea* pure culture has been shown to increase the mRNA levels of AMO and HAO (Sayavedra-Soto et al., 1996). In addition, in a *N. europaea* pure culture, NH_3 was required for the *de novo* synthesis of polypeptides, despite the presence of NH_2OH as an electron source (Hyman and Arp, 1995). It is possible that, in the absence of NH_3, genes coding for NH_2OH oxidation are under expressed causing the overall reduction in N transformation activity. However, this should be confirmed with transcriptomic analysis.

Collectively, our findings suggest that NH_4^+ has physiological importance and its presence is mandatory for high NO_2^- and N_2O production activity. However, it is not possible to conclusively determine the specific regulatory role of NH_4^+ in the N transformation metabolic pathways of AOB in this study.

5 Conclusions

Continuous NH_2OH loading in the presence and absence of ammonium rendered interesting observations, which contribute to the current understanding of ammonia oxidation by AOB and its related N_2O production pathways:

- NH_2OH overloading in the presence of ammonium slows down overall N transformation activity, and can be partly alleviated by providing higher DO concentrations;
- In the presence of ammonium, nitrifier denitrification is likely the dominant N_2O producing pathway during co-supply of NH_2OH, whilst the NH_2OH oxidation pathway was considered dominant when NH_2OH was supplied in the absence of NH_4^+;
- The effects of DO and NO_2^- on the NO_2^- and N_2O production rates are different in the presence and in the absence of NH_4^+, suggesting that NH_4^+ has a strong physiological role for ammonia oxidizing bacteria;
- The absence of NH_4^+ during NH_2OH loading results in a 10-fold lower ammonia oxidation rate, likely produced by the NH_2OH oxidation related pathways.

6 Acknowledgements

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Chapter 4 Biocathodic nitrous oxide removal in a bioelectrochemical system

Abstract

Nitrous oxide (N_2O) emission from humane sewage treatment is estimated to contribute up to 3.2% of the total anthropogenic N_2O emission. Furthermore, the contribution from BNR treatment of manure and industrial effluents likely contributes significantly more. Therefore, mitigation of these emissions is warranted since N_2O is a strong greenhouse gas and important ozone-depleting compound. Hence, its impact on the carbon footprint of the wastewater treatment plant can be very significant. Until now, only physicochemical technologies have been applied to mitigate point sources of N_2O, and no biological treatment technology has been developed so far. In this study, a bioelectrochemical system (BES) with an autotrophic denitrifying biocathode was considered for the removal of N_2O. The high N_2O removal rates obtained ranged between 0.76 and 1.83 kg N m^{-3} Net Cathodic Compartment (NCC) d^{-1} and were proportional to the current production, resulting in cathodic coulombic efficiencies near 100%. Furthermore, our experiments suggested the active involvement of microorganisms as the catalyst for the reduction of N_2O to N_2, and the optimal cathode potential ranged from -200 to 0 mV vs Standard Hydrogen Electrode (SHE) in order to obtain high conversion rates. Successful operation of the system for more than 115 days with N_2O as the sole cathodic electron acceptor strongly indicated that N_2O respiration yielded enough energy to maintain the biological process. To our knowledge, this study provides for the first time proof of concept of biocathodic N_2O removal at long-term without the need for high temperatures and expensive catalysts.

Chapter redrafted after: *Desloover, J., Puig, S., Virdis, B., Clauwaert, P., Boeckx, P., Verstraete, W. & Boon, N. (2011). Biocathodic nitrous oxide removal in bioelectrochemical systems. Environmental Science & Technology, 45(24), 10557-10566.*

1 Introduction

The contribution of N_2O emissions derived from biological nitrogen removal to the total anthropogenic N_2O emission has been addressed previously (Chapter 1). Furthermore, it has been demonstrated that the impact on the CO_2 footprint on the scale of the wastewater treatment plant itself can be very significant (Chapter 1 and 2). Next to preventive measures, also curative treatment technologies need to be explored to mitigate these emissions. However, until now only physicochemical removal technologies have been developed such as thermal decomposition and selective catalytic reduction, requiring high temperatures (500-1000°C) and expensive catalysts (Centi et al., 2000, Kapteijn et al., 1996). Therefore, a curative biological treatment technology for N_2O removal was investigated in this chapter.

Bioelectrochemical systems (BES) with a denitrifying biocathode have been described previously and can provide a more sustainable alternative to physicochemical approaches, since N_2O is an intermediate of the denitrification pathway (Clauwaert et al., 2007a, Virdis et al., 2008). The latter comprises the stepwise reduction of oxidized nitrogen compounds nitrate and nitrite (NO_3^- and NO_2^-) to nitric oxide (NO), nitrous oxide (N_2O), and dinitrogen gas (N_2), performed by denitrifying microorganisms (Zumft, 1997). BES are electrochemical devices where the oxidation of an electron donor at the anode is coupled with the reduction of an electron acceptor at the cathode, using bacteria to catalyse one or both reactions (Rabaey and Rozendal, 2010). In a standard configuration, anode and cathode compartments are separated by an ion exchange membrane. The concept of using a solid-state electrode to supply electrons for biological nitrate reduction to nitrite was reported for the first time by Gregory and co-workers (Gregory et al., 2004). However, it has been only in recent years that the cathodic bioelectrochemical denitrification has been coupled with a bioanode to supply electrons (Clauwaert et al., 2009, Clauwaert et al., 2007a, Virdis et al., 2010, Virdis et al., 2008, Virdis et al., 2009, Xie et al., 2011). In particular, the reduction of N_2O to N_2 represents a respiratory process in its own (reaction 1) (Zumft, 1997). Yet, this final denitrification step has not been investigated in detail at a biocathode (R. 4.1).

$$N_2O + 2\ e^- + 2H^+ \rightarrow N_2 + H_2O; \quad E°' = +1.36\ V\ vs\ SHE\ (\Delta G°' = -262\ kJ \cdot mol^{-1}) \quad (R.\ 4.1)$$

From a thermodynamic point of view, N_2O should be a more favourable electron acceptor compared to the other oxidized nitrogen species of the denitrification pathway (Thauer et al.,

1977). However, the denitrifying microbial community is not often prone to take advantage of this since significant amounts of N_2O can be released in microbiological active environments (Richardson et al., 2009). At present, a strong debate exists in regards to the biochemical energy conservation of this reaction (Richardson et al., 2009, Richardson, 2000, Wasser et al., 2002, Zumft, 1997).

Given the current lack of knowledge existing in regards to biocathodic N_2O reduction, this study aimed at investigating whether N_2O can be effectively removed by a denitrifying biocathode. Therefore, the performance and efficiency of a N_2O reducing biocathode was characterized in terms of its volumetric removal rate, current production and cathodic coulombic efficiency. Furthermore, the possible role of microorganisms present in the cathode was indicated by experiments at open circuit, fixed cathodic potential and abiotic controls. Finally, the advantage of biocathodic N_2O removal and the main challenges for further development are discussed.

2 Material and methods

2.1 BES construction

A 2-chambered BES was made of two polycarbonate frames (8.0 x 8.0 x 1.9 cm^3) placed side by side. Anodic and cathodic compartments (0.121 L each) were filled with granular graphite (type 00514, diameter 1.5-5 mm, Mersen, Wemmel, Belgium). As a result, the net anodic (NAC) and net cathodic (NCC) compartment, was 0.060 L for each compartment. Contact to the external electrical circuit was guaranteed by placing two graphite rods (5 mm diameter, Morgan, Belgium) in intimate contact with the granular matrix. A cation exchange membrane (CEM; Ultrex CMI7000, Membranes International Inc., USA) was used to separate the anodic and cathodic compartments. The same BES was used during the entire experimental period except for the abiotic cathode controls for which an identical cell was used with similar anode operating conditions.

2.2 Inoculum and synthetic medium

The anodic compartment was inoculated with anode effluent from an already active BES treating acetate present in our laboratories, whilst the cathodic compartment was inoculated with activated nitrifying denitrifying sludge (Ossemeersen municipal WWTP, Ghent, Belgium). The anodic and cathodic liquid streams consisted of an autoclaved and nitrogen-

purged modified M9 medium with addition of trace elements as previously described (Clauwaert et al., 2007a). No nitrogen source was added in the M9 medium.

2.3 Operational conditions

The experimental study was divided into three main periods, comprising an 82-days continuous feeding period with nitrate as the electron acceptor at the cathode (day 0-81), followed by a batch feeding period of 70 days with N_2O as the electron acceptor (day 82-151) and finally a continuous N_2O feeding period of 46 days (day 152-197). The anode was always fed continuously with sodium acetate and M9 medium. A summary of the operational conditions is given in Table 4.1 and a scheme of the reactor setup is presented in Fig 4.1.

Table 4.1 Summary of the operational conditions applied to the BES during 197 days of operation. N/A: Not Applicable; HRT: Hydraulic Retention Time; Cont.: Continuous.

Time (days)	Description	Anode				Cathode				External resistance (Ω)
		Mode	Electron Donor	Loading rate (kg COD m^{-3} NAC d^{-1})	HRT (h)	Mode	Electron Acceptor	Loading rate (kg N m^{-3} NCC d^{-1})	HRT (h)	
0-81	**Continuous nitrate feeding period** (Table 4.2)	Cont.	Acetate	1.19	1.6	Cont.	Nitrate	0.21	1.6	5 (d 0-69) 10 (d 69-82)
82	**Batch test nitrate** (Fig. 4.2)	Cont.	Acetate	2.39	1.6	Batch	Nitrate	N/A	N/A	10
82-151	**Batch tests N_2O**									
85	**A**: Gas + liquid phase N_2O removal (Table 4.2 & Fig. 4.3)	Cont.	Acetate	2.39	1.6	Batch	N_2O	N/A	N/A	10
120	**B**: 2h open and closed circuit (Table 4.2 & Fig. 4.4)	Cont.	Acetate	2.39	1.6	Batch	N_2O	N/A	N/A	10
125	**C**: 24 h open circuit (Table 4.2 & Fig. 4.5)	Cont.	Acetate	2.39	1.6	Batch	N_2O	N/A	N/A	10
140	**D**: 24 h open and closed circuit with abiotic cathode (Table 4.2 & Fig. 4.6)	Cont.	Acetate	2.39	1.6	Batch	N_2O	N/A	N/A	10
110	**E**: Fixed cathode potential (Table 4.2 & Fig. 4.7)	Cont.	Acetate	2.39	1.6	Batch	N_2O	N/A	N/A	Potentiostat
152-197	**Continuous N_2O feeding period** (Table 4.2)	Cont.	Acetate	2.39	1.6	Cont.	N_2O	2.10	7.3	10

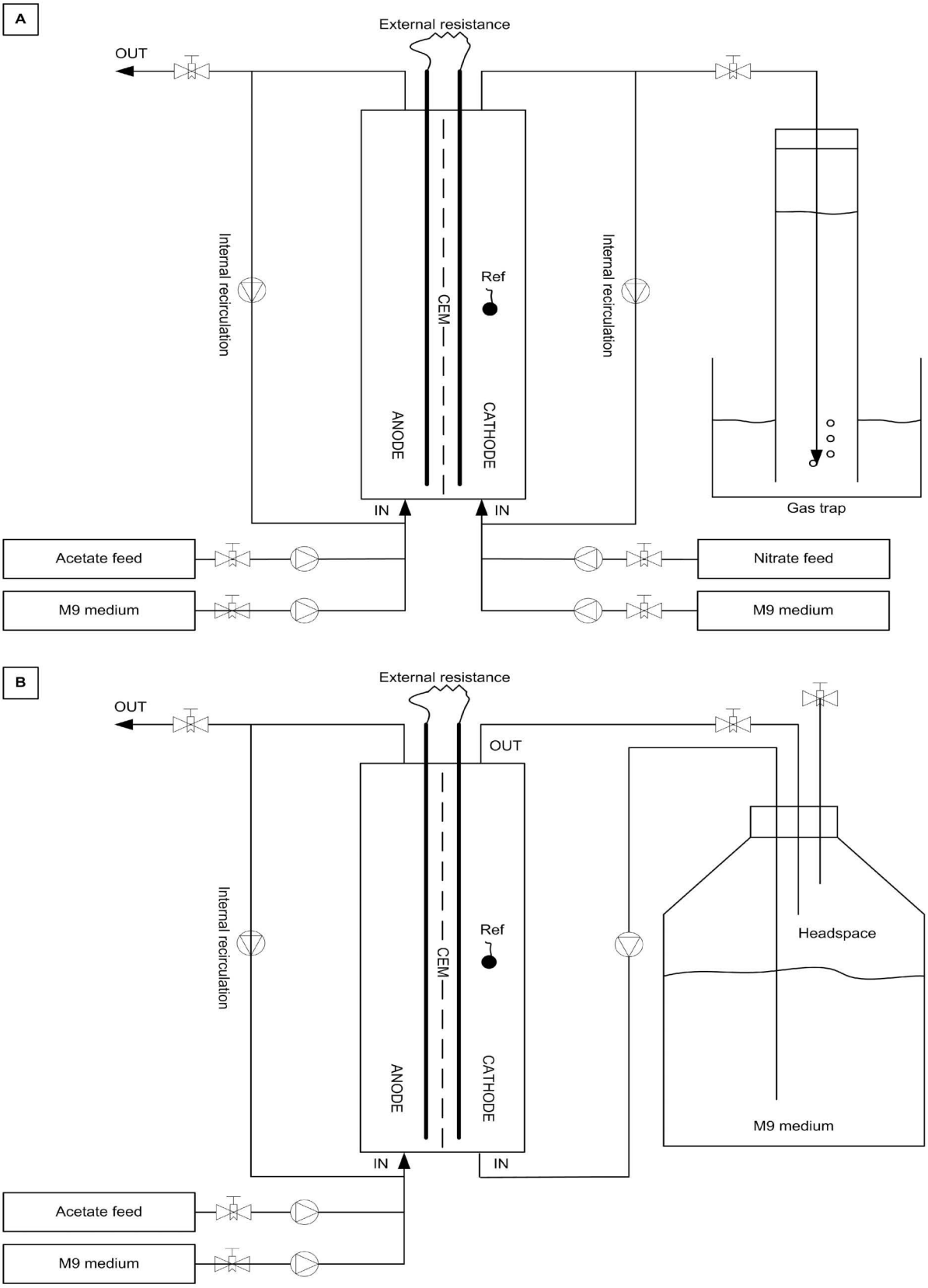

Figure 4.1 Scheme of the reactor setup during the continuous nitrate feeding period (A) and the experimental period during which all the batch tests were performed (B).

2.3.1 Continuous nitrate feeding period

A continuous nitrate feeding period was maintained in order to develop a denitrifying biocathode. During the nitrate-feeding period (day 0-82), both anode and cathode compartments were operated continuously. In order to obtain the desired loading rate, concentrated solutions of sodium acetate (1.11 g L^{-1}) and sodium nitrate (2.07 g L^{-1}) were injected with a syringe pump (8.3 mL d^{-1}) in the influent stream (M9 medium; 0.9 L d^{-1}) of the anode and cathode compartment, respectively. The hydraulic retention time (HRT) for each compartment was 1.6 hours (Table 4.1). To minimize concentration polarization in the anode, the loading rate of the anode was always twice as much as the loading rate of the cathode on a coulombic basis, giving 1.19 kg COD m^{-3} NAC d^{-1} and 0.21 kg NO_3^--N m^{-3} NCC d^{-1} for the anode and cathode compartment, respectively. By doing so, the anode was expected to be non-limiting in the performance of the BES, and was verified by performing polarization curves on a weekly basis. Finally, in order to guarantee well-mixed conditions within the compartments and thus avoid concentration gradients, both anolyte and catholyte were recirculated at a rate of 2.4 L h^{-1}. In order to collect the gases that would have accumulated during the operation of the reactor, the effluent tubes of both compartments were connected to a gas trap.

2.3.2 Batch operation tests with nitrate and N_2O

Batch operation tests with nitrate or N_2O were performed in the cathode in order to assess the production and removal of denitrification intermediates (nitrate, nitrite and N_2O; nitrate batch test), and to characterize the performance and efficiency of the biocathode under conditions where N_2O is the sole electron acceptor present (batch operation tests with N_2O).

Therefore, from day 82nd onwards, the cathode compartment was switched to a batch operation mode, whilst the anode operation remained continuous at an acetate loading rate of 2.39 kg COD m^{-3} NAC d^{-1}. Prior to each batch test, residual oxidized nitrogen species present in the catholyte and recirculation vessel were removed by maintaining the system at closed circuit. The latter was verified by measuring the concentration of nitrate, nitrite and N_2O, and also by allowing the cell voltage to reach almost zero (5 - 10 mV). Meanwhile, autoclaved M9 medium was prepared in a new recirculation vessel and flushed with nitrogen gas for 15 minutes prior to connection with the cathode compartment. The latter rendered a total cathodic liquid and headspace volume of 0.860 and 0.350 L, respectively. Approximately 30 minutes following connection, the desired amount of nitrogen was injected

in the recirculation vessel by dosing a certain volume of a concentrated solution of sodium nitrate (nitrate batch test), or by adding a volume of 100% N_2O gas in the headspace of the recirculation vessel with a syringe (N_2O batch tests). All batch tests were run over a 10 Ω external resistor, except when a potentiostat was used (batch test E).

2.3.3 Control experiments: open circuit and abiotic cathode

Control experiments were performed to determine the electron donor for the cathodic removal of N_2O (batch test B and C), and to elucidate the role of the microorganisms present in the biocathode (batch test D).

Open circuit tests were identical to closed circuit tests, except that for these cases the external electrical circuit was interrupted at the moment of N_2O addition, preventing migration of electrons from anode to cathode.

Tests with an abiotic cathode were done by placing autoclaved graphite granules in the cathode compartment. By autoclaving, microbiological activity was assumed to be absent. The amount of granules was equal to the amount present during the tests with a denitrifying biocathode.

2.3.4 Batch tests with N_2O under fixed cathodic potential

Batch tests were performed at fixed cathode potentials of -200, 0 and +100 mV vs Standard Hydrogen Electrode (SHE) (batch test E). At the start of each batch test, a certain volume of 100% N_2O gas was injected in the headspace of the recirculation vessel. Electrodes were connected with a potentiostat, which kept the system at the desired fixed cathode potential (PAR Bi-Stat Potentiostat, Princeton Applied Research, France; three electrode set-up). The current and cathodic potential were measured and recorded every 5 seconds. Tests were performed in triplicate in the same BES.

2.3.5 N_2O feeding during batch operation

N_2O was supplied each time the cell voltage reached almost zero (5-10 mV) during the time periods between the different performed batch experiments. Furthermore, the recirculation buffer was replenished at least three times a week in order to prevent possible interference of microbial products (e.g. redox mediators) and to keep the pH constant (7.2±0.1).

2.3.6 Continuous N_2O feeding period

The operation of the cathode was switched to a continuous mode on day 152 after a batch operation period of 70 days. A 100% N_2O saturated solution (0.64 g L^{-1} N_2O-N) was fed to the cathode at a rate of 200 mL d^{-1}, giving a loading rate of 2.1 kg N_2O-N m^{-3} NCC d^{-1}, or 10 mA (167 A m^{-3} NCC) when recalculated to current production (see R. 4.1).

2.4 Electrochemical monitoring

The cell voltage over a fixed resistor and the cathode potential were recorded every minute with a Data Acquisition Unit (HP 34970A, Agilent, USA). The cathodic potential was monitored with an Ag/AgCl reference electrode (3.5 M KCl; + 0.206 V vs SHE). Polarization curves were obtained according to Clauwaert and co-workers (Clauwaert et al., 2007a). Briefly, the polarization program consisted of an open circuit stabilization period of 15 min after which the cell voltage was gradually decreased till 0 mV at a rate of 0.2 mV s^{-1}.

2.5 Calculations

Current and power production was calculated according to Ohm's law. The volumetric current density could be expressed as a theoretical nitrogen removal rate (D; kg N m^{-3} NCC d^{-1}) according to the equation (Eq. 4.1) reported in Clauwaert et al (Clauwaert et al., 2007a).

$$D = \frac{IM}{Fn}\frac{86400 \times s \times d^{-1}}{1000 \times g \times kg^{-1}} = 2.507 \times 10^{-3} I \qquad \text{(Eq. 4.1)}$$

I = the volumetric current density (A m^{-3} NCC), M = the molar mass of nitrogen (14 g N mol^{-1}), F = Faraday's number (96485 C mol^{-1}), and n = the moles of electrons exchanged per mole nitrate (5 moles of electrons) or mole N_2O-N (1 mole of electrons) reduced.

A gas trap was used to assess the gas production during the continuous nitrate feeding period. The cathodic coulombic efficiency ($\varepsilon_{cathode}$) was evaluated as the ratio of the coulombs produced and the theoretical amount of coulombs needed based on the oxidized nitrogen compounds removed at the cathode.

Nitrogen removal rates were expressed relative to the net cathodic volume (0.060 L). Removal rates reported with R^2 were obtained after fitting a linear regression curve to the data enclosed in the given time interval.

2.6 Chemical analysis

The concentration of NO_3^- and NO_2^- were determined using an Ion Chromatograph (Compact IC 761 with conductivity detector, Metrohm, Switzerland). Ammonium (NH_4^+) concentration was determined according to the colorimetric Nessler procedure (Greenberg, 1992).

Gas-phase N_2O concentration was measured with a gas chromatograph (14B, Shimadzu, Japan) fitted with a ^{63}Ni electron capture detector (detection limit 300 ppbv) according to Roobroeck and co-workers (Roobroeck et al., 2010).

Measurements of the concentration N_2O in the liquid-phase (solubility: 0.029 M atm^{-1} at 20°C; (Sander, 1999)) were performed according to Chapter 2 section 2.3.

3 Results

3.1 The development of a denitrifying biocathode

Continuous feeding with nitrate as cathodic electron acceptor was maintained during an 82 days period in order to selectively enrich for denitrifying microorganisms at the cathode. After inoculation (day 0), a start-up period of 46 days was observed. From then on, an average nitrogen removal rate of 0.21±0.01 kg NO_3^--N m^{-3} NCC d^{-1} was obtained, resulting in 100% removal efficiency (Table 4.2). No residual nitrite was detected in the effluent and the concentration of N_2O in the gas trap of the cathode compartment was always below 10 μL L^{-1}. Considering the average gas production together with the nitrogen load during the stable nitrate removal period, the N_2O emission represented only 0.001 to 0.002% of the nitrogen load.

An additional nitrate batch test was performed at the cathode in order to quantify the production and removal of denitrification intermediates during the process (Fig. 4.2). An average nitrogen removal rate and cathodic coulombic efficiency of 0.51±0.02 kg N m^{-3} NCC d^{-1} and 95% were obtained, respectively (Table 4.2). Nitrite accumulation was observed during the first eight hours of the experiment, representing up to 55% of the nitrate initially added. In contrast, no such effect could be observed for N_2O as the average concentration measured throughout the nitrate batch test amounted only for 0.025±0.022% of the nitrogen initially injected. Furthermore, NH_4^+ was not detected in the cathodic liquid during both the continuous operation period and the nitrate batch test.

Table 4.2 Summary of results obtained during continuous nitrate feeding, batch operation tests with nitrate and N_2O, and continuous N_2O feeding. N indicates the number of replicates, average ± standard deviation. N/A: Not applicable.

Experiment	**Nitrogen Removal Rate**[a] (kg N m^{-3} NCC d^{-1})	**Removal Efficiency** (%)	**Current Production** (mA)	(A m^{-3} NCC)	**Coulombic Eff. Cath** (%)	**Cathode Potential** (V vs SHE)
Continuous Nitrate Feeding	0.21±0.01[b]	100±0	5.05±0.57	84±9	100±11	-0.206±0.027 (Rext = 5Ω) -0.167±0.015 (Rext = 10 Ω)
Nitrate Batch Test	0.51±0.02 (R^2 = 0.9869)	100	10.64±3.10	177±52	95	-0.053±0.060
N_2O Batch Test A	1.83±0.08 (R^2 = 0.9931)[d]	100	7.32[c]	122[c]	99	-0.138[c]
N_2O Batch Test B						
Closed Circuit		70	10.28[c]	167[c]	77	-0.079[c]
(0-1h)	3.26±0.02 (R^2 = 0.9985)					
(1-2h)	1.63±0.02 (R^2 = 0.9995)					
Open Circuit		27	N/A	N/A	N/A	+0.110[c]
(0-1h)	1.74±0.04 (R^2 = 0.9899)					
(1-2h)	0.32±0.10 (R^2 = 0.8384)					

N_2O Batch Test C						
Open Circuit		50	N/A	N/A	N/A	+0.087[(c)]
(0-1h)	1.24[(e)]					
(1-24h)	0.07±0.01 (R^2 = 0.9954)					
N_2O Batch Test D						
Closed circuit		35	-0.33±0.20	-5±3	N/A	-0.427±0.021
(0-1h)	1.02±0.20 (R^2 = 0.9271)					
(1-24h)	0.04±0.01 (R^2 = 0.9607)					
Open circuit		41	N/A	N/A	N/A	-0.462±0.012
(0-1h)	0.73±0.28 (R^2 = 0.7749)					
(1-24h)	0.07±0.01 (R^2 = 0.9954)					
N_2O Batch Test E						
-200 mV vs SHE (n=3)	1.45±0.12 (R^2 = 0.9730)	60±3	7.01±0.39	117±7	95±6	Fixed
0 mV vs SHE (n=3)	0.85±0.04 (R^2 = 0.9884)	35±2	3.82±0.05	64±1	94±14	Fixed
+100 mV vs SHE (n=3)	0.65±0.08 (R^2 = 0.9374)	26±1	0.11±0.01	2±0	3±0	Fixed

Continuous N_2O Feeding	0.76±0.26[(f)]	36±12[(g)]	3.63±1.25	61±21	N/A	-0.204±0.055

[(a)] : Nitrogen removal rates were obtained from a linear regression curve including the data points of the addressed time period of the experiment, and were presented as mean value ± standard deviation unless stated otherwise

[(b)] : Nitrogen removal rate calculated as mean ± standard deviation of 5-weekly samples

[(c)] : Maximum current production or cathode potential obtained during test

[(d)] : Nitrogen removal rate obtained during first 2.5 h of the test

[(e)] : Nitrogen removal rate calculated from 2 data points (at 0 and 1 h)

[(f)] : Nitrogen removal rate recalculated from the current production

[(g)] : Assumed that the loading rate was constant (influent 100% saturated with N_2O, giving a loading rate of 2.10 kg N m^{-3} NCC d^{-1})

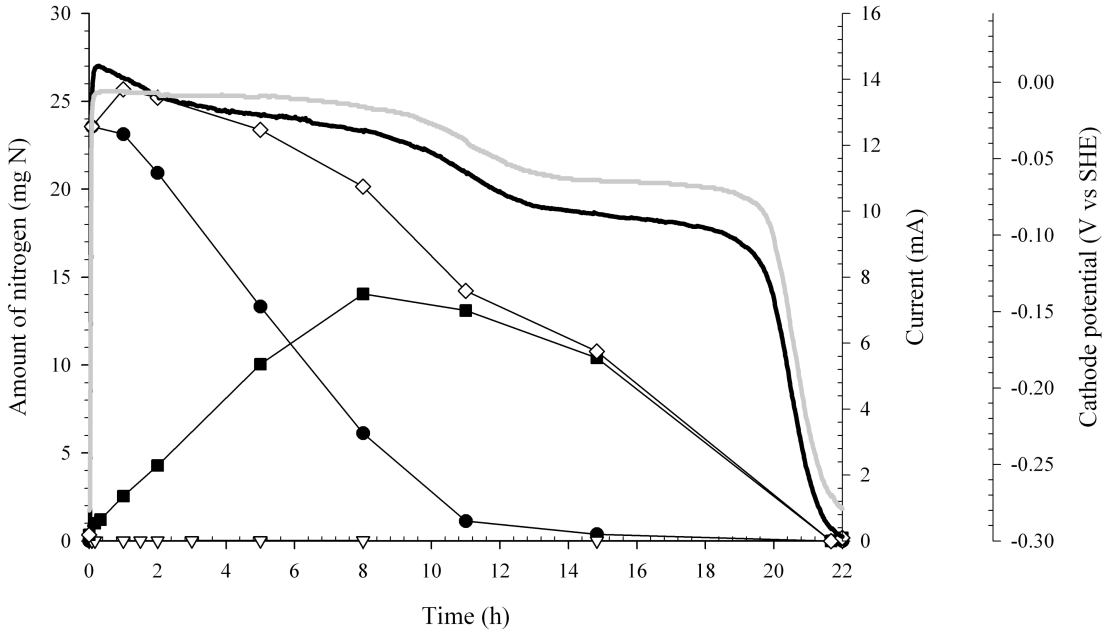

Figure 4.2 Nitrate batch test. Amount of nitrate (circles; black), nitrite (squares; black), gas-phase N_2O (triangles; white), total amount of nitrogen (diamonds; white), current production (solid line; black) and cathode potential (solid line; grey) in function of time. 24 mg of NO_3^--N was added at the start of the experiment (0h).

3.2 A denitrifying biocathode with N_2O as the sole electron acceptor

After successful enrichment with nitrate, the latter was replaced by N_2O as the sole electron acceptor in the cathode compartment. The ability of the biocathode to reduce N_2O was investigated in a batch experiment (batch test A, Fig. 4.3 and Table 4.2). In order to assess accurately the fate of N_2O at the cathode, both gas- and liquid-phase concentrations of N_2O were measured. The total N_2O removal rate clearly followed the same trend as did the current production and the cathodic potential. After reaching maximum current production at 2.5 h, a gradual decrease of the N_2O removal rate, current production and cathode potential could be seen until complete depletion of N_2O. The cathodic coulombic efficiency of the batch test amounted to 99%. No NH_4^+, NO_3^- or NO_2^- were detected in the catholyte.

Changing from nitrate to N_2O as the sole electron acceptor present also had an influence on the diversity of the microbial community. Overall, a decrease in bacterial diversity was observed during N_2O reduction. This is in line with the fact that a significant fraction of the denitrifying population does not have the genetic capacity to reduce N_2O (Hallin et al., 2011). Therefore, bacteria lacking the *NosZ* gene might have been outcompeted, resulting in a decreased bacterial diversity. Most likely, the dominant bacteria can be found in the groups of Proteobacteria, Firmicutes and Chloroflexi as shown by an extensive microbiological study

on denitrifying cathodes (Wrighton et al., 2010). Furthermore, Virdis and co-workers showed that *Paracoccus* and *Pseudomonas* ssp. were amongst the most abundant denitrifying organisms at a cathode (Virdis et al., 2011).

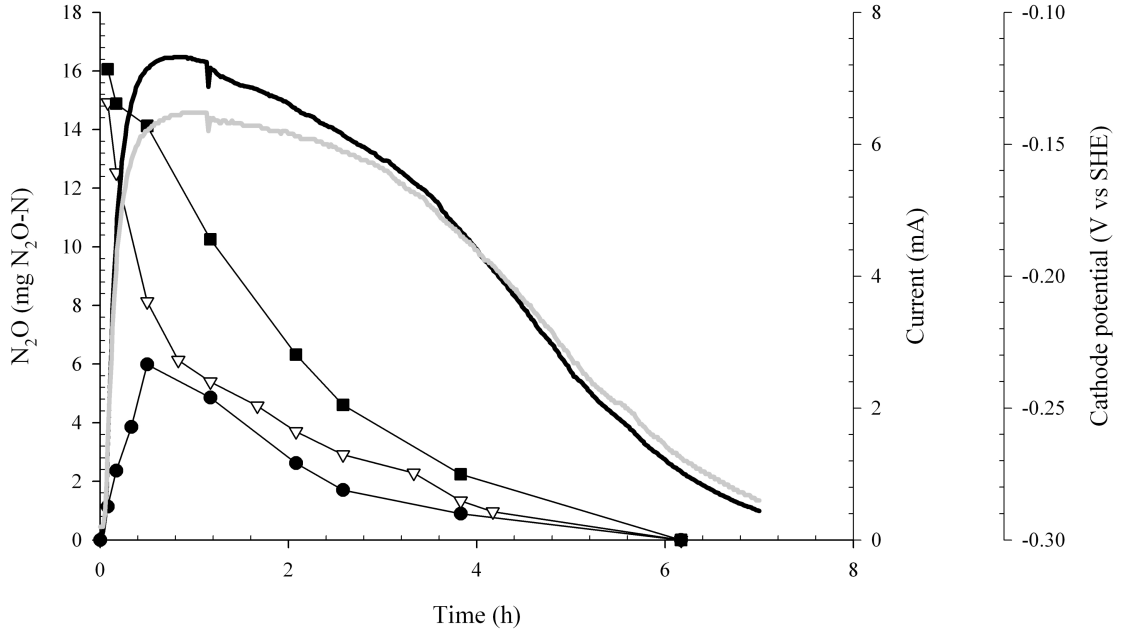

Figure 4.3 N_2O batch test A. Amount of N_2O total: squares; gas-phase: triangles; liquid-phase: circles), current production (solid line; black) and cathode potential (solid line; grey) in function of time.

3.3 Open versus closed circuit removal of N_2O

Comparing closed and open circuit experiments allowed the investigation of the removal of N_2O with and without the supply of electrons derived from the anode (batch test B, Fig. 4.4 and Table 4.2). N_2O was monitored in both gas- and liquid-phase for 2h in order to assess the nitrogen removal during open and closed circuit operation. The N_2O removal rate during the second hour of the closed circuit experiment (Fig. 4.4A) decreased with approximately 50% compared to the first hour. Overall, a cathodic coulombic efficiency of 77% was obtained.

During open circuit operation (Fig. 4.4B), 23% of the N_2O initially injected was removed during the first hour. Overall, 27% of the N_2O initially added was removed after 2 h. An additional open circuit experiment was performed over 24 h (batch test C) in order to assess the N_2O removal over a longer period (Table 4.2 and Fig. 4.5). At the end of the experiment, 50% of the added N_2O was removed of which 19% during the first 2 h.

No NH_4^+, NO_3^- or NO_2^- were detected in the cathodic liquid both during the open and closed circuit experiment.

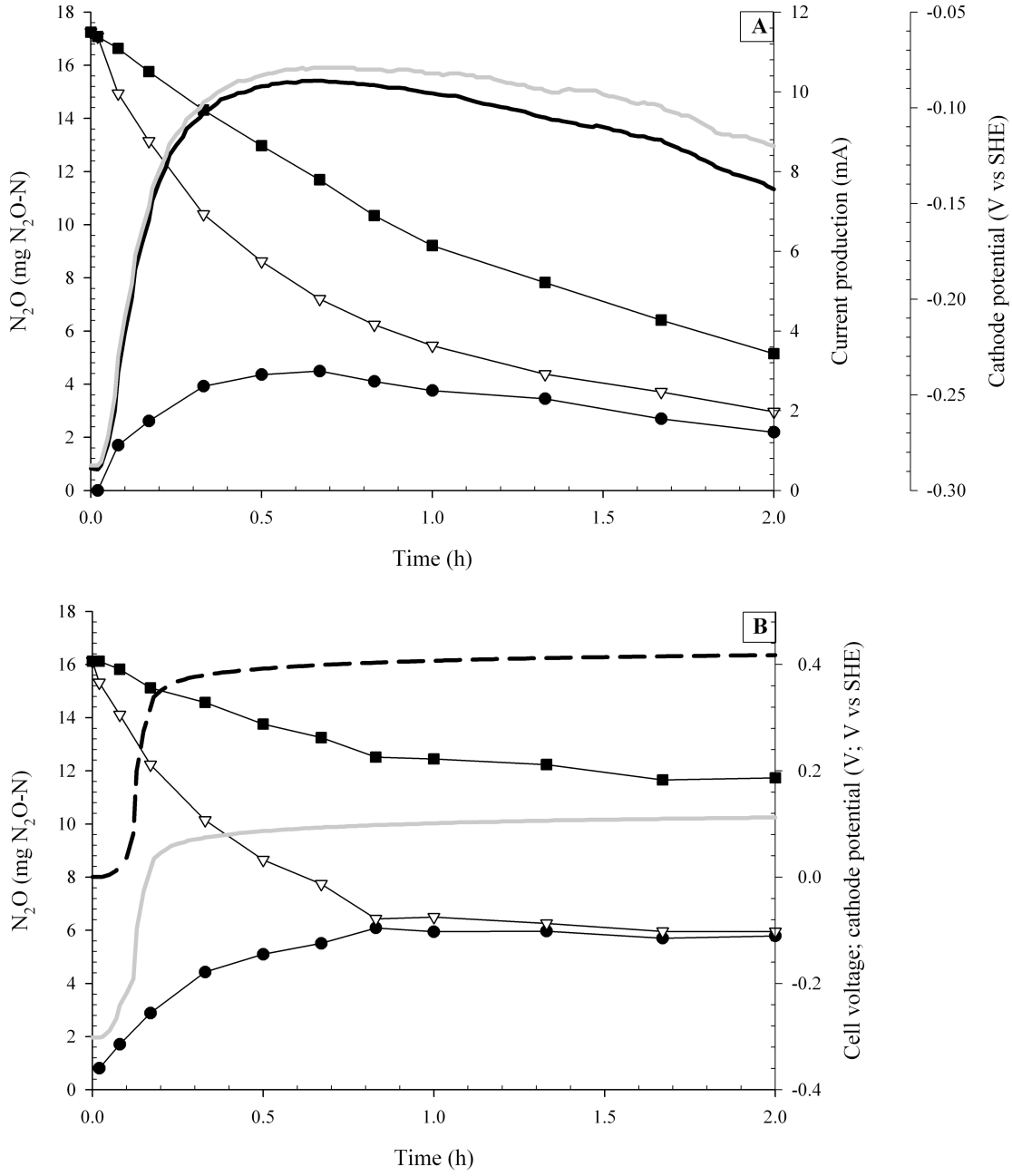

Figure 4.4 N_2O batch test B. Amount of N_2O (A and B, total: squares; gas-phase: triangles; liquid-phase: circles), current production (A, solid line; black), cell voltage (B, dashed line; black) and cathode potential (A and B, solid line; grey) in function of time during closed (Fig. 4.4A) and open (Fig. 4.4B) circuit operation mode.

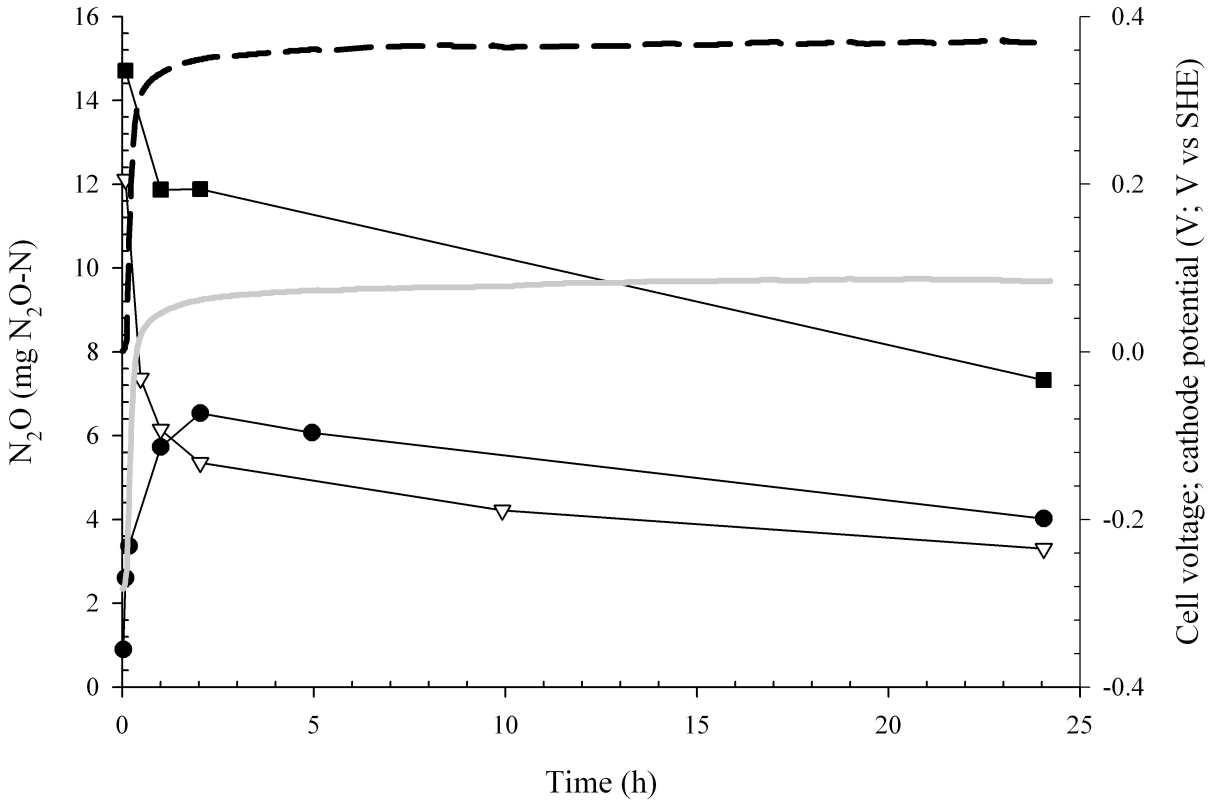

Figure 4.5 N_2O batch test C. Amount of N_2O (total: squares; gas phase: triangles; liquid phase: circles), cell voltage (dashed line; black) and cathode potential (solid line; grey) in function of time during the 24 h open circuit experiment.

3.4 N_2O removal in an abiotic cathode

In order to address the catalysing role of the denitrifying microorganisms in the N_2O removal process, a 24 h experiment under closed and open circuit conditions (Fig. 4.6) was performed with an abiotic cathode filled with autoclaved granules (batch test D; Table 4.2). In total, 35% of the added N_2O was removed during the closed circuit experiment, from which 18% during the first hour. For the open circuit experiment, 41% of the N_2O initially added was removed, of which 14% during the first hour.

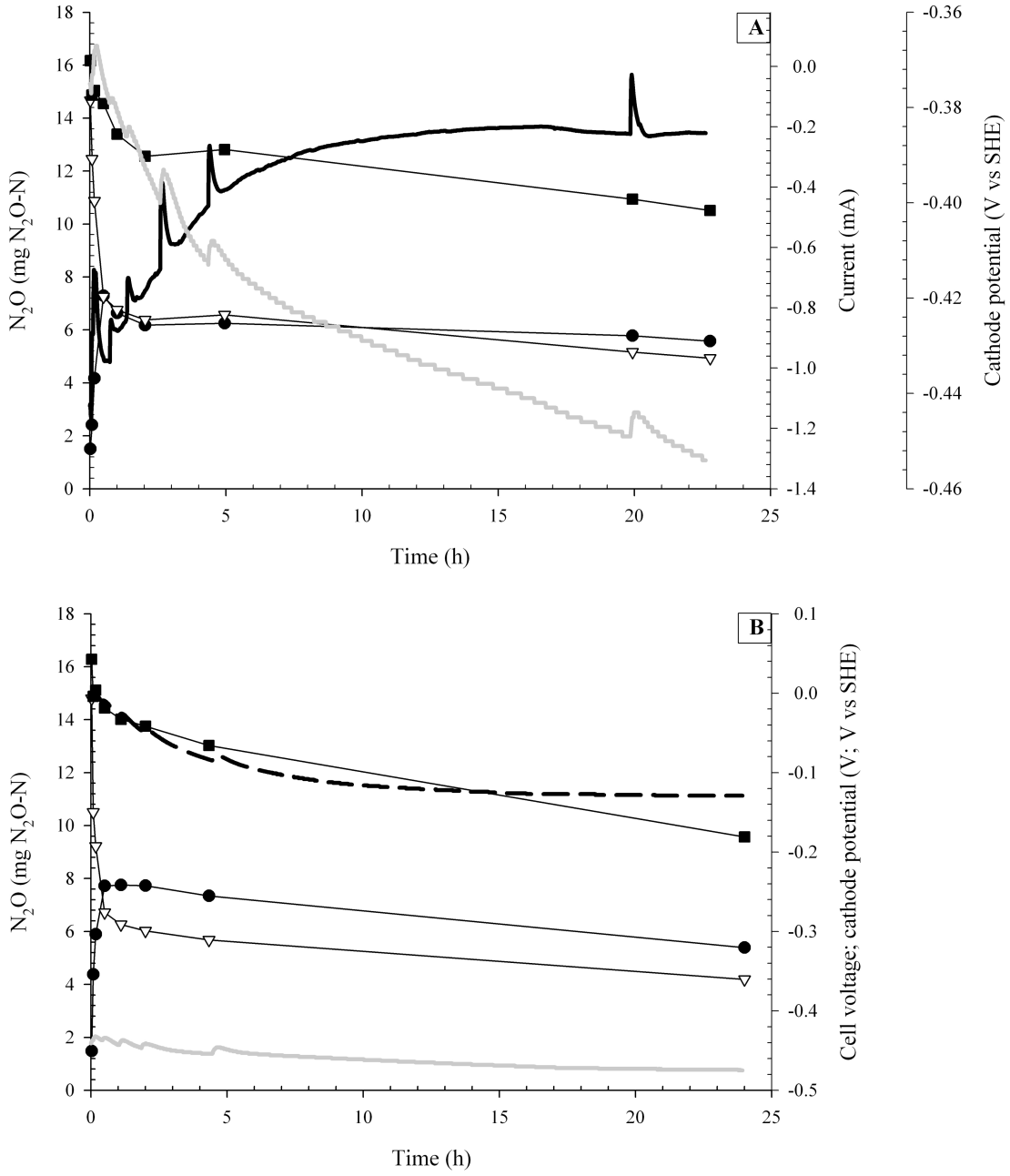

Figure 4.6 N_2O batch test D. Amount of N_2O (total: squares; gas phase: triangles; liquid phase: circles), current (solid line, black), cell voltage (dashed line; black) and cathode potential (solid line; grey) in function of time during the 24 h open circuit experiment with an abiotic cathode (A: closed circuit; B: open circuit).

3.5 N_2O removal at different cathodic polarizations

The removal of N_2O was assessed at three different poised cathode potentials (-200, 0 and +100 mV vs SHE; Batch test E; Fig. 4.7 and Table 4.2). By doing so, the theoretical energy gain, determined by the voltage difference between the cathode and the redox potential of the final electron acceptor (N_2O; E°' = +1.36 V vs SHE), could be controlled.

In general, lower N_2O removal rates were observed at higher cathodic potentials. The removal rates obtained at -200 and 0 mV vs SHE correlated well with the average current production expressed as a nitrogen removal rate, equal to 1.46±0.08 kg N m^{-3} NCC d^{-1} and 0.80±0.01 kg N m^{-3} NCC d^{-1}, respectively. In contrast, almost no current was produced at +100 mV vs SHE (Fig. 4.7A), although N_2O removal was also observed. The mass balance was verified by relating the amount of N_2O removed expressed as coulombs with the amount coulombs produced through current generation, giving 95±6%, 94±14% and 2.9±0.3% cathodic coulombic efficiency for the tests performed at a fixed cathode potential of -200, 0 mV and + 100 mV vs SHE, respectively (Fig. 4.7B). No NH_4^+, NO_3^- and NO_2^- were detected in the catholyte.

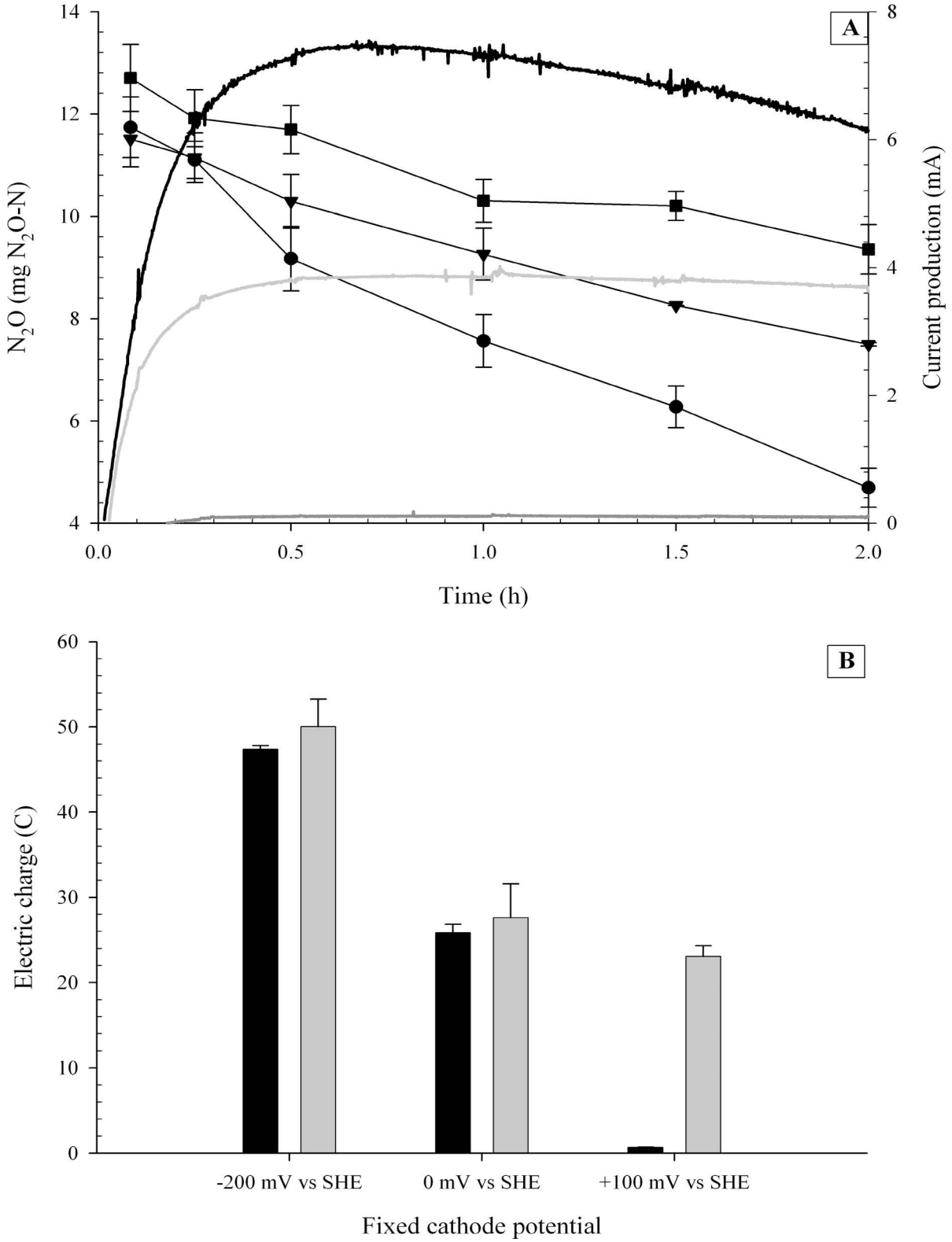

Figure 4.7 N_2O batch test E. A: Total amount of N_2O (gas + liquid phase) and current production in function of time at -200 (circles; black solid line), 0 (triangles; grey solid line) and +100 mV vs SHE (squares; dark grey solid line). B: Coulombs produced (black bars) and amount of N_2O removed recalculated to coulombs removed (grey bars). Error bars indicate standard deviation of triplicate experiments.

3.6 Long-term performance

The cathode of the BES was fed with N_2O in a batch operation mode over a period of 70 days (days 82-151). From day 152 onwards, the BES was operated in continuous mode for 45 days in order to assess the long-term performance of the system. During this period, cell voltage, current production and cathode potential were monitored continuously. From day 156 onwards, an average current production of 3.62±1.25 mA (61±21 A m^{-3} NCC) was obtained, which corresponded to a calculated N_2O removal rate of 0.76±0.26 kg N m^{-3} NCC d^{-1} (Table 4.2). The average cathode potential during this period was -0.204±0.055 V vs SHE (Table 4.2).

Polarization curves were obtained on a weekly basis during the batch and continuous N_2O feeding periods as an additional means to monitor the performance of the reactor (Fig 4.8). From day 95 till day 150 (batch operation period), maximum current and power production decreased from 217 to 73 A m^{-3} NCC and from 25 to 7.6 W m^{-3} NCC, respectively. However, after 20 days of continuous N_2O feeding (day 172), maximum current and power production increased again to 104 A m^{-3} NCC and 11 W m^{-3} NCC, respectively.

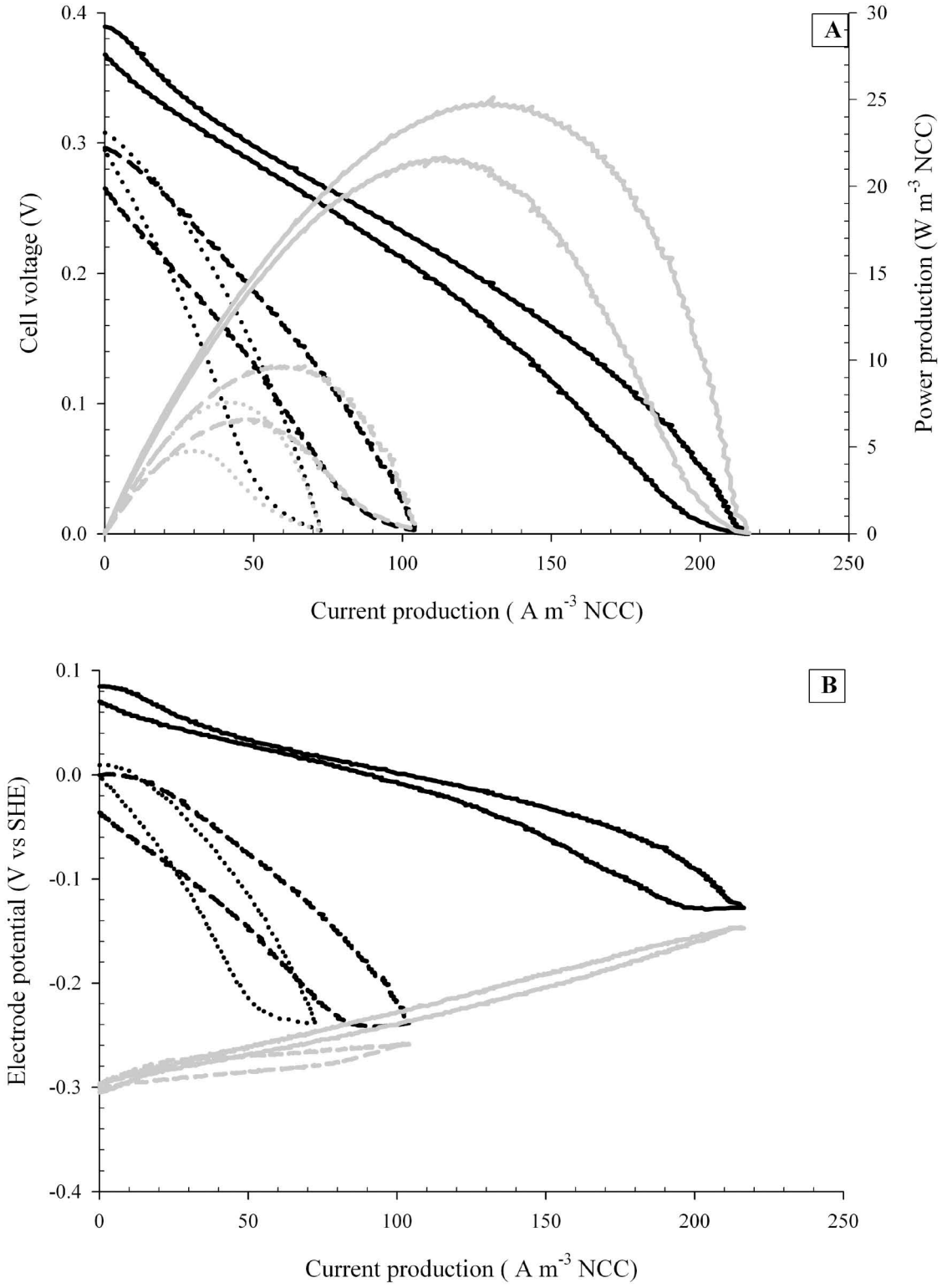

Figure 4.8 Polarisation curves (forward and backward profiles; 0.2 mV s^{-1}) obtained at day 95 (solid lines), 150 (dotted lines) and 172 (dashed lines) of the experimental period. A: Cell voltage (black) and power production (grey) in function of current production. B: Cathode potential (black) and anode potential (grey) in function of current production.

4 Discussion

4.1 Autotrophic nitrate removal: performance and evolution of nitrogen intermediates

A denitrifying biocathode was enriched with nitrate for 82 days as a step to the development of an N_2O reducing biocathode. The obtained removal rate and cathode potential during the 35-day (days 46-81) stable period during continuous nitrate-feeding (Table 4.2) were in the same range as the values reported in other studies concerning autotrophic nitrate removal in BES (Clauwaert et al., 2009, Clauwaert et al., 2007a, Virdis et al., 2008, Virdis et al., 2009). During the nitrate batch experiment (Fig. 4.2), nitrite accumulation was observed. This was in accordance with the results obtained by Puig and co-workers (2011), and can be explained from both a thermodynamical and kinetic perspective. The lower redox potential of nitrite ($E°'$ = +0.35 V vs SHE) compared to nitrate ($E°'$ = +0.43 V vs SHE), implies that theoretically, less energy is available for the microorganisms when nitrite is the electron acceptor (Thauer et al., 1977), and the nitrate reduction rate of the present microbial community appeared to be higher compared to that of nitrite.

The N_2O emission observed during both the continuous and batch operation with nitrate was low compared to the values reported in previous studies on bioelectrochemical nitrogen removal (Virdis et al., 2010, Virdis et al., 2008, Virdis et al., 2009). The production and emission of N_2O are strongly influenced by many parameters, among which the concentration of ammonium and nitrite, as well as the dissolved oxygen levels are the most important (Kampschreur et al., 2009b). Most likely, the absence of oxygen and ammonium was beneficial for the low N_2O emission in this case. Furthermore, the nitrate batch test revealed a high cathodic coulombic efficiency of 95%, and indicated that the cathode was the principle electron donor for nitrate reduction.

4.2 N_2O as the sole electron acceptor in a denitrifying biocathode: establishment, performance and efficiency

After 35 days of stable nitrate removal, the biocathode was investigated for its ability to treat N_2O as the sole electron acceptor present (batch test A, Fig. 4.3 and Table 4.2). The N_2O removal rate was up to 2.6 times higher than the nitrate removal rate observed during the nitrate batch test. This is not surprising since fewer electrons are needed to achieve reduction

to N_2 (Thauer et al., 1977). Therefore, the microbial community might have compensated the shorter electron transport chain by a higher activity.

The obtained N_2O removal rates in the order of 1 kg N_2O-N m^{-3} NCC d^{-1} can be considered as relatively high. For comparison, the obtained removal rates in biological nitrate removal systems range from 0.03 to about 1.5 kg N m^{-3} d^{-1} (Kim et al., 2009, Pedros et al., 2008, Vlaeminck et al., 2010). The latter could also explain the absence of N_2O emission or accumulation during both the continuous nitrate feeding period and the nitrate batch test. Furthermore, a high cathodic coulombic efficiency near 100% was observed and indicated that the electrons originating from the anode were the main electron donors for N_2O reduction, and that complete removal of N_2O could be established.

4.3 Removal mechanisms and biocatalysis

At open circuit, anode and cathode are not electrically connected. Consequently, no cathodic reduction reaction should be observed and the concentration of N_2O should remain constant. Surprisingly, the N_2O level also decreased in open circuit. However, this seemed to be mainly a temporary effect since the removal of N_2O decreased significantly after 1 h during the open circuit experiments (batch test B, Fig. 4.4B and Table 4.2; batch test C, Fig. 4.5 and Table 4.2). Indeed, the total N_2O removal rate observed from 1 – 24 h during the 24 h open circuit experiment was ca. 20 times lower compared to closed circuit experiments and can therefore most likely be accounted as gas diffusion loss from the reactor. Interestingly, the abovementioned temporary N_2O removal mechanism was also observed during the closed circuit experiments since the 2 h closed circuit experiment (batch test B, Fig. 4.4A and Table 4.2) showed a 2 times higher N_2O removal during the first hour compared to the second hour, and a relatively low cathodic coulombic efficiency of 77% was observed. The latter observations thus suggest a temporary alternative removal mechanism during the first hour of both the open and closed batch experiments. Virdis and co-workers explained a similar phenomenon with the capacitance of the graphite granular matrix constituting the electrodes (2009). A steep rise of the cathode potential, observed at the beginning of each experiment, results from the discharge of the electrode during which electrons are liberated. This local flow of electrons can subsequently be used by bacteria to endure N_2O reduction. However, this does not result in net current production since these electrons were already present in the charged graphite granules in the cathode compartment.

A closed and open circuit experiment with an abiotic cathode (Batch test D; Fig. 4.6 and Table 4.2) suggested that microorganisms catalyse the reduction reaction since the obtained

removal rates obtained between 1 and 24 hours were similar to the rates obtained in open circuit with a biocathode. However, when looking to the N_2O profile during the first hour of both the closed and open circuit abiotic experiment, a 10 to 20 times higher N_2O removal was observed. Since no steep rise of the cathode potential took place, a discharge effect of the electrode was likely to be absent and N_2O was removed through another mechanism. The absence of ammonium, nitrate and nitrite throughout all the batch tests with N_2O as an electron acceptor excludes any other reductive or oxidative removal pathway. A plausible explanation would be an initial adsorption effect of N_2O to the graphite granules. The latter could also have played a role during the experiments with a biocathode. Most likely, both processes occurred simultaneously.

4.4 Energetic constraints and energy conservation

A batch test performed under poised cathode potentials (Batch test E, Fig. 4.7 and Table 4.2) revealed that the N_2O removal rate increased with a factor 2.2 when the cathode potential was decreased from +100 to -200 mV vs SHE. The latter is corroborated by the thermodynamics of the process, since a lower cathode potential increases the theoretical energy gain for the microorganisms. Virdis and co-workers performed similar experiments with nitrate, and observed N_2O accumulation, indicating lower specific N_2O removal rates as compared with nitrate removal rates (Virdis et al., 2009). In this study, N_2O was the sole electron acceptor present, and the observed N_2O removal rates were 1.9 to 2.5 times higher compared to the values reported by Virdis et al (2009). This is a strong indication that a specialized N_2O reducing community was present in this study. The current production was proportional to the N_2O removal rate for the applied cathode potentials of -200 and 0 mV vs SHE, giving cathodic coulombic efficiencies near 100% (Table 4.2). However, no current production was observed at a poised cathode potential of +100 mV vs SHE, and indicated that the microorganisms were not able to take up electrons from the cathode at that potential. Nevertheless, N_2O removal was observed without concomitant current production, explaining the low cathodic coulombic efficiency observed. The latter suggests again the involvement of an alternative N_2O removal mechanism. The removal rate observed here was 10 times higher than what was previously accounted for as gas diffusion loss from the reactor, and leads to a capacitance or adsorption effect as possible explanations.

Interestingly, much debate exists regarding the energy conservation associated with the reduction of N_2O to N_2. Wasser and co-workers stated that the energy derived from N_2O

reduction is generally dissipated as heat (2002). In contrast, Zumft and Richardson reported that each reaction in the denitrification pathway is catalysed by an enzyme that is coupled to the production of proton motive force and thus energy conservation (Richardson, 2000, Zumft, 1997). The lack of N_2O reduction makes 20% difference to the bioenergetics of denitrifying bacteria (Richardson et al., 2009).

This aspect was investigated on long-term tests by continuing batch feeding of the cathode for 70 days, followed by a continuous feeding period of 45 days. The current production during the continuous feeding period was 61 ± 21 A m^{-3} NCC (Table 4.2). In addition, the cathodic potential of around -200 mV vs SHE that was observed during continuous operations was similar to those reported in similar studies treating nitrate or nitrite (Clauwaert et al., 2009, Clauwaert et al., 2007a, Puig et al., 2011). Analysis of the obtained polarization curves (Fig. 4.8) revealed a decreasing performance of the BES over the batch operation period, and was almost entirely due to a decreasing activity of the biocathode (Fig. 4.8B). However, operating the cathode continuously resulted in an increased performance of the biocathode, and suggests that the microbial community prefers a continuous feeding over a feast and famine regime. The latter was, together with the fact that the biocathode was fed for 115 days with N_2O as the sole electron acceptor, a strong indication that the microorganisms could conserve energy from the reduction of N_2O without the need for higher oxidized nitrogen species.

5 Conclusions

The results clearly show that an N_2O reducing biocathode can be sustained at long-term and high activity. The main advantages are that BES can remove N_2O at ambient temperatures, and no additional expensive catalysts are needed to perform the reduction reaction. Furthermore, these systems are able to decouple oxidation and reduction processes and can be operated at lower carbon to nitrogen ratios compared to conventional denitrification systems (Ahn, 2006, Virdis et al., 2008), leading to a lower demand for organic carbon. Nevertheless, it is worth noting that this technology faces a number of scale-up challenges that need to be resolved prior to the development of practical applications. Furthermore, the O_2 sensitivity of the enzyme catalysing N_2O reduction (Korner and Zumft, 1989) can be considered as a major constraint. However, biocathodic denitrification at high dissolved oxygen concentration is feasible (Virdis et al., 2011) and species like *P. stutzeri* TR2 have been shown to denitrify efficiently at high oxygen partial pressures (Miyahara et al., 2010). Once these limitations can be solved, niches could be found in the biological nitrogen removal sector where N_2O can be released in significant amounts (Ahn et al., 2010b, Desloover et al., 2011, Foley et al., 2010b,

Kampschreur et al., 2009b). Hereby aiming at minimizing N_2O emissions by the treatment of streams containing dissolved N_2O or N_2O-rich off-gases.

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Chapter 5 Isolation of dominant anoxic and aerobic N_2O reducers and pathway of N_2O consumption in *P. stutzeri*

Abstract

The microbial consumption of nitrous oxide (N_2O) is the only known biochemical pathway able to mitigate N_2O emission during BNR. The consumption of N_2O results from its reduction to dinitrogen gas (N_2) as part of the denitrification process. However, the enzyme catalysing N_2O reduction is very oxygen sensitive, thereby often impeding this pathway to act as an N_2O sink. Furthermore, there is on-going debate regarding an alternative pathway, namely reduction of N_2O to NH_4^+, or assimilatory N_2O consumption. To date, this pathway is poorly investigated and lacks unambiguous evidence.

In this study, enrichment of activated sludge rendered a mixed culture capable of anoxic and oxic N_2O consumption. Dilution plating, isolation and DNA fingerprinting identified a collection of *Pseudomonas stutzeri* strains as dominant N_2O consumers in both anaerobic and aerobic enrichments. A detailed isotope tracing experiment with a *P. stutzeri* isolate showed that consumption of N_2O via assimilatory reduction to NH_4^+ was absent. Conversely, respiratory N_2O reduction was directly coupled to N_2 fixation.

Chapter redrafted after: *Desloover, J., Roobroeck, D., Heylen, K., Puig, S., Boeckx, P., Verstraete, W. and Boon, N. Pathway of N_2O consumption in isolated Pseudomonas stutzeri strains under anoxic and oxic conditions, submitted.*

1 Introduction

The denitrification process is widely dispersed within the domain of Bacteria and appears to be dominant within Proteobacteria (Shapleigh, 2006). Often but not always, denitrifying bacteria possess an N_2O reductase enzyme (NOS) (Schmidt et al., 2004), which completes the final reduction step in the denitrification pathway (Zumft, 1997) and is generally considered the sole enzyme able to interact with N_2O. The occurrence of N_2O reduction, however, depends on several abiotic conditions. In this, the O_2 concentration is considered the most important determinant next to pH and carbon availability (Richardson et al., 2009). Nitrous oxide is often described as the major end product during aerobic denitrification (Morley et al., 2008, Otte et al., 1996, Takaya et al., 2003). This can be attributed to the greater oxygen sensitivity of NOS compared to other denitrification enzymes (Knowles, 1982).

Various authors have suggested the existence of an alternative N_2O consumption pathway in which N_2O is directly reduced to ammonium (NH_4^+) by nitrogenase, the enzyme involved in N_2-fixation (Burgess and Lowe, 1996, Jensen and Burris, 1986, Yamazaki et al., 1987). During regular N_2-fixation, a nitrogen assimilation pathway exclusively performed by prokaryotes, nitrogenases catalyse the conversion of N_2, protons and electrons to ammonium and hydrogen (Martinez-Romero, 2006). Furthermore, N_2-fixation is also an oxygen-sensitive process (Martinez-Romero, 2006) and the presence of NH_4^+ can switch off nitrogenase activity (Bergersen, 1991, Desnoues et al., 2003). Interestingly, both nitrous oxide reductase and nitrogenase are found in denitrifiers of the genera *Rhodobacter*, *Hyphomicrobium*, *Frankia*, *Azospirillum* and *Azoarcus* (Shapleigh, 2006) and also in *Pseudomonas* strains such as *Pseudomonas stutzeri* (Lalucat et al., 2006).

Despite the anticipated enzymatic potential for direct reduction of N_2O to NH_4^+, only one empiric test has been performed based on ^{15}N-N_2O tracing in soils (Vieten et al., 2008). Unfortunately, due to low analytical resolution of that study (i.e., bulk N analysis, identical ^{15}N ratios of N_2O and N_2), their disproof of assimilatory N_2O reduction is ambiguous. Given the interest in microbial N_2O consumption, conclusive information is needed to elucidate the possible pathways of N_2O reduction. These are conceptually visualised in Fig. 5.1.

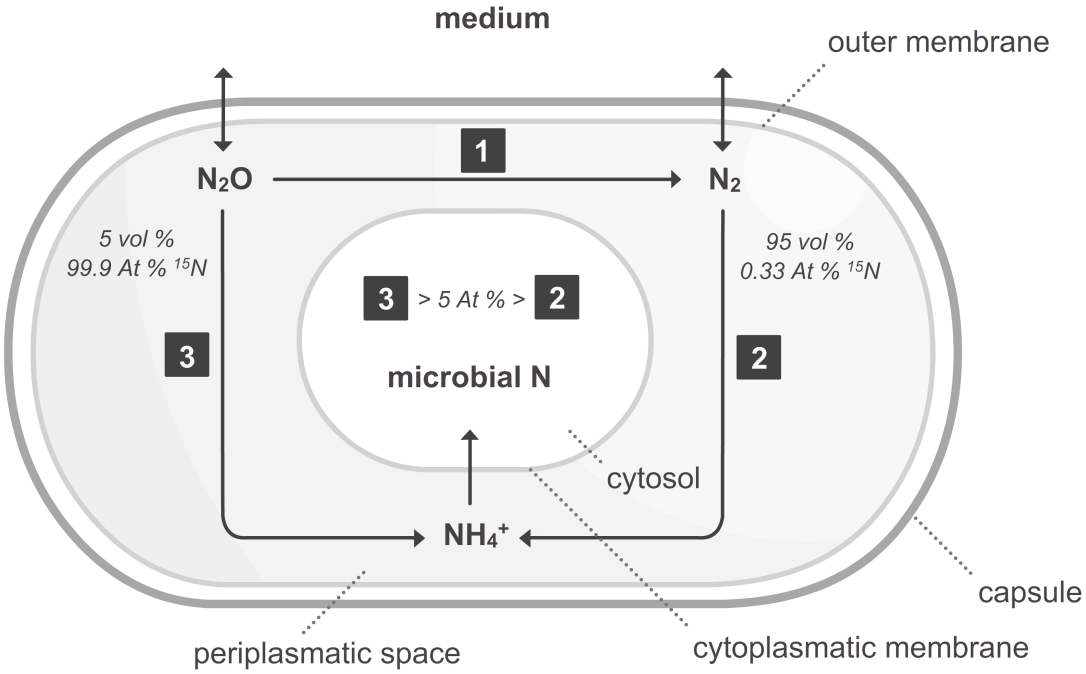

Figure 5.1 Hypothesized pathways of N_2O reduction and resulting ^{15}N atom% of microbial biomass. (1) respiratory reduction of N_2O to N_2. (2) fixation of N_2 to NH_4^+ plus incorporation in microbial biomass. (3) assimilatory N_2O reduction to NH_4^+ plus incorporation in microbial biomass.

Therefore, the aims of this study were to (1) enrich and isolate dominant N_2O-consuming bacteria from activated sludge in both oxic and anoxic conditions; (2) investigate the effects of oxygen and ammonium on N_2O consumption; and (3) determine the pathways of N_2O reduction in a pure culture by means of ^{15}N-N_2O tracing.

2 Material and methods

2.1 Medium composition

Enrichment of microbial biomass, N_2O batch experiments and ^{15}N-N_2O tracing were performed using a modified nitrogen-free M9 medium (6 g L^{-1} $Na_2HPO_4.2H_2O$, 3 g L^{-1} KH_2PO_4, 1 g L^{-1} $NaHCO_3$, 0.5 g L^{-1} NaCl, 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$, 16.5 mg L^{-1} $CaCl_2$) supplemented with trace elements, as previously described (Clauwaert et al., 2007b). Sodium acetate was added (6.1 mM) to the medium to serve as an electron donor and carbon source, rendering a C/N ratio of 1.48. This medium will be referred to as M9-Ac.

2.2 Enrichment procedure

Anaerobic and aerobic enrichments were performed starting from denitrifying activated sludge (municipal wastewater treatment plant, denitrification compartment, Ossemeersen, Gent, Belgium) as inoculum. Enrichments were shaken on an acclimatised planetary shaker at 20°C.

For the anaerobic enrichment, triplicate 120 mL serum flasks were filled with 40 mL of freshly autoclaved M9-Ac, and 1% v/v of activated sludge was inoculated into the medium. Subsequently, the bottles were sealed airtight and alternately evacuated and flushed with helium for 15 minutes. Finally, 4 mL of pure N_2O (4.66 mg N_2O-N) was added, resulting in an initial headspace concentration of 5% N_2O. After all N_2O was removed, the triplicates were consolidated and served as the inoculum for the next enrichment (each time 1% v/v). This enrichment procedure was repeated two additional times.

For the aerobic enrichment, a similar strategy was applied under oxic conditions (95% air, 5% N_2O). A biomass-free control enrichment was implemented to exclude abiotic N_2O removal during anaerobic and aerobic enrichments.

At the end of both the anaerobic and aerobic enrichments, samples were taken for PCR-DGGE and for isolation by dilution plating. The enrichments were kept active under similar conditions during their respective enrichment periods in order to monitor the microbial community over time.

2.3 Dilution plating and isolation

Serial dilutions of the anaerobic and aerobic enrichments were made in a physiological solution (8.5 g L^{-1} NaCl). One hundred microliters of each dilution was plated on Tryptic Soy Agar (TSA) plates and incubated at 20°C under a 100% N_2O atmosphere. Nine morphologically unique colonies at a 1:1000 dilution and 10 colonies at a 1:10.000 dilution of the anaerobic and aerobic enrichment, respectively, were picked for isolation on a freshly made TSA plate. Subsequently, all 19 isolates were re-grown anaerobically in M9-Ac medium at 20°C under a 5% N_2O atmosphere. Samples of each were taken for DNA extraction and further molecular analysis.

2.4 PCR and DGGE

The template for PCR amplification was obtained by extracting total genomic DNA from the enrichments using a procedure described previously (Boon et al., 2000). A 100 µL aliquot of the crude extract was further purified with the Wizard DNA Clean-Up kit as described by the manufacturer (Promega, Madison, USA). 16S rRNA gene fragments were amplified with an Applied Biosystems 2720 Thermocycler using the primers PRBA338fGC and P518r (Muyzer et al., 1993) and analysed using DGGE with a denaturing gradient ranging from 45 to 60% (Boon et al., 2002).

2.5 DNA fingerprinting of strain set

Genomic DNA was released from the bacterial cells through alkaline lysis. A small amount of cells was lysed in 20 µL alkaline lysis buffer (0.25% (w/v) SDS and 0.05 M NaOH) for 15 min at 95 °C. Subsequently, 180 µL sterile Milli-Q water was added, and lysates were immediately used for PCR. Detailed characterisation of the genetic variability among isolates belonging to *Pseudomonas* was achieved by DNA fingerprinting based BOX-PCR using the primer BOXA1R (Martin et al., 1992). PCR conditions were as described by Rademaker and de Bruijn (1997). Electrophoresis and data analyses were performed as described by Ghyselinck *et al.* (2011). Patterns were normalised and clustered according to the Pearson correlation coefficients by the unweighted pair group method with arithmetic averages and analysed with the cophenetic correlation cluster analysis in BioNumerics version 5.1 (Applied Maths, Sint-Martens-Latem, Belgium). Clustering of isolates at a subspecies to strain level was performed with a previously determined robust cut-off value for DNA fingerprinting of 86% (Ghyselinck et al., 2011), as well as by visual inspection of individual profiles.

2.6 Phylogenetic identification of isolates

For DNA sequencing, 16S rRNA gene fragments were amplified with an Applied Biosystems 2720 Thermocycler using the primers P63f (El Fantroussi et al., 1999) and P1378r (Heuer et al., 1997). DNA sequencing of the PCR fragments was carried out by AGOWA Genomics (Berlin, Germany). Homologies of the DNA sequences were searched with the Ribosomal Database Project Classifier (Wang et al., 2007). The identified *P. stutzeri* isolate used for the pure culture experiments (non-labelled batch experiment and labelled ^{15}N-N_2O experiment) is available at the Belgian Coordinated Collections of Microorganisms BCCM/LMG (Ghent University, Ghent, Belgium) under accession number LMG 26881.

2.7 Oxic incubations with anaerobic enrichment

Incubations with the anaerobic enrichment were performed by filling triplicate 120 mL serum flasks with 40 mL autoclaved M9-Ac and by inoculation of 1% v/v of the anaerobic enrichment. The bottles were sealed airtight and fitted with the correct headspace according to the experiment, that is, 95% v/v air and 5% v/v N_2O for the aerobic experiment and 85% v/v air, 10% v/v acetylene and 5% v/v N_2O for the acetylene experiment. Acetylene was administered to block N_2O reductase activity. For the incubations with ammonium, NH_4Cl was added to obtain an initial concentration of 35 mg NH_4^+-N L^{-1}.

2.8 Incubations with *P. stutzeri* isolate at different O_2 concentrations

Incubations with the *P. stutzeri* isolate were performed by growing stored isolate overnight on TSA plates under a 100% N_2O atmosphere. Distinct colonies were collected from the agar, inoculated into 40 mL autoclaved M9-Ac medium and incubated overnight in a 120 mL serum flask fitted with a headspace of 95% v/v N_2 and 5% v/v N_2O. Traces of nitrogen originating from the TSA medium were removed in this fashion. N_2O consumption in the pre-culture was verified by complete N_2O removal after 24 h. Subsequently, 1% v/v of the pre-culture was inoculated into 40 mL of the M9-Ac medium in triplicate 120 mL serum flasks and sealed airtight. The administration of different O_2 concentrations was achieved by fitting a 100% He headspace upon addition of ambient air and N_2O to obtain a final headspace concentration of 0, 5, 10 or 15% v/v O_2 and 5% v/v N_2O each time. For obtaining a 20% v/v O_2 headspace, flasks were left open to the air, sealed, and N_2O was added afterwards. This procedure caused different N_2 concentrations in the headspace. Incubations were shaken on an acclimatised planetary shaker at 20°C.

2.9 Chemical analysis

The concentrations of N_2O, CO_2, O_2 and N_2 in the headspace were analysed using a Compact GC (Global Analyser Solutions, Breda, The Netherlands) equipped with a Porabond pre-column and a Molsieve SA column. A thermal conductivity detector was used to quantify the concentration of the gases, and calibration was performed by injecting standards for each gas. The concentrations of nitrate (NO_3^-) and nitrite (NO_2^-) were determined using an ion chromatograph (Compact IC with conductivity detector, Metrohm, Switzerland). NH_4^+ was measured according to the colorimetric Nessler procedure (Greenberg, 1992).

2.10 ^{15}N-N_2O isotope tracing

The culture of *P. stutzeri* used for ^{15}N-N_2O probing was prepared in the same manner as for the pure culture batch experiments given above. The headspace of all samples was alternately evacuated and filled with N_2 for 15 minutes. Next, 4 mL of $^{15}N^{15}NO$ (99.95 atom% ^{15}N; Cambridge Isotope Laboratories, Andover, USA) was added to the headspace targeting a concentration of 5% v/v. Samples were shaken on an acclimatised planetary shaker at 20°C. The headspace and medium of three replicate samples were collected every 6 h over a 48 h period, starting at 0.25 h after the addition of ^{15}N-N_2O. Headspace samples were put in gas-tight exetainers (Labco, UK) and analysed within 24 h. The entire 40 mL of medium in each replicate was put in a plastic container, frozen in liquid nitrogen and stored in a freezer at -20°C until further processing. Three biomass-free samples were concurrently administered 5% v/v ^{15}N-N_2O and collected after 48 h as a control for the non-microbial fraction of organic N of inoculated samples. Three blank replicates of M9-Ac medium were included to scrutinize for any infestation during preparation and incubation.

2.11 Determination of ^{15}N in N_2 and microbial biomass

The ^{15}N ratio of N_2 in the headspace and microbial biomass in the medium were determined for each replicate sample. The ^{15}N ratio of N_2 was measured using a trace gas preparation unit (ANCA-TGII, Sercon, Crewe, UK) coupled to an Isotope Ratio Mass Spectrometer (20-20, Sercon). Both m/z 29 and m/z 30 of N_2 were measured given that $^{15}N^{15}NO$ was used. All ^{15}N was accounted for by m/z 30 of N_2.

The atom% ^{15}N is the fraction of ^{15}N compared to the sum of ^{14}N and ^{15}N, computed as follows:

$$atom\%^{15}N = \frac{R_{sample}}{R_{sample}+1} \times 100 \quad \text{(Eq. 5.1)}$$

Where R_{sample} is the isotopic ratio of ^{15}N compared to ^{14}N. The atom% ^{15}N of the microbial biomass in the pre-culture was calculated to determine the ^{15}N ratio of the microbial biomass at the start of the incubation.

The ^{15}N ratio of microbial biomass (MB) was determined as the difference between the ^{15}N content of organic N in inoculated samples and non-inoculated samples (Eq. 5.2).

$$atom\%^{15}N_{ON-MB} = \frac{atom\%^{15}N_{TON-sample} - \left(atom\%^{15}N_{ON-medium} \times f_{ON-medium}\right)}{f_{ON-MB}} \quad \text{(Eq. 5.2)}$$

Where the atom% $^{15}N_{ON\text{-}MB}$ is the atom% ^{15}N of organic N originating from microbial biomass, atom% $^{15}N_{TON\text{-}sample}$ is the atom% ^{15}N of the total organic N in inoculated sample, atom% $^{15}N_{ON\text{-}medium}$ is the atom% ^{15}N of the organic N in biomass-free sample, $f_{ON\text{-}medium}$ is the fraction of the total organic N comprised by organic N from biomass-free sample and $f_{ON\text{-}MB}$ is the fraction of the total organic N comprised by organic N from microbial biomass.

The ^{15}N content of organic N, in turn, was determined as the difference between the ^{15}N content of total dissolved N (TDN = organic N + inorganic N) and the ^{15}N content of inorganic N ($NO_2^- + NO_3^- + NH_4^+$) (Eq. 5.3).

$$atom\%^{15}N_{ON} = \frac{atom\%^{15}N_{TDN} - \left(atom\%^{15}N_{IN} \times f_{IN}\right)}{f_{ON}} \quad \text{(Eq. 5.3)}$$

Where the atom% $^{15}N_{ON}$ is the atom% ^{15}N of the total organic N, atom% $^{15}N_{TDN}$ is the atom% ^{15}N of the total dissolved N, atom% $^{15}N_{IN}$ is the atom% ^{15}N of the inorganic N, $f_{IN\text{-}sample}$ is the fraction of the total dissolved N comprised by inorganic N and $f_{TON\text{-}sample}$ is the fraction of the total dissolved N comprised by organic N.

The TDN in samples was assessed by subjecting a subsample of 2.5 mL to an alkaline persulphate oxidation, which converts all N-species to NO_3^- (Lachouani et al., 2010,

Vandenbruwane et al., 2007). NO_3^- + NO_2^- concentrations were measured by cadmium reduction and reaction with N-(1-naphthyl)ethylenediamine. NH_4^+ concentrations were determined using the salicylate–nitroprusside method (Mulvaney, 1996). Colorimetric measurements were performed using a continuous flow auto-analyser (AA3; Bran & Luebbe, Norderstedt, Germany). The concentration of NH_4^+ in inoculated and non-inoculated samples was negligible. The ^{15}N ratio of NO_3^- in persulphate- and non-persulphate-treated samples was determined after conversion to N_2O by *Pseudomonas aureofaciens* (Sigman et al., 2001). The ^{15}N ratio of N_2O was measured using a trace gas preparation unit (ANCA-TGII, Sercon) coupled to an Isotope Ratio Mass Spectrometer (20-20, Sercon).

3 Results

3.1 Enrichment and isolation of N_2O consuming diazotrophs

Microorganisms capable of reducing N_2O were enriched from activated sludge of a municipal wastewater treatment plant under both anoxic and oxic conditions by selectively administering the inoculum with N_2O and acetate in a mineral nitrogen-free medium. Anaerobic enrichments showed N_2O removal immediately after inoculation. N_2O removal rates increased five-fold (11 ± 1 to 56 ± 2 mg N_2O-N L^{-1} d^{-1}) over the three consecutive enrichments steps. The decrease of N_2O in the headspace coincided with an increase of CO_2 and N_2 in the headspace indicating acetate consumption and N_2 production.

Aerobic enrichments did not show N_2O removal until 25 days after inoculation, and only 3 months after inoculation all N_2O was removed from the headspace. With the two subsequent enrichment steps concurrent N_2O and O_2 consumption stepped in immediately after inoculation. For the third enrichment step, the N_2O and O_2 removal rate was respectively 14 ± 1 mg N_2O-N L^{-1} d^{-1} and 41 ± 1 mg O_2 L^{-1} d^{-1}. Biomass-free controls did not show removal of N_2O. The content of NO_3^-, NO_2^- and NH_4^+ was below the level of detection (0.02 mg N L^{-1}) for all experiments.

3.2 Influence of NH_4^+ and acetylene in oxic incubations with anaerobic enrichment

The dynamics of N_2O consumption of the anaerobic enrichment were investigated in oxic conditions (O_2 as alternative electron acceptor, 95% v/v air, 5% v/v N_2O) with or without NH_4^+ (alternative nitrogen source) or acetylene (disables N_2O reductase activity) addition

(Fig. 5.2). Removal of N_2O showed a lag phase of approximately 2 days when no NH_4^+ or acetylene was present. After this initial latency simultaneous N_2O and O_2 removal was recorded, and CO_2 production increased. When NH_4^+ or acetylene was administered, no N_2O removal was observed. The concentration of NH_4^+ decreased from 34 ± 4 to 25 ± 1 mg NH_4^+-N L^{-1} over the first day of incubation. Biomass-free controls without NH_4^+ or acetylene did not demonstrate N_2O removal. No detectable NH_4^+ was found in incubations without addition of NH_4^+ or acetylene. Furthermore, no NO_3^- and NO_2^- were detected when NH_4^+ was administered.

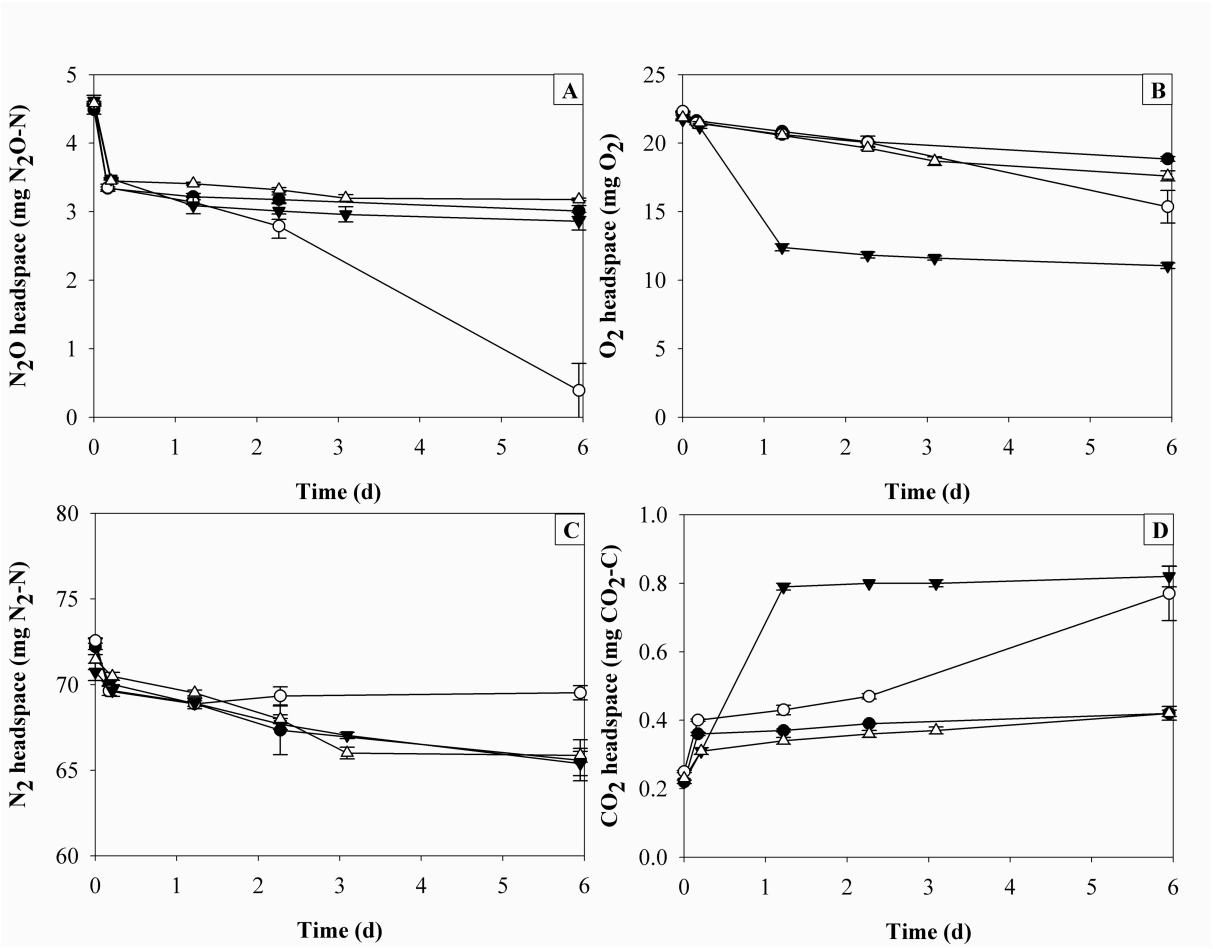

Figure 5.2 Performance of the anaerobic enrichment under oxic starting conditions (95% air, 5% N_2O; white circles), under starting oxic conditions in the presence of ammonium (35 mg N L^{-1}; black triangles), and under starting oxic conditions in the presence of acetylene (10% v/v; white triangles). Error bars indicate standard deviations of triplicate experiments (sometimes smaller than symbols). A biomass-free control experiment (black circles) was included to exclude abiotic N_2O removal. A, B, C and D respectively represent the amount of N_2O-N, O_2, N_2-N and CO_2-C in the headspace in function of time.

3.3 Microbial community analysis of the anaerobic and aerobic enrichment

Fingerprinting of 16S rRNA at the end of enrichment procedure as well as 50 days later revealed the same dominant DGGE band for both anaerobic and aerobic enrichments. Next, separate dilution plating was performed for the cultures of aerobic and anaerobic enrichments. Nine anaerobic and ten aerobic isolates were selected based on differences in colony morphology. The N_2O removal ability of all isolates was confirmed in anoxic incubations with the M9-Ac medium. 16S rRNA of each isolate was amplified by PCR, analysed on DGGE and compared with the enrichment cultures. The bands of all the isolates were found at the same position on the DGGE gel and were identical to the dominant band of the mixed cultures obtained from anaerobic and aerobic enrichments.

The complete 16S rRNA genome of isolates was sequenced and showed 99% similarity to that of *P. stutzeri*. Isolates were differentiated to subspecies level by BOX-PCR fingerprinting. Fingerprints could be attributed to six groups and four individuals based on a cluster cut-off value of 86% (Ghyselinck et al., 2011) and visual inspection.

3.4 *P. stutzeri*: N_2O consumption in presence of oxygen

The *P. stutzeri* isolate LMG 26881 was selected as a model organism and its N_2O consumption potential was investigated at different O_2 concentrations (Fig. 5.3). The isolated *P. stutzeri* simultaneously consumed N_2O and O_2 except for incubations with 20% v/v O_2 in the headspace where solely O_2 consumption was observed. Furthermore, the lag time of N_2O consumption and the variation between replicate samples increased with increasing O_2 concentration in the headspace. Biomass-free controls did not show N_2O removal after an initial equilibration (Fig. 5.3). Clearly, the N_2O consumption pathways became less functional as the N_2O consumption rate decreased with increasing O_2 concentration.

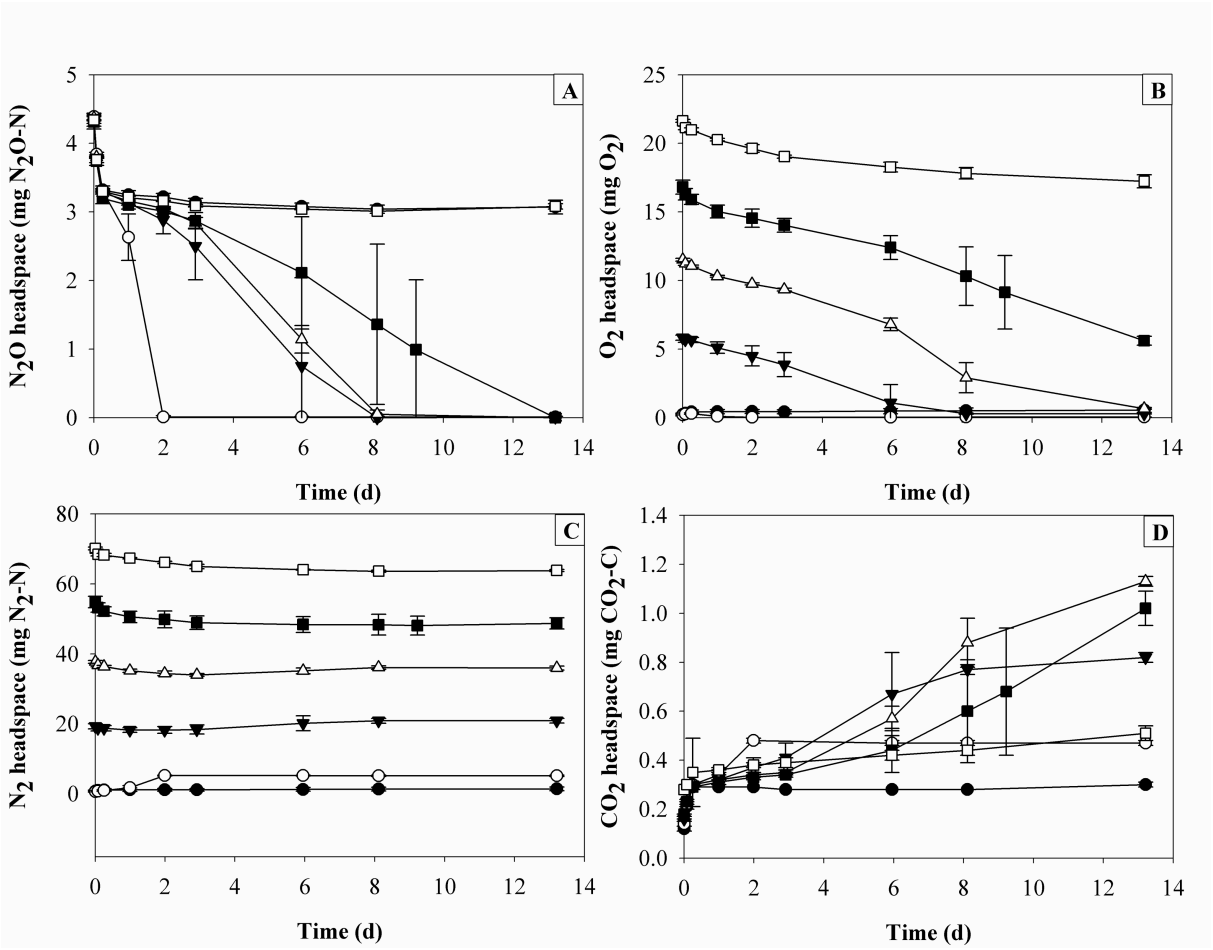

Figure 5.3 Ability of *P. stutzeri* to consume N_2O at different O_2 partial pressures (0% v/v O_2, white circles; 5% v/v O_2, black triangles; 10% v/v O_2, white triangles; 15% v/v O_2, black squares; 20% v/v O_2, white squares). Error bars indicate standard deviations of triplicate experiments (sometimes smaller than symbols). A biomass-free control (0% v/v O_2, black circles) was included to exclude abiotic N_2O removal. A, B, C and D respectively represent the amount of N_2O-N, O_2, N_2-N and CO_2-C in the headspace in function of time.

3.5 *P. stutzeri*: pathway of N_2O consumption

The pathways of N_2O consumption in the isolated *P. stutzeri* strain were studied by tracing ^{15}N labelled N_2O in microbial biomass-N during anoxic incubation. At the start of incubations, headspaces were administrated with 5% (v/v) N_2O at 99.95 atom% ^{15}N and 95% (v/v) N_2 at natural abundance (0.37 atom% ^{15}N). In this case N_2 could maximally reach ca. 5 atom% ^{15}N if all N_2O was reduced to N_2. The hypothesized pathways of N_2O reduction and resulting ^{15}N atom% of microbial biomass are shown in Fig. 5.1. With, (1) respiratory reduction of N_2O to N_2, (2) fixation of N_2 to NH_4^+ plus incorporation in microbial biomass, and (3) assimilatory N_2O reduction to NH_4^+ plus incorporation in microbial biomass. Accordingly, ^{15}N atom% in microbial biomass would not exceed that of N_2 when N_2O was first reduced to N_2 and left the cells before being fixed. Direct assimilatory N_2O reduction, on

the contrary, would result in a ^{15}N ratio of microbial biomass above that of N_2 in the headspace.

The ^{15}N content of the microbial biomass was assessed by persulphate digestion of total N to NO_3^- and subsequent bacterial conversion to N_2O to facilitate isotope ratio measurement. Microbial N comprised on average more than 98% of the total N present in the medium. Biomass-free samples administered with ^{15}N-N_2O were found to contain 0.56 ± 0.03 atom% ^{15}N in the organic N fraction. This blank contamination likely originates from the oxidation of dissolved N_2O by persulphate used to assess total organic N. Furthermore, the medium itself also contained organic N. Correcting for these non-microbial fractions of organic N is very much required given that the amount of microbial N less than doubled over the 48h incubation, i.e. only 84%.

The atom% ^{15}N in headspace N_2 and organic N of isolated *P. stutzeri* measured on various times during incubation (Fig. 5.4) reveals how N from N_2O flows to microbial N. Averaged over all time points the headspace N_2 contained 2.34 atom% of ^{15}N, whereas the microbial biomass only contained 1.90 atom%. This confirms that N_2O is reduced to N_2 and leaves the cell before being incorporated into microbial biomass of the studied *P. stutzeri* strain. The higher atom% ^{15}N in microbial biomass compared to N_2 in the headspace during the first 18 hours of incubation is likely owed to an offset in the ^{15}N atom% of N_2 between the inside of microbial cells and headspace at the start of the incubation due to retarded exchange of N_2 between the cell and headspace. Assimilatory N_2O reduction cannot cause this initial offset given that it should render the $^{15}N/^{14}N$ ratio of microbial biomass to approach the 99.95 atom% ^{15}N of N_2O.

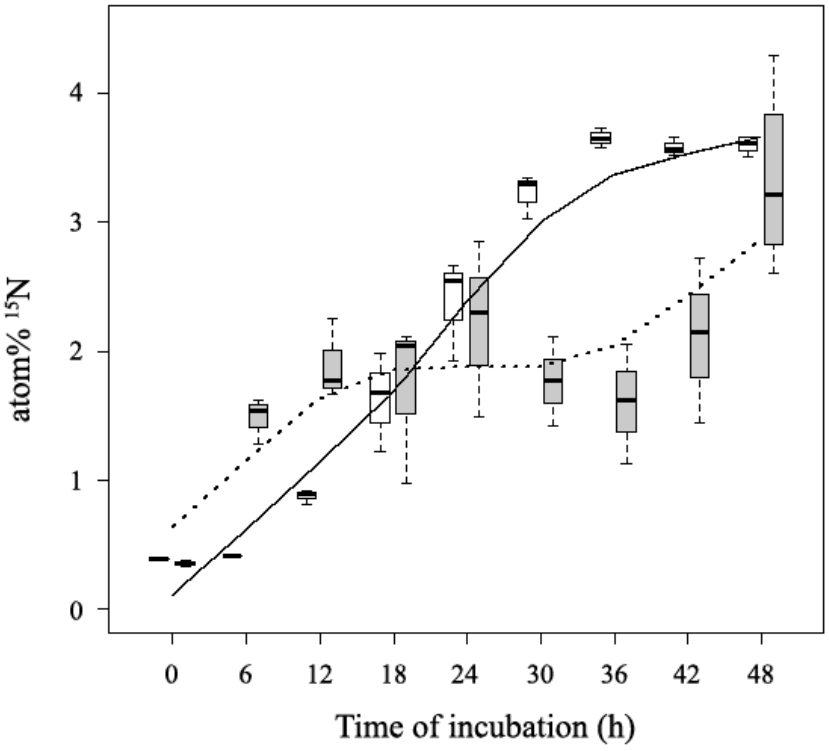

Figure 5.4 Atom% ^{15}N of N_2 in the headspace (white boxes) and microbial biomass of inoculated samples (grey boxes) during incubation with 99.95 atom% double-labelled ^{15}N-N_2O.

4 Discussion

4.1 N_2O consumption under anoxic and oxic conditions

Both aerobic and anaerobic mixed culture enrichments consumed N_2O at an oxygen concentration of 20% (v/v) in the headspace (Fig. 5.2), whereas in the isolate of *P. stutzeri* N_2O consumption stopped at an O_2 concentration higher than 15% (v/v) (Fig. 5.3). Aerobic microorganisms positioned at the surface of the flocs formed in mixed cultures may keep O_2 outside the centre of the flocs resulting in lower O_2 sensitivity for N_2O consumption. Floc formation was not observed during incubation with pure *P. stutzeri* cultures, which might explain absence of N_2O consumption at higher O_2 partial pressures.

The N_2O consumption by *P. stutzeri* isolates under oxic conditions in this study supports previous findings that the *NosZ* gene can be expressed at high O_2 concentrations (Miyahara et al., 2010). Nevertheless, aerobic N_2O consumption remains remarkable because the N_2O reductase is postulated to be very sensitive to O_2 (Knowles, 1982, Mckenney et al., 1994, Otte et al., 1996). However, it should be noted that the observed aerobic N_2O consumption might have been driven by the high N_2O concentration applied in this experiment. A headspace concentration of 5% (v/v) N_2O typically renders a concentration of 44 mg L^{-1} N_2O-N in water as compared to 9 mg L^{-1} O_2 with 20% v/v O_2 in the headspace (using Henry coefficients of 0.029 and 0.0014 M atm^{-1} for N_2O and O_2, respectively, at 20 °C). Consequently, the initial concentration of N_2O in the medium was five-fold that of O_2.

The negative effect of NH_4^+ on N_2O consumption under oxic conditions seems surprising. Indeed, respiratory N_2O reduction should be feasible using NH_4^+ instead of N_2 as a nitrogen source for growth (Lalucat et al., 2006, Miyahara et al., 2010). Furthermore, there are no reports indicating that NH_4^+ has a regulatory role on N_2O reductase activity. *P. stutzeri* is a facultative anaerobic specie that can also use O_2 as terminal electron acceptor, however, microaerophilic conditions have to be established when they are cultured as diazotrophs (Lalucat et al., 2006) as nitrogenases are sensitive to high concentrations of O_2 (Newton, 2007). By the addition of NH_4^+, the need to create microaerophilic conditions appeared to be absent as no floc formation could be observed when NH_4^+ was added. Ammonium is known to suppress nitrogenase activity (Desnoues et al., 2003) as a ^{15}N-N_2 tracing study by Krotzky and Werner (1987) showed that N_2-N incorporation by *P. stutzeri* decreased five-fold when

NH_4^+ was administered during incubation. Furthermore, NH_4^+ assimilation is less energy-intensive compared to N_2 fixation (Martinez-Romero, 2006). As a result, the enrichment shifted to an aerobic metabolism with NH_4^+ as nitrogen source and O_2 as terminal electron acceptor, thereby using all acetate as carbon and electron donor. N_2 fixation by nitrogenase was suppressed by NH_4^+ and O_2. Respiratory N_2O consumption by N_2O reductase was likely impeded by a too high O_2 concentration.

The cessation of N_2O consumption under a headspace containing 10% v/v acetylene (Fig. 5.2) is attributed to the inhibition of N_2O reductase activity by acetylene (Balderston et al., 1976). Furthermore, the lack of microbial growth when acetylene is administered shows that N_2 fixation was also blocked. This was probably due to competition from acetylene as a substrate for the nitrogenase (Hardy and Knight, 1966).

4.2 Dominance of *P. stutzeri*

The identification of a diverse collection of *P. stutzeri* strains in aerobic and anaerobic enrichments originating from the activated sludge from a domestic wastewater treatment plant performing biological nitrogen removal can be explained by the fact that N_2O consumption is generally attributed to denitrifying bacteria, which include *P. stutzeri* species (Heylen et al., 2006). Furthermore, as mentioned earlier, *P. stutzeri* species are also capable of consuming N_2O under oxic conditions (Miyahara et al., 2010). However, as wastewater usually contains mineral N, diazotrophic microorganisms would not be expected to be present (Newton, 2007) and yet N_2 fixation does occur in wastewater treatment plants, mostly when mineral N availability is low (Kargi and Ozmihci, 2004, Reid et al., 2008, Slade et al., 2011). Moreover, different species of *P. stutzeri* possess the functional genes for N_2 fixation (Krotzky and Werner, 1987, Lalucat et al., 2006, Vermeiren et al., 1999, Yan et al., 2008).

4.3 Pathway of N_2O consumption

Tracing of ^{15}N-N_2O in microbial biomass-N of the *P. stutzeri* strain (Fig. 5.4) provided important information on the proposed N assimilation pathways (Fig. 5.1), and gave insight into the provoked question whether these pathways have a functional linkage. Indeed, next to N_2 fixation by nitrogenase, the existence of direct N_2O fixation or assimilatory N_2O reduction by nitrogenase has been suggested by various authors (Burgess and Lowe, 1996, Jensen and Burris, 1986, Yamazaki et al., 1987). However, this pathway has been refuted in this specific experiment, as the $^{15}N/^{14}N$ ratio of the microbial biomass would have exceeded that of N_2 in this case. The average atom% ^{15}N of the microbial biomass was 0.44 atom% lower than that

of N_2 in the headspace (Fig. 5.4). This shows however that respiratory N_2O reduction can be coupled to N_2 fixation as N_2O is first reduced to N_2, before being partly further reduced to NH_4^+ and incorporated into cell protein. The stagnation of atom% ^{15}N in biomass N after 24h was due to the absence of electron acceptor as all N_2O was respired by then. Moreover, the very high N_2O concentrations (5% v/v) in the incubations as compared to ambient conditions (320 ppbv) reduce the likelihood of assimilatory N_2O reduction in the studied *P. stutzeri* strain. Furthermore, the apparent growth of *P. stutzeri* with N_2O as the sole electron acceptor suggests that N_2O reduction provides sufficient energy for N_2 fixation. The question remains whether the demonstrated coupling of respiratory N_2O reduction and N_2 fixation in this model microorganism plays a significant role in N cycling of natural ecosystems, and therefore deserves further investigation.

5 Conclusions

Studying the pathways of N_2O consumption is critical to acquire a more comprehensive understanding of the net N_2O fluxes and to assess their responses to environmental changes. The combination of analytical techniques adopted in this study allows for correct investigation of N_2O cycling by microbial isolates. ^{15}N-N_2O tracing conclusively showed that the studied strain of *P. stutzeri* consumes N_2O by respiratory reduction to N_2, not by assimilatory reduction to NH_4^+. However, the generality of this pathway should be investigated due to the anticipated enzymatic potential for assimilatory N_2O reduction. In regard of this thesis, the obtained enrichment shows potential for curative N_2O treatment.

6 Acknowledgements

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Chapter 6 Electrochemical nitrogen recovery

Abstract

Direct nitrogen recovery from wastewater could supply a significant amount of the current reactive nitrogen demand. Several agricultural and industrial liquid waste streams contain nitrogen concentrations up to several grams per liter. For these streams, nitrogen recovery could be a more sustainable and economical attractive technology compared to biological nitrogen removal (BNR), as the latter becomes very energy-intensive. Moreover, with this approach significantly less nitrogen has to pass through a BNR system, which would result in a much lower overall N_2O emission from BNR processes. As a result, recovery not only allows to recycle valuable nutrients, it is also a preventive measure to mitigate BNR derived N_2O emissions.

A typical example of such an interesting waste stream is digestate, a product from the anaerobic digestion of organic material. In this lab-scale study, an electrochemical cell was used to investigate the NH_4^+ flux from the anode compartment, loaded with the waste stream, to the cathode compartment. Furthermore, subsequent recovery of nitrogen from the cathode was also evaluated. Under optimal conditions with synthetic wastewater, an NH_4^+ charge transfer efficiency of 96% and NH_4^+ flux of 120 g N m^{-2} d^{-1} could be obtained at a concomitant electricity input of 5 kWh kg^{-1} N removed. A more selective NH_4^+ transfer could be established by maintaining a high concentration of other cations in the cathode compartment. Comparable NH_4^+ fluxes could be obtained with digestate at an electrical power input of 13 kWh kg^{-1} N removed and 41% current efficiency. The ammonium level in the digestate could be lowered from 2.1 to 0.8 – 1.2 g N L^{-1}. Interestingly, also potassium fluxes of up to 241 g K^+ m^{-2} d^{-1} could be obtained at 23% current efficiency. As the cathode can be operated at high pH without the need for chemical addition, stripping and absorption of dissolved ammonia could reach 100% efficiency.

Chapter redrafted after: *Desloover, J., Abate, A., Verstraete, W., Boon, N. & Rabaey, K. (2012). Electrochemical resource recovery from digestate to prevent ammonia toxicity during anaerobic digestion. Environmental Science & Technology, 46(21), 12209-12216.*

1 Introduction

Given the unlimited amount of nitrogen present in the atmosphere (78% v/v), direct recovery of nitrogen from waste streams seems unnecessary. However, up to 50% of the reactive nitrogen produced through the Haber-Bosch process eventually ends up in waste streams (agricultural, industrial and domestic effluents; Chapter 1). The latter does not only indicate the strong market potential for direct nitrogen recovery, it could also become a more sustainable way of handling valuable nutrients. However, from an economical point of view, only waste streams containing up to several grams per liter of nitrogen are considered suitable for recovery (Mulder, 2003), such as digestates.

Digestate is a product from anaerobic digestion, a technology that enables the degradation and stabilization of organic waste streams with concomitant production of renewable energy in the form of methane (Holm-Nielsen et al., 2009). During this process, ammonium is set free during the degradation of nitrogenous matter such as proteins and urea (Kayhanian, 1999). As a result, depending on the feedstock, the digestate can contain nitrogen concentrations up to several grams per liter.

At this stage, digestates are usually treated in a BNR plant to remove excess organic matter and nutrients. However, it is known that the biological treatment of these kinds of streams is very energy-intensive (Mulder, 2003). Furthermore, it was shown that the N_2O emission derived from the biological treatment of these highly loaded waste streams can amount up to several percentages of the nitrogen load (Chapter 2). As a result, nitrogen recovery can be considered as an interesting alterative, thereby not only allowing nutrient recovery, but also prevention of the related N_2O emission when the stream would be treated in a BNR plant.

Next to this, it has also been widely documented that methanogens, responsible for the final step in the anaerobic digestion process, have a moderate tolerance to ammonia (Chen et al., 2008), a compound in equilibrium with ammonium according to the pH. This toxicity issue, inherent to the digestion of nitrogenous material, exposes digesters to a risk of process instability and limits the loading and thus biogas production rate (Angelidaki and Ahring, 1993).

Various techniques have been investigated to remove and/or recover ammonium from nitrogen-rich waste streams such as biological nitrogen removal (Verstraete et al., 2012), air or steam stripping (Lei et al., 2007, Siegrist, 1996), chemical precipitation (Siegrist, 1996), ion exchange or adsorbants (Tada et al., 2005), electrodialysis (Ippersiel et al., 2012), reverse osmosis (Mondor et al., 2008) and microbial fuel cells (Kuntke et al., 2012). While mainly

ammonia stripping has been investigated in the context of inhibition (De la Rubia et al., 2010, Walker et al., 2011, Zhang et al., 2012), high energy requirement, inefficient stripping and the need for chemicals have impeded full scale application.

In this chapter we investigated an electrochemical cell as a possible alternative approach to recover ammonium from nitrogen-rich waste streams. Such a system consists of an anode (oxidation) and cathode (reduction) compartment, separated by an ion exchange membrane (Anglada et al., 2009). By applying a voltage across the cell, electrical current flows from anode to cathode. In order to remain charge neutrality, this flux of electrons needs to be compensated by an equal flux of cations or anions, depending on the type of membrane that is used. Ammonium could be a suitable charge transporter in case a cation exchange membrane is used (Cord-Ruwisch et al., 2011). We hypothesize that by maintaining a high ionic strength of cations other than ammonium at the cathode side the ammonium flux over the membrane is improved. As NH_4^+ is converted to NH_3 in the alkalifying cathode and further removed by stripping, only other ions such as Na^+ would be available to exchange towards the anode. This leads to a net flux of for instance sodium from cathode to anode compensated by an increased NH_4^+ flux towards the cathode. The importance of the current is to drive a net increased flux towards the cathode and to generate the alkaline conditions that enable conversion of NH_4^+ to NH_3 and its stripping using formed (and recirculated) hydrogen gas. Hydrogen gas as the reductive cathode product can be used to increase the calorific value of the biogas from the digester or be fed into the digester for conversion to methane. Inefficiencies in the system lead to KOH or NaOH formation, these products can be used to remove CO_2 from biogas (and generate e.g. K_2CO_3) or be used elsewhere.

The aim of this lab-scale study is to map the ammonium flux in an electrochemical system by investigating the influence of operational parameters such as current density, pH, ionic strength and nitrogen concentration. Whereas the parameter experiments were performed on synthetic wastewaters, also digestate was used as a proof of concept to evaluate the applicability of this technology.

2 Material and methods

2.1 Experimental setup

The experimental setup comprised a 2-chambered electrochemical cell of which the cathode compartment was coupled to a stripping and absorption unit, depending on the performed experiment. A schematic overview of the setup is presented in Fig. 6.1 and Fig. 6.2.

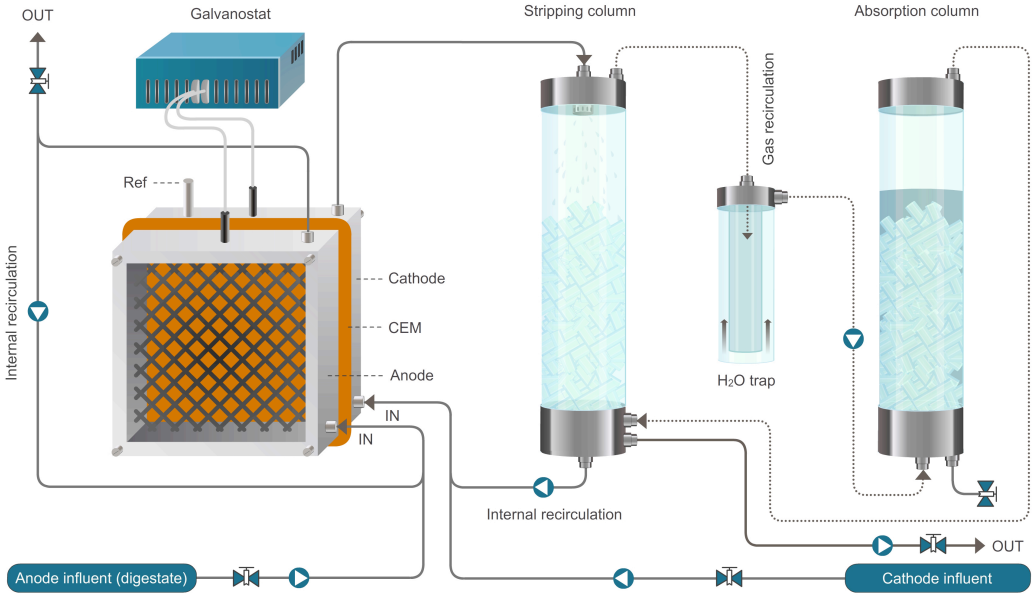

Figure 6.1 Schematic overview of the experimental setup. Solid line: liquid streams. Dashed lines: gas streams. Ref: reference electrode. CEM: Cathode Exchange Membrane.

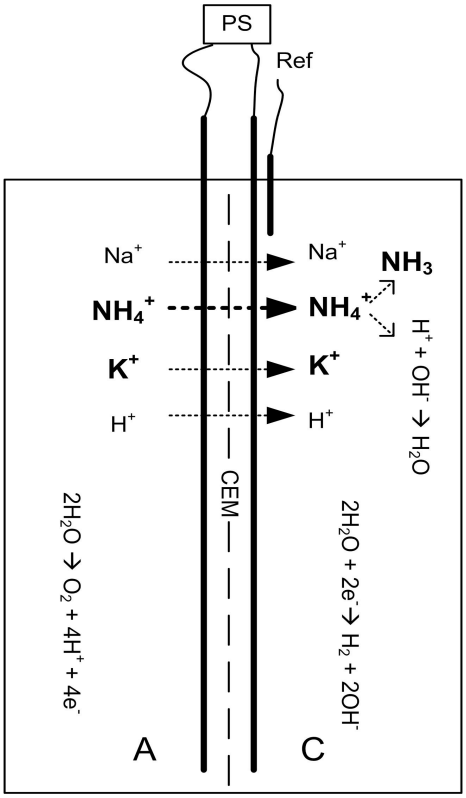

Figure 6.2 Indication of electrode reactions and flow direction of cations. A: anode compartment, C: Cathode compartment, CEM: Cation Exchange Membrane, PS: Power Supply, Ref: Reference electrode.

2.1.1 Electrochemical cell

Two square Perspex frames were used as anode and cathode compartments (internal dimensions: 8 x 8 x 1.9 cm, 2 cm wall thickness) and separated by a cation exchange membrane (Ultrex CMI-7000, Membranes International Inc., USA). The frames were bolted together between two square Perspex plates. The total volume for each compartment was 122 mL. The anode used was a Ti electrode coated with Ir MMO (dimensions: 7.8 x 7.8 cm; 1 mm thickness; specific surface area 1.0 m^2 m^{-2}, Magneto Special Anodes , the Netherlands). The cathode used was a 316L stainless steel mesh (dimensions: 7.8 x 7.8 cm; mesh width: 564 μm; wire thickness: 140 μm, Solana, Belgium). Both the anode and cathode had a projected electrode surface area of 64 cm^2 (corresponding to 52 m^2 per m^3 reactor compartment) and were placed in close contact to the membrane.

2.1.2 Stripping and absorption unit

Two types of stripping and absorption units were used. Type 1 was used during the experiments with synthetic wastewater and type 2, an improved version, was used for the experiments with digestate.

The type 1 unit consisted of two tubular columns (height: 0.7 m; width: 0.03 m) both filled with Kaldnes K1 packing material for 0.5 m. The liquid volume at the bottom of the stripping column was 25 mL. Type 2 consisted of two tubular columns (height 1 m; width 0.07 m), both filled with Raschig rings (dimensions: 4 x 4 mm; Saillart, Belgium) for 0.5 m. The liquid volume at the bottom of the stripping column was 80 mL. The spraying of the catholyte over the type 2 stripping column was improved by installing a nozzle at the top of the column. Furthermore, a gas chamber was foreseen above the liquid level at the bottom of the stripping column to improve the gas distribution over the column. The absorption column was filled in both types with 500 mL of 1 M H_2SO_4.

2.1.3 Reactor operation

All experiments were performed at room temperature (20±2 °C). Anode and cathode compartments were operated continuously at a flow rate of 4 L L^{-1} reactor compartment d^{-1} (0.5 L d^{-1}) unless stated otherwise, and an internal recirculation (49 L L^{-1} reactor compartment h^{-1} or 6 L h^{-1}) was provided in both compartments to ensure sufficient mixing. The hydraulic retention time (HRT) in the anode compartment was 6 h whilst the HRT in the cathode compartment was 7 or 9.6 h, in case the cathode compartment was connected to the

type 1 or type 2 stripping and absorption unit, respectively. Coupling was provided by integrating the stripping unit in the internal recirculation of the cathode compartment (Fig. 6.1). The gas phase was internally circulated in a closed loop (air flow rate: 2.5 - 4.5 L min^{-1}) over the stripping and absorption column, counter current to the liquid recirculation flow in the stripping column (6 L h^{-1}), resulting in an air/liquid ratio of 25 - 45. In case no stripping and absorption unit was used, the cathode HRT was equal to the anode HRT. For electrochemical control, a VSP multipotentiostat was used (Bio-Logic, France). The cathodic half-cell potential was measured by placing an Ag/AgCl reference electrode (assumed +0.197 V vs SHE, Bio-Logic, France) in the cathode compartment. The anode potential was calculated as the difference between cell voltage and cathode potential (not corrected for ohmic resistance).

The duration of closed circuit experiments with synthetic wastewater was 2 days and executed in duplicate. Four steady-state samples were taken from the anode, cathode and acid trap. The duration of closed circuit experiments with digestate was 4 days (not duplicated). Eight steady-state samples were taken from the anode, cathode and acid trap. Open circuit experiments were executed for 2 days to investigate diffusion driven processes. An overview of the operational conditions of the experiments performed to investigate different operational parameters is given in Table 6.1.

Table 6.1 Overview of the variable and fixed settings during experiments with synthetic wastewater and digestate. Characteristics of the type 1 and 2 stripping and absorption units are explained in the materials and methods section. N/A: Not Applicable.

Tested parameter	**Current Density** ($A\ m^{-2}$)	**Anode NH_4^+** ($g\ NH_4^+$-$N\ L^{-1}$)	**Cathode NaOH** (M)	**Cathode NaCl** (M)	**Type Stripping & Absorption Unit**
Experiments with synthetic wastewater					
Current density	Variable	5	0.1	N/A	Type I
NH_4^+ anode	20	Variable	0.1	N/A	Type I
pH cathode	10	5	Variable	N/A	Type I
Ionic strength cathode	10	5	N/A	Variable	N/A
Anode pH	10	5	N/A	0.1	N/A
Experiments with digestate					
Current density	Variable	± 2	N/A	0.1	Type II

2.2 Medium composition

For the anode, either synthetic wastewater or digestate was used. The synthetic wastewater contained per litre: 0.58 g KH_2PO_4, 1.03 g $Na_2HPO_4.2H_2O$, 0.10 g $MgSO_4.7H_2O$ and 0.02 g $CaCl_2.2H_2O$. This medium (pH neutral) was amended with the required amount of $(NH_4)_2SO_4$ to obtain a final concentration of 1, 3 or 5 g N L^{-1}, depending on the experiment. The digestate, after filter press and centrifugation, was obtained from a DRANCO installation (Brecht, Belgium) of which the general composition can be found in Table 6.2. The cathode feed consisted of either NaOH (0.01, 0.1 or 1 M) or NaCl (0.01, 0.1 or 1 M).

Table 6.2 General composition of the digestate obtained from a Dranco installation (Brecht, Belgium) after filter press and centrifugation.

Parameter	Value
TSS (g L^{-1})	21.4
VSS (g L^{-1})	13.3
COD (g L^{-1})	21.8
BOD (g L^{-1})	5
VFA (mg L^{-1})	3600
Kjeldahl-N (mg N L^{-1})	3435
NH_4^+-N (mg NH_4^+-N L^{-1})	2120
pH (-)	7.5-8
Alkalinity (meq L^{-1})	205
Conductivity (mS cm^{-1})	17.05
Na^+ (mg L^{-1})	2580
K^+ (mg L^{-1})	5097
Ca^{2+} (mg L^{-1})	65
Mg^{2+} (mg L^{-1})	41
Cl^- (mg L^{-1})	2093

2.3 Chemical analysis

Sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) were measured using Flame-AAS (AAS, AA-6300, Shimadzu). All samples were acidified with either HCl or HNO_3 before measurement and 2% of a 1 g L^{-1} lanthanum standard solution (Chem-Lab, Belgium) was added for the measurement of calcium or magnesium to suppress chemical interference with other elements. Nitrate (NO_3^-), nitrite (NO_2^-), sulfate (SO_4^{2-}), phosphate (PO_4^{3-}) and chloride (Cl^-) ions were determined with a 761 compact ion chromatograph equipped with a conductivity detector (Metrohm, Switzerland). Total solids (TS), volatile suspended solids (VSS), Kjeldahl nitrogen (KN), ammonium (NH_4^+), chemical oxygen demand (COD), biological oxygen demand (BOD), alkalinity, pH, and conductivity were determined according to Standard Methods (Greenberg, 1992). Volatile fatty acids (VFA)

were, after extraction in diethyl ether, analyzed with a flame ionization detector (FID) gas chromatograph (GC-2014, Shimadzu).

2.4 Calculations

Formulas used to calculate fluxes and efficiencies are given below. Current efficiencies always reflect efficiency for ammonium transfer unless stated otherwise. Current densities and fluxes were reported relative to the projected membrane-electrode surface area.

The nitrogen flux (J_N, g N m^{-2} d^{-1}) from anode to cathode was calculated as:

$$J_N = \frac{\left(C_{An,in} - C_{An,out}\right) \times Q}{A} \quad \text{(Eq. 6.1)}$$

where $C_{An,in}$ (g N L^{-1}) and $C_{An,out}$ (g N L^{-1}) are the measured ammonium concentrations coming in and out the anode compartment, respectively. Q (L d^{-1}) is the anode flow rate and A (m^2) is the membrane surface area (equal to projected anode and cathode surface area).

The nitrogen flux can be presented as a current density (I_N, A m^{-2}) by:

$$I_N = \frac{J_N \times z_{NH_4^+} \times F}{M \times 86400\, s/d} \quad \text{(Eq. 6.2)}$$

where z_{NH4+} (-) is the charge of NH_4^+, F the Faraday constant (96485 C mol^{-1}) and M the molecular weight of nitrogen (14 g mol^{-1}).

The current efficiency (CE, %) was calculated as:

$$CE = \frac{I_N}{I_{Applied}} \times 100 \quad \text{(Eq. 6.3)}$$

Where $I_{Applied}$ (A m^{-2}) is the applied current density.

The theoretical maximum nitrogen flux ($J_{N,max}$, g N m^{-2} d^{-1}) was calculated as:

$$J_{N,\max} = \frac{I_{Applied} \times M \times 86400\, s/d}{F \times z_{NH_4^+}} \quad \text{(Eq. 6.4)}$$

The theoretical maximum nitrogen removal efficiency (RE_{max}, %) was calculated as

$$RE_{\max} = \frac{J_{N,applied} \times A}{C_{An,in} \times Q} \times 100 \quad \text{(Eq. 6.5)}$$

where $J_{N,applied}$ (g N m^{-2} d^{-1}) is the applied current density expressed as a nitrogen flux.
The power input per kg of nitrogen transferred to the cathode (P_N, kWh kg^{-1} N) was calculated as:

$$P_N = \frac{Ecell \times I_{Applied} \times 1000 \times 86400}{J_N \times 3600000\, J/kWh} \quad \text{(Eq. 6.6)}$$

where E_{cell} is the cell voltage.

3 Results

3.1 Synthetic wastewater: influence of operational parameters

Synthetic wastewater was used as the anode feed to investigate the influence of operational parameters on the nitrogen flux to the cathode compartment. An overview of the results is presented in Table 6.3, and the effect of each variable operational parameter on the nitrogen flux is presented in Fig. 6.3.

3.1.1 Influence of applied current density

In the absence of current (Open Circuit, OC), the nitrogen flux was already 69% of that of the closed circuit (CC) flux at 10 A m^{-2}. The nitrogen flux increased by 49% when the applied current was increased from 10 to 30 A m^{-2} (Fig. 6.3A). A current efficiency of 77% could be reached at 10 A m^{-2}, meaning that the transfer of every mol of electrons to the cathode was accompanied by 0.77 mol NH_4^+.

3.1.2 Influence of NH_4^+ concentration

At a current density of 20 A m^{-2}, the nitrogen flux increased 3-fold by increasing the ammonium concentration in the anode feed from 1 to 5 g NH_4^+-N L^{-1}. Also the current efficiency increased from 37 to 51%. In contrast, the OC nitrogen flux did not increase further at a nitrogen concentration in the anode of 3 g NH_4^+-N L^{-1} (Fig. 6.3B).

3.1.3 Influence of pH and ionic strength of the cathode

The combined influence of cathode ionic strength and pH on the nitrogen flux was investigated by using different concentrations of NaOH as the cathode feed. Interestingly, the CC nitrogen flux showed an optimum at a cathode pH of 13 (0.1 M NaOH), whereas the OC showed a similar profile (Fig. 6.3C). This aspect was further investigated by applying different NaCl concentrations to the cathode compartment. Furthermore, the cell was not coupled to the stripping and absorption unit to prevent substantial pH differences in the cathode as the stripping process consumes hydroxyl ions. In this case, NaOH was produced *in-situ* due to the cathodic reduction of 2 H_2O to H_2 and 2 OH^-, resulting in a pH of 10.1-10.5 in the cathode compartment (Table 6.3). This time, no peak of the nitrogen flux was observed at 0.1 M NaCl, i.e. equal ionic strength as 0.1 M NaOH. Instead, a linearly increasing trend of the nitrogen flux could be observed within the range tested (Fig. 6.3D).

3.1.4 Influence of the anode pH

Under all CC conditions, the bulk pH in the anode compartment consequently decreased to approximately 1.56-1.98 (Table 6.3). While this implies that the proton concentration is a factor 10-20 lower compared to the other cations (except NH_4^+) present in the anode feed, it needs to be considered that the protons are made adjacent to the membrane and need to diffuse out to the bulk. Moreover, protons have a higher mobility relative to the other cations, implying that protons could play an important role in balancing the electron flux. The anode flow was increased from 4 to 66 and 131 L L^{-1} anode compartment d^{-1}, which resulted in a pH in the anode compartment of 4.1 and 6.1, respectively (Table 6.3). The significantly lower proton concentration in case of pH 6 had a clear effect on the nitrogen flux, which increased with about 26% (Fig. 6.3E), and a current efficiency of 96% could be obtained.

3.1.5 Power input characteristics

The required cell voltage was dependent on the applied current density and varied from 2.55 to 3.46 V (Table 6.3). This variation was due to varying anode potentials (+1.95±0.34 V vs SHE, not corrected for ohmic resistance) and cathode potentials (-1.13±0.09 V vs SHE). An example graph with electrode potentials over time during experiments with synthetic wastewater is presented in Fig. 6.4A.

The power input needed to drive the nitrogen flux ranged from 5.0 to 40.9 kWh per kg of nitrogen removed from the anode compartment, and was lowest in case of high anode flow (16 L d^{-1}) and thus higher anode pH, high ammonium concentration (5 g N L^{-1}) and low current density (10 A m^{-2}) (Table 6.3). This amount of energy has to be added to the energy needed for subsequent stripping and absorption.

Table 6.3 Overview of the results obtained with synthetic wastewater and digestate. N/A: Not Applicable

Mode	Anode NH_4^+	Cathode NaOH or NaCl	Theor. Anode N Removal Efficiency	Appl. Current Density	N flux as Current Density	Anode N Removal Efficiency	pH Effl. Anode	pH Effl. Cathode	Cell Voltage	Power Input
	(g N L^{-1})	(M)	(%)	(A m^{-2})	(A m^{-2})	(%)	(-)	(-)	(V)	(kWh kg^{-1} N rem.)
Synthetic wastewater (Fig. 6.3A-E)										
		NaOH								
Current density (Fig. 6.3A)										
OC	5	0.1	N/A	0	5.57±0.13	19±1	7.12±0.04	12.27±0.18		N/A
CC	5	0.1	31	10	7.65±0.44	25±1	1.82±0.13	12.55±0.24	2.73±0.06	6.7±0.4
CC	5	0.1	62	20	10.1±0.5	34±1	1.62±0.08	12.87±0.26	2.99±0.08	11.1±0.6
CC	5	0.1	91	30	11.3±0.6	41±2	1.56±0.14	12.92±0.08	3.35±0.21	16.8±1.4
N concentration (Fig. 6.3B)										
OC	1	0.1	N/A	0	2.24±0.13	38±2	8.54±0.01	13.00±0.04		N/A
CC	1	0.1	>100	20	3.32±0.17	57±3	1.66±0.01	13.07±0.07	4.04±0.52	40.9±5.6
OC	3	0.1	N/A	0	5.32±0.13	28±1	7.37±0.18	12.62±0.01		N/A
CC	3	0.1	>100	20	7.40±0.50	41±3	1.59±0.06	13.00±0.04	3.26±0.25	18.9±1.9
OC	5	0.1	N/A	0	5.57±0.13	19±1	7.12±0.04	12.27±0.18		N/A
CC	5	0.1	62	20	10.1±0.5	34±1	1.62±0.08	12.87±0.26	2.99±0.08	11.1±0.6

NaOH concentration (Fig. 6.3C)										
OC	5	0.01	N/A	0	1.02±0.28	3±1	6.55±0.01	11.93±0.04		N/A
CC	5	0.01	31	10	5.65±0.25	18±1	1.80±0.12	11.97±0.09	3.28±0.16	11.0±0.7
OC	5	0.1	N/A	0	5.57±0.13	19±1	7.12±0.04	12.27±0.18		N/A
CC	5	0.1	31	10	7.65±0.44	25±1	1.82±0.13	12.55±0.24	2.73±0.06	6.7±0.4
OC	5	1	N/A	0	3.61±0.56	11±2	8.68±0.21	13.93±0.07		N/A
CC	5	1	31	10	5.54±0.53	19±2	2.07±0.12	13.90±0.04	2.73±0.07	9.3±0.9
		NaCl								
NaCl concentration (Fig. 6.3D)										
OC	5	0.01	N/A	0	1.58±0.23	6±1	6.86±0.23			N/A
CC	5	0.01	31	10	5.95±0.26	21±1	1.93±0.07	10.57±0.09	2.69±0.04	8.5±0.2
OC	5	0.1	N/A	0	3.02±0.30	10±1	6.71±0.15			N/A
CC	5	0.1	31	10	6.95±0.23	23±1	1.98±0.10	10.12±0.15	2.56±0.07	6.9±0.4
OC	5	1	N/A	0	4.47±0.30	16±1	6.78±0.28			N/A
CC	5	1	31	10	7.77±0.39	27±1	1.95±0.19	10.19±0.03	2.59±0.06	6.3±0.4

Flow anode (Fig. 6.3E)										
CC	5 (0.5 L d^{-1})	0.1	31	10	7.65±0.44	25±1	1.82±0.13	12.55±0.24	2.73±0.06	6.7±0.4
CC	5 (8 L d^{-1})	0.1	2	10	9.08±0.29	2±0	4.15±0.07	10.49±0.01	2.55±0.01	5.3±0.2
CC	5 (16 L d^{-1})	0.1	1	10	9.64±0.19	1±0	6.09±0.01	10.45±0.07	2.57±0.03	5.0±0.1
Digestate (Fig. 6.3F)										
		NaCl								
OC	2	0.1	N/A	0	0.33±0.22	3±2	7.90±0.01	8.27±0.38		
CC	2	0.1	91	10	4.09±0.21	38±2	6.95±0.07	12.67±0.22	2.83±0.14	13.1±0.9
CC	2	0.1	>100	20	7.15±0.33	58±3	5.62±0.24	12.73±0.06	3.17±0.08	16.7±0.9
CC	2	0.1	>100	30	7.53±0.16	63±1	4.75±0.03	12.88±0.04	3.46±0.07	26.0±0.7

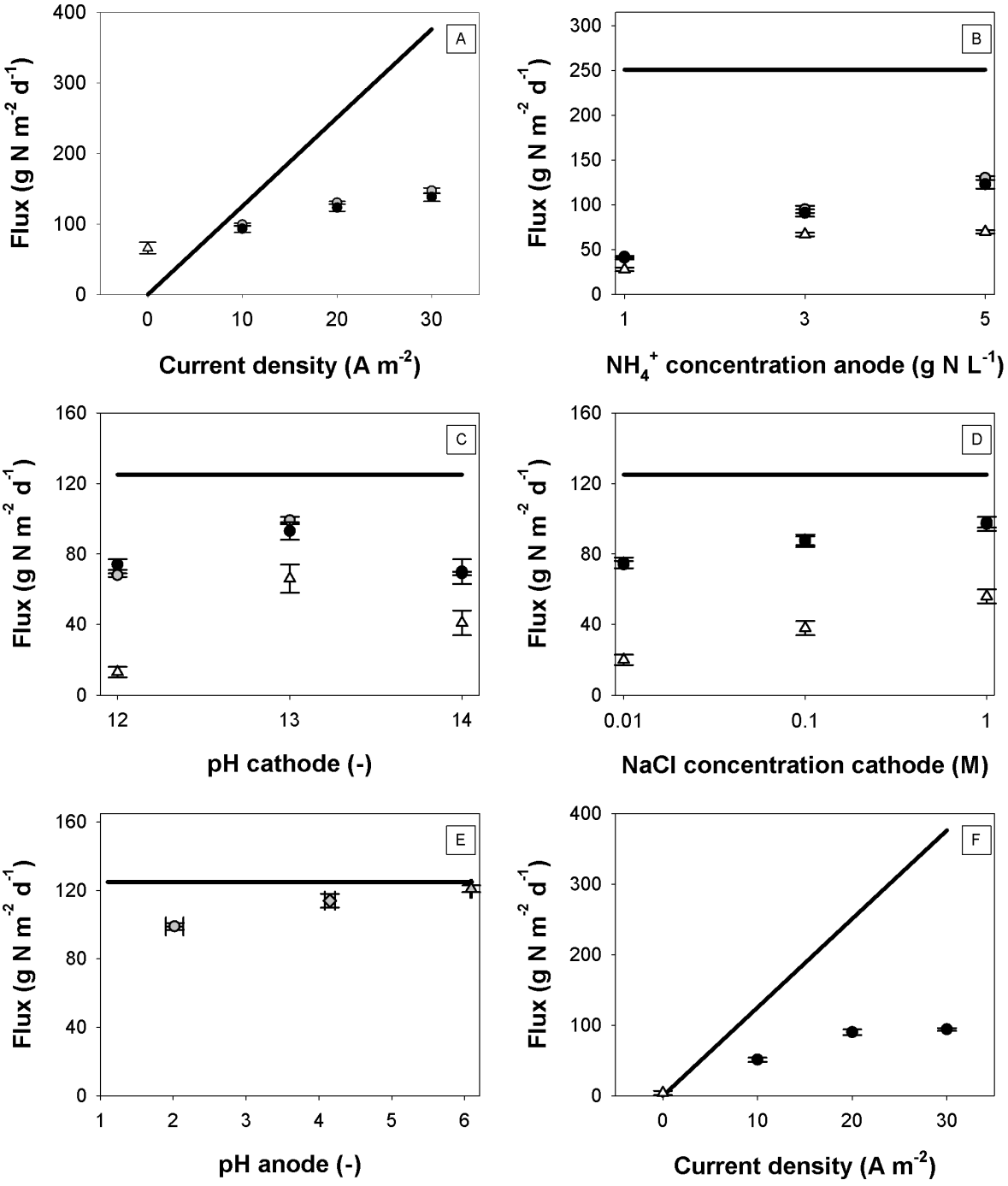

Figure 6.3 Influence of current density (A), anode NH_4^+ concentration (B), cathode pH (C), cathode NaCl concentration (D) and anode pH (E) on nitrogen flux during experiments with synthetic wastewater. F: influence of the current density on nitrogen flux during experiment with digestate. The black solid line indicates 100% current efficiency for NH_4^+ transfer to the cathode. Symbols: A-D: triangles: open circuit flux, black and grey circles: closed circuit flux (duplicated); E: circle: anode flow 4 L L^{-1} anode compartment d^{-1}, square: anode flow 66 L L^{-1} anode compartment d^{-1}, triangle: anode flow 131 L L^{-1} anode compartment d^{-1}; F: triangle: open circuit flux, black circles: closed circuit flux.

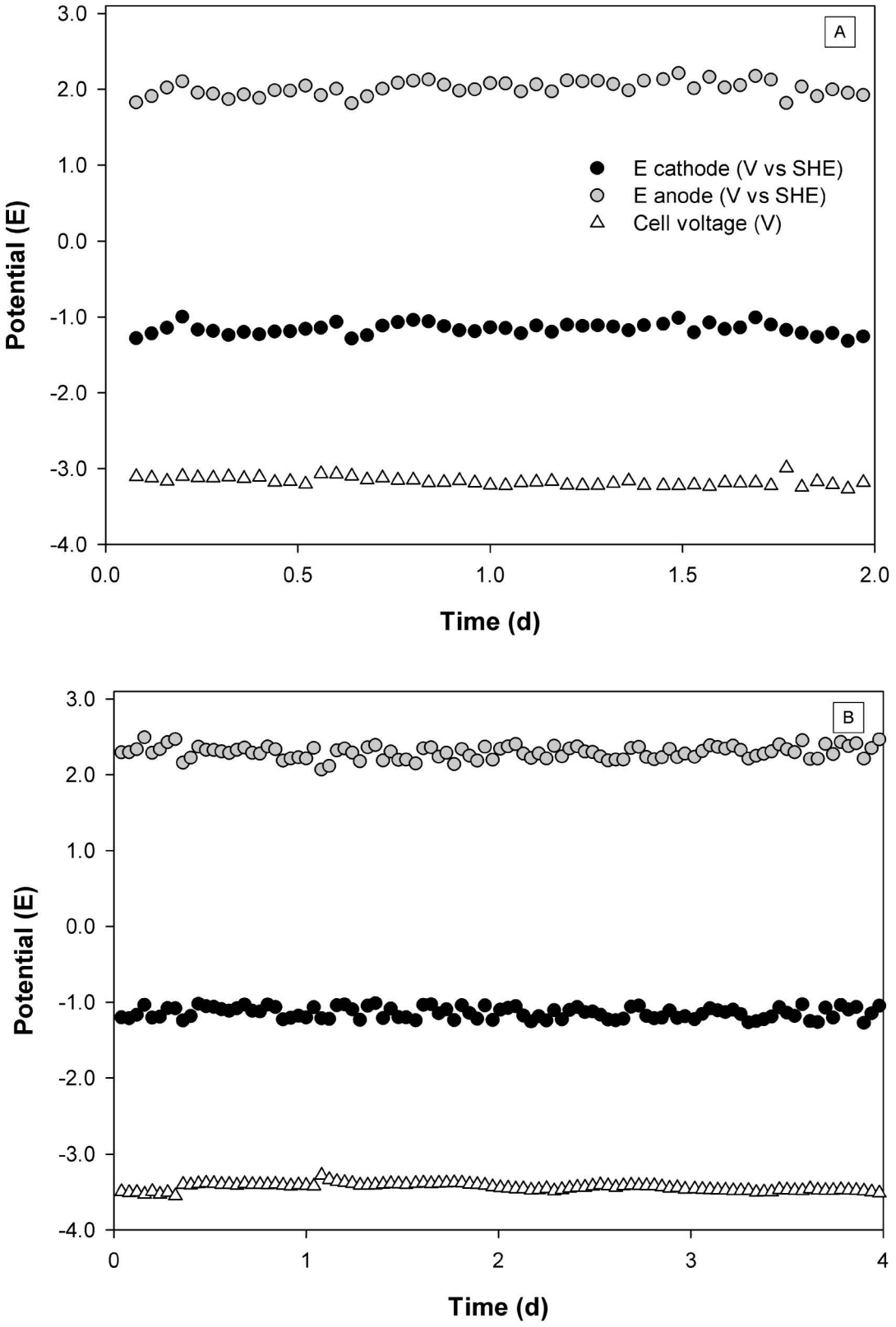

Figure 6.4 Electrode and cell potential in function of time. A: experiment with synthetic wastewater at 30 A m^{-2}, anode 5 g N L^{-1} and cathode 0.1 M NaOH. B: experiment with digestate at 30 A m^{-2} and cathode 0.1 M NaCl.

3.2 Digestate: influence of current density on the nitrogen flux and charge balance

After investigating the effect of the operational parameters on synthetic wastewater, digestate was used as anode feed to investigate the nitrogen flux in a real matrix.

The attainable nitrogen flux with digestate (± 2 g N L^{-1}) at different applied current densities was comparable to the fluxes obtained with synthetic wastewater (Fig. 6.3A and 6.3F). For instance, at 20 A m^{-2} with synthetic wastewater containing 3 g N L^{-1}, a flux of 95 g N m^{-2} d^{-1} was obtained compared to 90 g N m^{-2} d^{-1} with digestate containing ± 2 g N L^{-1}. In contrast, the OC flux with digestate was negligible compared to synthetic wastewater. The relative contribution of the different cations that balance the electron flux are visualized in Fig 6.5. This figure demonstrates that current efficiencies of 40% can be obtained for NH_4^+, and that charge balancing by protons or reversed hydroxyl flux from cathode to anode increases with increasing current densities. Furthermore, the Na^+ flux is relatively low despite a similar concentration to NH_4^+ in the digestate (Table 6.2). Potassium was removed at a rate of 130 – 243 g K^+ m^{-2} d^{-1}, depending on the applied current density, leading to K^+ removal efficiencies of 33 – 68%. Next to cation transport, also electroosmosis must have occured whereby ions drag water molecules each time they move through the membrane. However, this effect was not measurable.

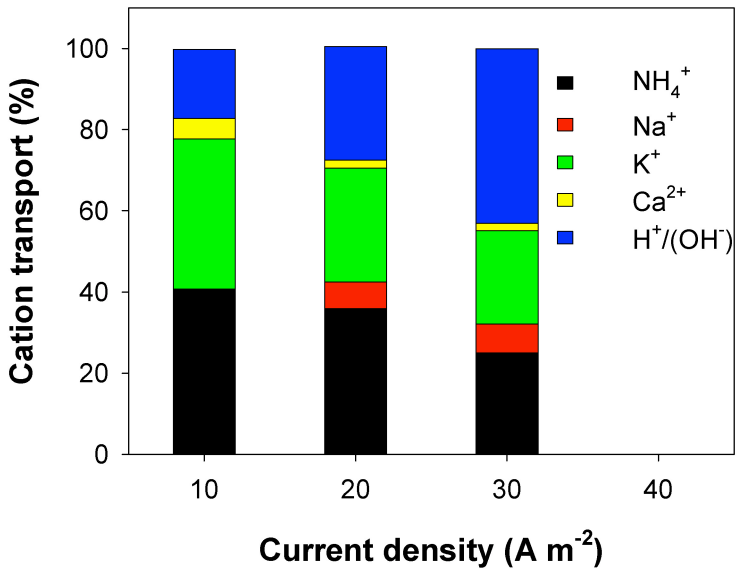

Figure 6.5 Relative contribution of cations to the charge balance in function of the applied current density during operation with digestate.

3.2.1 Power input characteristics

An example graph with electrode potentials over time during experiments with digestate is presented in Fig. 6.4B.

The electrical power input increased from 13 to 26 kWh kg^{-1} N transferred from the digestate to the cathode compartment, depending on the applied current density (Table 6.3). This amount of energy has to be added to the energy needed for subsequent stripping and absorption.

3.3 Nitrogen recovery efficiency and nitrogen mass balance

For the experiments with synthetic wastewater, the type 1 stripping and absorption unit was coupled to the cathode compartment (except for the experiments performed with different NaCl concentrations). At the beginning, a stripping/absorption efficiency of 67% could be obtained and decreased linearly over time to approximately 32% due to a decreasing gas recirculation. The latter was caused by a decreasing performance of the air recirculation pump. However, this did not have a noticeable influence on the nitrogen flux. Taking all experiments with synthetic wastewater into account, the average nitrogen mass balance was 90±13%. The relatively small gap in the balance was likely due to ammonia loss from the vacuum pump and measurement error (flows, concentrations).

For the experiments with digestate, the type 2 stripping and absorption unit was used. At the beginning, a stripping/absorption efficiency of 100% was obtained but decreased again to 83% for the same reason as mentioned above. Overall, the nitrogen mass balance could be closed for 90±8%.

4 Discussion

4.1 Creating a high and selective NH_4^+ transfer at high current efficiency

Overall, the flux of ions in an electrochemical cell is determined by the interplay of two different processes, that is, diffusion and electro-migration. These two transport mechanisms are responsible for charge balancing and can be described by the Nernst-Planck equation (Sokalski et al., 2003). Transport of water due to osmosis or electro-osmosis was not observed (flows measured daily).

Under OC conditions, only diffusion plays a role, which is a concentration-driven process and dependent on the diffusion coefficients of the ions in the solution and the membrane

(Harnisch et al., 2009). This aspect is clearly demonstrated in Fig. 6.3B for an increase from 1 to 3 g N L^{-1} in the anode, as the nitrogen flux increased significantly. No further significant increase was observed at 5 g N L^{-1}. The diffusion rate of NH_4^+ to the cathode under OC conditions was also dependent on the concentration of cations in the cathode compartment, in this case Na^+ (Fig. 6.3D). Indeed, it has been demonstrated that by maintaining a higher concentration of Na^+ in the cathode compared to the anode, a higher flux of NH_4^+ can be obtained due to ion exchange with Na^+ (Cord-Ruwisch et al., 2011). Interestingly, this linear increase in flux of NH_4^+ with increasing Na^+ concentration in the cathode was not observed in case NaOH was the catholyte instead of NaCl (Fig 6.3C). The latter showed a decrease of the NH_4^+ flux at pH 14 (1 M OH^-) which was most likely due to the diffusion of OH^- through the CEM to the anode compartment, thereby establishing part of the charge balancing (Rozendal et al., 2006, Sleutels et al., 2009). Surprisingly, in the case where digestate was investigated under OC conditions, almost no diffusion of NH_4^+ could be observed (Fig 6.3F), and this was also true for other cations such as K^+, despite its high concentration in the digestate (Table 6.2). Most likely, polyelectrolyte added during filter press and centrifugation of the digestate complexated the cations during open circuit conditions.

Under CC conditions, electro-migration comes into play, which is depending on the valence, concentration and diffusion coefficient of the ionic species, as well as the strength of the electrical field (Harnisch et al., 2009). The latter is determined by the applied current density and dielectric constant of the electrolytes and the membrane. The positive correlation between the NH_4^+ flux and the current density was demonstrated for both synthetic wastewater (Fig. 6.2A) and digestate (Fig. 6.3F). When comparing the NH_4^+ fluxes under OC and CC conditions, two aspects have to be taken in mind. First, the current density is directly related to the oxidation rate and thus proton production. This results in an acidification at the anode (Rozendal et al., 2006) and in this case particularly near the membrane surface where the electrode is positioned. This increase in proton concentration with increasing current density (Table 6.3) has a direct effect on the overall cation flux as the ion mobility of H^+ is about 5-6 times higher compared to the mobility of NH_4^+, K^+ and Na^+ (Lide, 2001, Rozendal et al., 2006). This explains the relatively small difference in NH_4^+ flux between OC and 10 A m^{-2} (Fig. 6.3A) as the proton concentration is 4 orders of magnitude higher under CC conditions. It was demonstrated that by increasing the anode flow and thus alkalinity load, the pH decrease could be lowered, which eventually resulted in a higher NH_4^+ flux (Fig. 6.3E).

Second, the electrical field drives the migration of cations from anode to cathode, and thus works opposite to the concentration-driven diffusion of Na^+ from cathode to anode. For

synthetic wastewater, the latter could be confirmed by the lower net diffusion of Na^+ to the anode under CC compared to OC conditions, but still resulted in a selective, albeit less pronounced, NH_4^+ transfer by applying a high Na^+ concentration in the cathode (Fig 6.3C and 6.3D).

The influence of pH and alkalinity of the anode feed on the one hand and electrical field and ionic strength of the cathode on the other hand, were visualized in a charge balance graph for the experiments performed with digestate (Fig. 6.5). This figure clearly demonstrates the selective transfer of NH_4^+ and K^+ over Na^+, due to the similar Na^+ concentration in both compartments. Even though Na^+ was 28% of the cationic strength in the anode, it represented only 0 to 7.1% of the flux towards the cathode, depending on the applied current density. Furthermore, a higher current density shows the increased participation of protons, for charge balancing due to a decreasing pH of the digestate. Alternatively, also diffusion of hydroxyl ions from cathode to anode could have played a role in charge balancing the electron flow, as shown in the experiment with synthetic wastewater and a pH of 14 in the cathode.

The performed experiments demonstrated that high current efficiencies can be achieved in case three conditions are met: (i) a significant part of the charge balance is governed by concentration-driven diffusion, (ii) the pH in the anode is around neutral and (iii) cations in the digestate that are not of interest for recovery are present at a similar concentration in the cathode. Obtaining higher fluxes, however, requires higher applied current densities, which was shown to be inversely related to the current efficiency.

4.2 Power input characteristics

The standard electrode potentials (pH = 7 and T = 25 °C) for water oxidation at the anode and proton reduction at the cathode are respectively +0.8 V and -0.4 V vs SHE, resulting in a theoretical required cell voltage of 1.2 V (Thauer et al., 1977). During the experiments, electrode potentials were stable (see example Fig. 6.4) and the required cell voltage during the experiments (2.5 - 3.5 V) was determined by the applied current density, pH in the electrode compartments (59 mV per pH unit difference), anodic and cathodic overpotentials and ohmic losses. This voltage input together with the efficiency determines the energy need to extract NH_4^+ from the anode to the cathode compartment (Table 6.3). Our power requirement can be considered low compared to conventional electrodialysis, but so are the current densities and thus fluxes. For example, it has been demonstrated that NH_4^+ could be removed from swine manure in an electrodialysis stack batch setup at 70% current efficiency at a current density of 400 A m^{-2} and 17.5 V operating voltage (Ippersiel et al., 2012). However, the authors

obtained very low stripping efficiencies (±10%) unless by chemical dosage the pH of the concentrate was increased. In this study, high pH is generated *in situ* due to the fact that the cathode is placed in the concentrate compartment – we are thus performing electrolysis rather than electrodialysis. Current densities of an order of magnitude higher are technically feasible with our setup (Radjenouic et al., 2011), however the current efficiency is expected to decrease further at these current densities due to higher proton transfer. Possibly, spacers between membrane and electrode could partly alleviate this. The use of a biocatalysed anode could further decrease voltages provided low current densities of ~10 A m^{-2} are observed (Rabaey et al., 2010). While the system can also be operated as a microbial fuel cell, generating low amounts of energy (Kuntke et al., 2012), the obtained current densities and fluxes were a factor 100 lower compared to this study. It should be recognized that subsequent stripping and absorption of the catholyte requires a considerable energy input. The energy requirement for conventional air stripping and $(NH_4)_2SO_4$ production is 9 kWh kg^{-1} N, next to the need for CaO dosage for pH adjustment (Maurer et al., 2003). As mentioned above, chemical addition is not required in our case. It may be feasible to apply a gas diffusion electrode as cathode, whereby stripping costs could be considerably decreased. An interesting aspect is that a large K^+ flux was obtained towards the cathode when using digestate, thereby reducing overall energy input per kg of nutrient recovered. Potassium is an attractive fertilizer and upon reaction with the biogas the produced KOH can be converted into K_2CO_3 for use in agriculture.

5 Conclusions

The presented proof of concept allows to recover nitrogen without addition of chemicals, and to lower the amount of nitrogen that has to be treated through biological nitrogen removal processes. Hence, N_2O production inherent to BNR processes can be partly omitted. It has to be acknowledged however that the energy requirement for this direct recovery concept might be higher compared to refixation of nitrogen through the HB process. However, this concept has the potential to create multiple benefits such as lowering NH_3 toxicity during anaerobic digestion and the simultaneous recovery of other valuable nutrients such as potassium. The latter will lower the overall energy input per amount of nutrient recovered.

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Chapter 7 General discussion

The fact that wastewater treatment plants (WWTPs) are a potential source of N_2O has been known for many years. Considerable uncertainty existed, however, about the actual magnitude of BNR-derived N_2O emissions. Lab-scale studies reported N_2O emissions ranging from 0-95% of the nitrogen load and the first full-scale studies in 1995 reported values ranging from 0.035% to 14.6% (Kampschreur et al., 2009b). The high variability was attributed to the dynamic nature of N_2O emissions in combination with grab sampling, which is now considered as a non-accurate sampling strategy for N_2O measurements. A second aspect was the lack of understanding of the actual microbial processes responsible for N_2O production, and of the mechanisms involved and the influencing operational parameters. Initially, most researchers were convinced that denitrification was the main responsible, as N_2O is an intermediate of the denitrification pathway (Zumft, 1997). However, recent research has clearly demonstrated that in most cases nitrification is the dominant N_2O producing process. Finally, a third research gap was the lack of mitigation strategies that lower the overall N_2O emission from wastewater treatment.

This work has contributed to each of these aspects, that is, quantification (Chapter 2), understanding (Chapters 2 and 3) and mitigation (Chapters 4, 5 and 6). In this general discussion, the assessment of N_2O emission from BNR and the impact from a global versus local perspective were considered. Furthermore, the dominant N_2O producing process, nitritation by AOB, was investigated in terms of the different N_2O producing pathways involved. Finally, an overview of mitigation strategies through process optimisation was provided and discussed, and the practical applicability of novel mitigation technologies was elaborated.

1 Assessment and impact of N_2O emission from WWTPs

1.1 Assessment of N_2O emission

Nitrous oxide emission from WWTPs treating domestic wastewater is generally estimated by applying IPCC guidelines (IPCC, 2006). These guidelines implicate that no nitrogen is removed during wastewater treatment if no dedicated BNR technology is present. In this case, N_2O is assumed to be emitted from the receiving water bodies at 0.5% of the nitrogen content of the effluent. In case of advanced WWTPs with controlled nitrification/denitrification steps, a much lower factor of 3.2 g N person^{-1} year^{-1} is applied. The latter represents about 0.035% of the nitrogen load of a WWTP, and is based on solely one grab sampling study by Czepiel et al. (1995). On top, the studied WWTP was not even designed for nitrogen removal. The IPCC emission factor can be a large over- or underestimation given that accurate on-line measurements from full-scale domestic WWTPs report N_2O emission factors ranging from 0.001% up to 1-3% of the nitrogen load (Table 1.2).

Clearly, the IPCC methodology has to be reconsidered. At this stage, long-term (± 1 year) on-line or grab sampling measurements at full-scale are considered to deliver reliable data regarding the N_2O emission factor of a WWTP (Daelman et al., 2013a). However, due to the large variation in reported emission factors so far, applying an emission factor based on these data would be inaccurate as well. An interesting alternative that has gained a lot of interest in recent years is the development of a model that can estimate the N_2O emission (Corominas et al., 2012, Ni et al., 2011, Ni et al., 2013a). However, there is quite some debate whether these models will ever be sensitive enough to estimate N_2O emissions adequately as they suffer from parameter uncertainty.

Another important aspect to address regarding IPCC methodology is that this only implies the treatment of domestic wastewater. The methodology does not include N_2O emissions from industrial sources, unless these are co-discharged with domestic wastewater in the sewer system. Nevertheless, these industrial effluents are often highly loaded nitrogen streams, and have been reported to be significant sources of N_2O when treated in a BNR system (Table 1.2 and Chapter 2). On top, also certain types of manure (e.g. pig manure) and landfill leachates are often treated in BNR systems, and are currently omitted in the account for N_2O emission.

1.2 Impact: global versus local scale

In 2004, the total anthropogenic N_2O emission, when expressed in CO_2 equivalents, was estimated to account for 7.9% of the global anthropogenic GHG emission (IPCC, 2007). About 3% of these anthropogenic N_2O emissions are considered to be derived from domestic wastewater treatment (USEPA, 2006), and as a result ranked as the sixth largest contributor. Furthermore, these emissions are expected to increase by approximately 13% between 2005 and 2020. The overall contribution from BNR will likely be significantly higher when including emissions from the treatment of industrial effluents, manure and landfill leachates. However, the overall contribution from wastewater treatment can be considered as rather low compared to e.g. the agricultural sector, which represents about 80% of the anthropogenic N_2O emission (IPCC, 2007). Nevertheless, BNR derived N_2O emission has a quantitatively significant contribution at a global scale and should therefore be investigated, accounted for and mitigated if possible.

The impact of BNR derived N_2O emissions becomes much more apparent at a more local level. For example, direct N_2O emissions from wastewater treatment were estimated to contribute up to 10% of the GHG footprint of the water cycle in the Netherlands (Frijns, 2012). Another study in the Netherlands investigated the GHG emission (electricity, natural gas, CH_4 and N_2O) of three full-scale municipal WWTPs (STOWA, 2010). The contribution of N_2O to the GHG balance was low for two of the three plants (2-3%). However, the N_2O emission from the third plant contributed 21 to 83%, depending on the monitoring period. A model study (de Haas and Hartley, 2004) showed that an emission factor of 1% would already increase the carbon footprint of a domestic WWTP by approximately 30%. Also, for an industrial treatment plant it was recently demonstrated that the N_2O emission contributed 83% to the operational CO_2 footprint of the complete installation (Chapter 2).

The abovementioned studies clearly demonstrate that N_2O emission from BNR systems need to be integrated into a general impact assessment methodology in order to understand the actual environmental impact at both global and more local levels, and for reasons of benchmarking. It was recently demonstrated how GHG emissions could be included during benchmarking of wastewater treatment plants control strategies (Flores-Alsina et al., 2014, Guo et al., 2012). Furthermore, a standardized common carbon footprint assessment methodology defining clearly the scope and system boundaries is necessary (Frijns, 2012).

Unfortunately, common emission factors for GHG and indirect emissions such as chemicals are still lacking, hampering accurate carbon footprint assessments.

Minimizing one type of environmental impact might however simultaneously increase other environmental impacts. Therefore, there has been an emerging interest for life cycle impact assessment (LCIA), a more holistic tool, where the carbon footprint is only one of various impact categories that integrate the impact of all N_2O emissions during the whole life cycle of wastewater treatment. The advantage of LCIA is that the interlinked effects of N_2O emission abatement strategies can be quantified, and its impact compared relative to other environmental impacts. As such, an LCIA simulation study by de Haas (2008) for a number of WWTPs in Australia showed that GHG emissions represent only a small impact (<1%) of the normalised total environmental impacts from WWTP operations. In contrast, eutrophication and human health or ecotoxicity impacts related to the disposal of biosolids were dominant. However, the impact of N_2O might strongly increase in case the ozone depleting potential (ODP) of this compound is taken into account. As such, Lane and Lant (2012) demonstrated that in case their proposed ODP value for N_2O would be included in the LCA of a WWTP, the contribution of WWTPs to global ODP would rank 4th, and the impact would be in the range of other important impacts such as global warming and ecotoxicity.
Despite the interesting potential of LCA for decision-making purposes, several risks are associated concerning the interpretation of LCIA-results in general. Firstly, the scope and boundary conditions of the environmental or carbon footprint assessment is highly important and can significantly change the outcome of the assessment (Frijns, 2012). Secondly, the accuracy and sensitivity of input and background data need to be analysed, especially since local differences (e.g. emission rates) might highly influence the outcome as well. Given the high variability of reported N_2O emissions and its heavy weight in impact calculations, it is important to take real emission values and variability into account (Foley et al., 2010a) since the default values in commercially available life cycle inventories for wastewater treatment plants might be inadequate (Pasqualino et al., 2009). Thirdly, the harmonization of methods used for classification and characterization is still incomplete since many different methods are ISO 14040-14044 compatible (JRC European Commission, 2010). Finally, normalization and weighting is only optional according to the ISO standards, so conclusions drawn from these results are case specific and at least partly subjective.

2 Nitritation: the dominant N_2O producing process

The literature review (Chapter 1) and the research presented in this work (Chapter 2) clearly demonstrated that nitritation by ammonia-oxidising bacteria (AOB) is the dominant N_2O producing process during biological nitrogen removal. Current research in this area is devoted to unravel the N_2O production mechanisms of AOB, as well as elucidating the dominant N_2O producing pathway at different operational conditions typical for BNR systems.

At present, four approaches are being investigated in order to be able to understand and distinguish between the different N_2O producing pathways, that is, modelling, tracing isotopic signatures and genomic/proteomic studies.

Firstly, limited effort has been made to develop models describing N_2O dynamics. Recently, a few models have been developed that capture most N_2O production and consumption mechanisms in nitrogen removal processes (Mampaey et al., 2013, Ni et al., 2013b), and can be considered as a first step towards a powerful tool enabling to understand the effect of operational parameters on N_2O production dynamics. These models however describe the N_2O production and consumption pathways separately. Thus, a unified model implementing all the pathways, especially N_2O production through nitrifier denitrification and NH_2OH oxidation, is mandatory in order to come to a full understanding of the processes.

Secondly, isotopomer analyses of N_2O can significantly increase the resolution with which sources and sinks of N_2O can be identified, but also the relative contribution of the involved microbial communities and their pathways (Santoro et al., 2011, Sutka et al., 2006, Yoshida and Toyoda, 2000). As such, Wunderlin et al. (2013) recently investigated the sources and sinks of N_2O from an activated sludge microbiome by analysing the nitrogen isotope composition and intramolecular distribution of ^{15}N on the central (α) and terminal (β) positions of the asymmetric N_2O molecules. As a result, the authors found that most N_2O was produced through nitrifier denitrification, with a minor contribution from NH_2OH oxidation. However, it was assumed that denitrification and especially N_2O reduction was absent since the experiments were performed at a high DO (2-3 mg O_2 L^{-1}). However, at a lower DO concentration these pathways come into play and their related ^{15}N site preference value is close to the one reported for nitrifier denitrification. Thus, source partitioning between the different pathways becomes extremely difficult under such conditions.

Thirdly, as demonstrated by Chandran et al. (2011) for AOB, genomic and proteomic studies are extremely useful as they allow to reconstruct the metabolic pathways. Furthermore, integration of these reconstructed pathways with field and lab studies suggested that WWTPs

achieving low effluent aqueous nitrogen concentrations also minimize gaseous nitrogen emissions.

The fourth approach to investigate N_2O production dynamics by AOB is to perform experiments with dosage of NH_2OH, an important metabolic intermediate (Kim et al., 2010, Wunderlin et al., 2012). In Chapter 3, we demonstrated that investigating the continuous loading of NH_2OH in the absence or presence of NH_4^+ significantly contributes to the current understanding of AOB dependent N_2O production. Especially the presence of NH_4^+ was shown to be mandatory for active NO_2^- production and N_2O production through nitrifier denitrification. Furthermore, electron overloading by co-supply of NH_2OH in presence of NH_4^+ rendered interesting observations regarding the encountered electron jam in the oxidation pathway, and the ability of O_2 to at least partly alleviate this issue.

Collectively, the research regarding this specific aspect has thus far often led to non-conclusive and contradictory results. The latter can be explained by the fact that most studies integrate only one of the abovementioned approaches. However, in order to come to a full understanding with conclusive results, comprehensive studies integrating all above described techniques will be necessary.

3 N_2O mitigation through process optimisation

To date, only a few lab-scale mitigation studies have been demonstrated, and their effectiveness remains yet to be demonstrated at full-scale. However, an important output of the ongoing N_2O research is a clear overview of guidelines aiming at minimal N_2O emissions during BNR treatment. Therefore, possible mitigation measures are summarized in Table 7.1 and the reasoning behind these measures is elaborated below. The key focus of N_2O mitigation is prevention of its emission, which can be accomplished by minimizing N_2O production and, if it is formed, maximizing *in-situ* N_2O consumption.

3.1 Minimize aerobic N_2O production

Currently, nitritation by AOB is considered as the most important N_2O producing process. Low DO levels (< 1 mg O_2 L^{-1}), high NO_2^- concentrations (> 1 mg N L^{-1}) and shifts in NH_4^+ concentrations were identified as the main causes for N_2O production (Kampschreur et al., 2009b). Furthermore, it has frequently been reported that any sudden process perturbation causing rapid shifts in reactor pH, DO and NH_4^+ or NO_2^- concentration results in an immediate increase of N_2O production (Kampschreur et al., 2008a, Park et al., 2000, Sinha and Annachhatre, 2007). More in-depth studies showed that N_2O production by AOB increased in response to recovery from transient anoxic periods because of an imbalance in the two-step nitritation metabolism and concurrent accumulation of NH_2OH (Rassamee et al., 2011, Yu et al., 2010). Furthermore, a shift in the AOB metabolism from a low specific to a high specific activity (Yu et al., 2010) and simultaneous high NO_2^- and low DO levels have been identified as important N_2O precursors (Rassamee et al., 2011). Finally, also a pH around 8 during nitritation showed a maximum in the AOB ammonia oxidation rate and a concomitant maximum N_2O production rate (Law et al., 2011).

Thus, minimizing N_2O production from AOB nitritation should aim at operating this microbial process as stable as possible, thus avoiding any sudden strong fluctuations in substrate levels, DO and pH. Moreover, NO_2^- accumulation and overloading should be avoided at any time. However, it should be noted that this is a very challenging task in full-scale installations. Nevertheless, a successful mitigation strategy was demonstrated by (Yang et al., 2009), who obtained a 50% N_2O emission decrease from a lab-scale SBR by maintaining lower and less fluctuating NO_2^- and NH_4^+ levels through step-feeding. As a result, full-scale N/DN systems that are designed to operate under more stable process conditions, such as oxidation ditches (more uniform DO), are expected to be less susceptible

to N_2O production compared to plants that are inherently subject to more fluctuations, such as modified Ludzack – Ettinger plants or SBRs. Furthermore, it is postulated that the full-scale PN/A systems are prone to higher N_2O emission factors compared to N/DN systems due to the higher specific loading rates and higher substrate levels. Moreover, 1-stage PN/A systems are preferred over 2-stage systems as the latter are subject to very high NO_2^- concentrations.

Besides optimizing the physicochemical parameters, it is recommended for the aerobic stage to focus on the optimisation of AOB nitritation and avoiding transient uncoupling with NOB, AnAOB or HDN communities in order to overcome unwanted accumulation of the N_2O production precursor NO_2^-. The key role of NO_2^- but also of NH_2OH suggests a strong need for more intensive monitoring of these compounds. Although NO can also be seen as a universal N_2O precursor, the tracking of *in-situ* concentrations of dissolved NO on full-scale installations is even harder compared to NO_2^- and NH_2OH. Nevertheless, on a more fundamental level, NO microsensors can be very useful to clarify NO and N_2O dynamics (Schreiber et al., 2009). Next to tracking important precursors, also follow-up of AOB functionality by measuring AMO and HAO enzyme expression and activity can be of added value in order to develop effective control strategies maintaining N_2O production at a minimum. Furthermore, the AOB community has been shown to be very dynamic, often changing 20% per week, and regularly amounts to only 5-10% of the HDN community (Ofiteru et al., 2010). Hence, process control to preserve the functionality of this community above a minimum threshold level can be crucial. An increase in sludge retention time (SRT) would increase the biomass concentrations and decrease the nitrifier-specific loading rates. If this is not feasible, one could explore bio-augmenting the nitrification stage. As a result, the microbial diversity for specific functions such as nitritation and nitratation can increase (Wittebolle et al., 2009) and could prevent accumulation of important N_2O production precursors NH_2OH and NO_2^-.

3.2 Minimize aerobic N_2O emissions

It is important to note that N_2O production is not equal to emission, a physical mechanism governed by passive diffusion, mixing and atmospheric wind advection in non-aerated compartments, and additionally by stripping in actively aerated compartments. Obviously, most N_2O is emitted in aerated zones since the mass transfer coefficient of N_2O under these conditions can be an order of magnitude higher (Foley et al., 2010b). Indeed, higher aeration rates are suspected to lead to higher N_2O emission rates (Kampschreur et al., 2009a,

Kampschreur et al., 2008b). Minimizing the actual emissions of N_2O, a purely physical process, by influencing the parameters governing mass transfer of N_2O from liquid to gas phase is an interesting though poorly investigated approach. Especially the type of aeration system can have a very strong influence on the mass transfer characteristics. However, a trade-off will exist between minimal N_2O emission and sufficient oxygen transfer. This definitely requires further experimental investigation.

As such, Ye et al. (2013) recently demonstrated by a model approach that the volumetric N_2O mass transfer coefficient in the vicinity of surface aerators is significantly higher compared to the transfer coefficient related to bubble aerators. Therefore, floating chamber hoods for N_2O measurements are not applicable in case of surface aerators, as they will significantly underestimate the overall N_2O emission. However, stripping by surface aerators is very local whereas bubble aerators create a much more uniform stripping pattern over the entire reactor zone. As a result, elucidating the most suitable type of aeration regarding minimal overall N_2O emission is quite hard, and warrants further investigation. Also passive aeration technologies (e.g. rotating biological contactor; no emission data available) or bubbleless aeration systems (e.g. membrane aerated bioreactor (Pellicer-Nacher et al., 2010)) can lead to lower N_2O emissions but this awaits confirmation at full-scale. Nevertheless, it is important to note that lowering the actual emissions of N_2O by lowering the transfer to the gas phase can only be fulfilled if the HDN community efficiently consumes the dissolved N_2O.

3.3 Maximize anoxic N_2O consumption

During denitrification, high NO_2^- concentrations and a low COD/N ratio are the main parameters leading to N_2O production (Kampschreur et al., 2009b). The COD content entering the denitrification stage can be increased by lowering COD removal in preceding treatment stages (primary settler, aerobic stage/phase) or by external carbon addition. Furthermore, during transient oxygen exposure, the type of exogenous COD has been shown to have an indirect effect, rendering a higher N_2O production from an ethanol-fed denitrifying microbial community compared to a methanol-fed community (Lu and Chandran, 2010), so not only costs but also side emissions should be considered when choosing an external carbon source. Moreover, N_2O reduction is catalysed by NOS and is the final and most O_2 sensitive denitrification step (Zumft, 1997). Therefore, over-aeration in the preceding aerobic stage/phase must be prevented.

Enhancing copper availability, the metal cofactor of the N_2O reductase has been suggested to higher the N_2O consumption potential of the HDN community (Richardson et al., 2009).

Indeed, Zhu et al. (2013) obtained a 55-73% decrease of N_2O emission at lab-scale in a synthetic wastewater matrix by addition of Cu^{2+} (10-100 μg L^{-1}), and observed increased activities of nitrite and N_2O reductases. The latter was confirmed with municipal wastewater, where Cu^{2+} concentrations are generally lower than 10 μg L^{-1}. Also bio-augmentation with aerobic denitrifying species like *Pseudomonas stutzeri* is considered an interesting option to improve aerobic denitrification of NO_2^- and N_2O (Miyahara et al., 2010), and will be elucidated more into detail in section 4.1.2.

Table 7.1 Overview of N_2O mitigation strategies.

Objective	Approach	Outcome
Minimize aerobic N_2O production	Ensure stable substrate levels by gradual feeding regime, sufficient mixing and buffer volume capacity	Low NH_4^+ fluctuations
	Ensure sufficiently high DO (N/DN) or adapted aeration regime (1-stage PN/A)	Prevent NH_2OH and NO_2^- accumulation
	Ensure low free ammonia and low free nitrous acid (N/DN)	Prevent NO_2^- accumulation by NOB stimulation (N/DN)
	In case of high NO_2^-, ensure sufficiently high DO (N/DN)	Prevent NO_2^- accumulation
	Ensure constant DO (no repeated changes from anoxic to oxic), low NH_4^+, sufficiently high SRT, neutral pH, bio-augment with AOB	Prevent high sludge-specific activity and changes from low to high specific activity
	Bio-augment with AOB	Lower nitritation functionality dynamics
Minimize aerobic N_2O emission	In case of active aeration: lower aeration rate and choose optimal type of aeration system	Lower volumetric mass transfer coefficient (k_La)
	In case of passive aeration: limit turbulence	Lower volumetric mass transfer coefficient (k_La)
	In case of bubble-less aeration: preferable in terms of N_2O emissions, e.g. membrane aerated bioreactor (MABR)	Lower volumetric mass transfer coefficient (k_La)

Maximize anoxic N_2O consumption	Lower aerobic COD breakdown and COD-removing pre-settling (sewage), or provide external COD	Sufficiently high COD/N for complete denitrification
	In case of external COD dosage: choose carbon source carefully (e.g. N_2O emissions ethanol > methanol)	Prevent incomplete denitrification
	Ensure efficient aeration in preceding stage (no over-aeration) and provide sufficient anoxic HRT	No DO, stimulate complete denitrification
	Bio-augment with N_2O-consuming HDN *Pseudomonas stutzeri*	Increase N_2O reduction potential
	Ensure sufficient copper availability	Ensure N_2O reductase synthesis and activity

COD: chemical oxygen demand; DO: dissolved oxygen; HRT: hydraulic retention time; N/DN: nitrification/denitrification; PN/A: partial nitritation/anammox; SRT: sludge retention time; AOB: ammonia-oxidizing bacteria; NOB: nitrite-oxidizing bacteria; HDN: heterotrophic denitrifying bacteria.

4 Innovative N_2O mitigation technology: potential for practical application

In the previous section, the possibilities to mitigate N_2O through process optimization of BNR systems were discussed based on knowledge gained from literature research (Chapter 1), and research Chapters 2 and 3. Below, two curative mitigation approaches (Chapter 4 and 5) and one preventive mitigation technology (Chapter 6) will be discussed in terms of their relevance and potential for practical applicability.

4.1 Curative treatment technology

Preventive mitigation should always take the lead on curative measures. However, in certain situations end-of-pipe treatment of N_2O might be an interesting option to consider. For instance, in order to avoid odorous emissions to the environment and nearby located communities, WWTPs are sometimes fully covered. The latter is the case for a number of WWTPs in the Netherlands (e.g. Kralingsveer). As discussed in Chapter 4, until now only physico-chemical N_2O removal technologies have been established, especially for the chemical industry such as production plants of adipic and nitric acid (Centi et al., 2000, Kapteijn et al., 1996), and no biological treatment technology has been developed so far. In the light of N_2O emission from BNR, the potential of biological N_2O removal technologies was not yet investigated due to a number of reasons: (i) the O_2 sensitivity of the biological N_2O reduction step (Zumft, 1997), (ii) the relative minor contribution to the anthropogenic N_2O emission budget compared to other sources such as agriculture (Mosier et al., 1999), and (iii) the absence of an economic or legislative incentive. However, a recently suggested GHG crediting system rewarding minimal N emission to the environment (Wang et al., 2011) could change the mindset given the very strong GHG potential of N_2O.

4.1.1 BES

In Chapter 4, a BES with an N_2O reducing biocathode was developed and operated successfully for a period of 3 months. This was the first proof of concept demonstrating biological N_2O removal with N_2O as the sole electron acceptor present. Admittedly, these results were obtained by operating a 120 mL reactor in the absence of O_2, and in presence of relatively high concentrations of N_2O (5% v/v). Therefore, in order to bring this concept to a next level, the system has to be operated at realistic scale and operational conditions with O_2

and N_2O off-gas concentrations of 1-21% and 1-200 ppmv, respectively. Indeed, upscaling these systems is known to be one of the major bottlenecks related to this technology. Nevertheless, the potential for a number of interesting applications is discussed below.

4.1.1.1 N_2O off-gas treatment

Treating the N_2O containing off-gas of a covered BNR plant in a BES will be a challenging task. To date, conventional options for biological gas treatment are biofilters, biotrickling filters and bioscrubbers. However, in this context the filter will be the cathode compartment. Given the fairly low solubility of N_2O (1.64 dimensionless gas/liquid, (Sander, 1999)), a biofilter approach would be the preferred technology (Rabaey et al., 2012). When taking for example the off-gas of the nitritation reactor described in Chapter 2 (1000 m^3 h^{-1}, 60 ppmv N_2O, 24.5 L mol^{-1} at 25°C), the resulting loading rate would be 1.6 kg N d^{-1}. Hence, assuming a default empty bed contact time (EBCT) of 60s, a 17 m^3 cathode compartment would be required, which consequently results in a reasonable volumetric loading rate of 0.1 kg N m^{-3} d^{-1}. An alternative approach that would render a smaller cathode compartment is to apply the concept of a bioscrubber. Similarly to the well-established Thiopaq® technology (www.paques.nl) for H_2S removal, N_2O can be absorbed in a gas washer and subsequently dosed to the bioscrubber unit, in this case the cathode compartment. However, as N_2O has a 4 times lower solubility compared to H_2S (Sander, 1999), the required gas washer volume will be significantly higher.

One of the main bottlenecks remains the inhibitory effect of O_2 on the N_2O reduction process. The O_2 partial pressure in the off-gas can be estimated at 15-18% v/v as part of the O_2 is transferred to the aeration basins. The latter could be detrimental to the N_2O consuming microbial community. Nevertheless, it was demonstrated in Chapter 5 that N_2O consumption still occurred at air saturated condition. The latter was likely the result of anoxic zones within the microbial flocs.

Whilst this concept looks feasible from a technological perspective, the economics of this concept is less promising. Compared to a conventional biofilter or bioscrubber, the capital investment will be significantly higher due to the need for a membrane, anode electrode, and a conductive cathode that also has to function as packing material, such as graphite granules (Chapter 4). Alternatively, the recently development of gas diffusion electrodes might also hold potential for electrochemical gas treatment (Alvarez-Gallego et al., 2012), although they are very expensive.

4.1.1.2 Treatment of dissolved N_2O

Another potential approach is the electrochemical treatment of dissolved N_2O as the biocathode can be easily submerged in the liquid phase. Denitrification zones suffering a too low COD/N ratio are known to produce high amounts of N_2O (Chapter 1). Usually, external organic carbon source such as ethanol is dosed to promote complete denitrification (Metcalf and Eddy, 2003). The latter can be omitted due to the autotrophic nature of the biocathode, which only needs electrons and CO_2 to perform the reduction reaction. By this, the produced N_2O can be consumed prior to discharge or recycle to aerobic zones, where the N_2O can be emitted to the environment.

4.1.1.3 N_2O biosensor

An interesting future of BESs is that they can be applied as biosensors as it has been demonstrated that the concentration of a component, e.g. BOD, can be correlated to the current production by the cell (Kim et al., 2003). Although such a correlation still has to be demonstrated for N_2O, it shows potential to at least detect this compound. The latter could fit in a proposal of Butler et al. (2009) who demonstrated that detection of N_2O could be an interesting tool for early warning of nitrification failure. In their experiments, a peak in N_2O production preceded failure of the nitrification reaction. Indeed, it will be a challenge to tune the selectivity of the biocathode towards N_2O, as the biocathode will also respond to the presence of O_2. However, this could be resolved by scavenging the oxygen in e.g. an alkaline ascorbate solution (Andersen et al., 2001).

4.1.2 *Pseudomonas stutzeri* enrichment

The results obtained with the enriched mixed culture in Chapter 5 look interesting for application in biological N_2O abatement technologies. The identification of *Pseudomonas stutzeri* as the dominant N_2O reducing species in both anoxic and aerobic activated sludge enrichments was not a surprise as multiple studies have reported on *Pseudomonas stutzeri* as specialists for aerobic denitrification (Lalucat et al., 2006, Su et al., 2001, Takaya et al., 2003). As such, these species have been proposed recently as a kind of bio-augmentation strategy for BNR to lower the N_2O emission (Ikeda-Ohtsubo et al., 2013, Miyahara et al., 2010). Alternatively, this enrichment could also be of interest as inoculum for biofilters. As mentioned before, covered WWTPs usually send the off-gas through a biofilter to avoid odorous emissions to the environment. Conventional biological treatment technologies are mostly dedicated to NO removal instead of N_2O, and reported N_2O removal efficiencies are

usually quite low (0.7-60%) (Lopez et al., 2013). This rather poor performance is most likely due to the fact that these biofilters were not specifically designed for N_2O removal, and the specific microbial community specialised in aerobic N_2O removal was probably lacking. Therefore, inoculation with enrichment is an interesting option to explore.

In Chapter 5, the possibility of an alternative sink for N_2O, that is, assimilatory N_2O reduction was also investigated. Although no evidence was found for a direct assimilatory pathway, it was demonstrated that in the absence of mineral nitrogen, the *Pseudomonas stutzeri* isolate used N_2O indirectly as a nitrogen source. In this way, the need for a mineral nitrogen source could be omitted and part of the nitrogen in the off-gas can be incorporated into biomass instead of being wasted to N_2.

4.2 Preventive treatment technology: direct nitrogen recovery

To date, nitrogen recovery can be done through ammonia stripping, but often requires extensive addition of chemicals (Lei et al., 2007, Siegrist, 1996). In Chapter 6, a novel approach for electrochemical nitrogen recovery was investigated, which does not require any addition of chemicals and allows to recover other interesting nutrients as well. Along this line, electrodialysis is also an interesting option currently under investigation (Ippersiel et al., 2012). This concept fits perfectly in the upcoming trend where wastewater is considered as a valuable resource providing nutrients, energy and reuasable water (Verstraete et al., 2009, Verstraete and Vlaeminck, 2011). As discussed in Chapter 1, nitrogen recovery from waste streams could potentially deliver almost half of the nitrogen demand currently delivered by the Haber-Bosch process. It has to be acknowledged that the energy required for electrochemical nitrogen recovery will most probably be higher compared to the Haber-Bosch process. However, less nitrogen will end up in water systems. Furthermore, in the context of this work, the more nitrogen that can be recovered directly, the less nitrogen that has to be wasted to N_2 in BNR systems. As a result, this approach can also mitigate a significant amount of N_2O.

Next to nitrogen recovery and N_2O mitigation, this concept also could prevent ammonia toxicity events during anaerobic digestion. It was shown that the ammonium level in the digestate can be lowered from 2.1 to 0.8 – 1.2 g N L^{-1}, thereby allowing a lower ammonia level in the digester and thus a higher loading rate can potentially be maintained.

In Chapter 6 the ammonia was absorbed in an acid solution. However, ideally the produced biogas in the digester can be used as absorbent of the ammonia and hence produce ammonium carbonate ($(NH_4)_2CO_3$). Similarly, the recovered potassium can be converted to K_2CO_3. As a

result, the CO_2 content of the biogas can be lowered with about 5%. Furthermore, the cathodically produced H_2 can be joined with the biogas to increase the caloric value. Scaling at the cathode due to salt precipitation can cause problems at longer term, but could possibly be resolved by intermittent polarity reversal (Pikaar et al., 2011) or other membrane cleaning techniques such as acid treatment.

Clearly, this concept is not only promising from a technological perspective, it also seems attractive from an economical perspective given that useful and valuable products are generated. Therefore, the economics of this concept is briefly investigated for a 1000 m^3 digester (Table 7.2 and Fig. 7.1). Assuming that the biogas production rate can be increased with 5-15% if the nitrogen level in the digester is decreased from 3.0 to 2.5 g N L^{-1}, the technology becomes economically beneficial at current densities of 20 A m^{-2}, thereby also taking into account the value of the produced $(NH_4)_2CO_3$, K_2CO_3 and H_2 (increases caloric value of the produced biogas). These products deliver 82-94% of the benefits next to the extra produced biogas due to the high market value of K_2CO_3. Assuming 40 m^2 membrane electrode assembly per m^3 reactor and 3.4 V cell voltage at 30 A m^{-2} operational, this results in a required reactor volume of 5.6 m^3, operating at 1.2 kA m^{-3} or 4.1 kW m^{-3} reactor. Clearly, the produced side-products, especially potassium, need to be validated to turn this concept into an economical viable technology.

Table 7.2 Values used to calculate the economics of a 1000 m^3 digester coupled to an electrochemical system to recover N/K, lower ammonia toxicity, improve the biogas production rate and generate side-products.

Digester	
Volume	1000 m^3
Loading rate	5 kg COD m^{-3} d^{-1}
Residence time	15 d
Concentration NH_4^+	3 g N L^{-1}
Recycle ratio (to N removal unit)	0.25
N to remove to bring N level digester from 3.0 to 2.5 g N L^{-1}	42 kg N d^{-1}
[1]Biogas production rate	1500 m^3 d^{-1}
CH_4 content	60%
[2]Electricity production	3650 kWh d^{-1}
Electrochemical system	
[3]Current efficiency N and K	40 %
Current density	10 – 50 A m^{-2}
[4]Cell voltage (depending on current density)	2.6 to 4.2 V
[5]Energy cost	0.07 € kWh^{-1}
[6]Capital cost	3000 € m^{-2} installed reactor
Depreciation time	15 years (5% interest)
Stripper + absorber	
[7]Energy requirement	5 kWh kg^{-1} N
[8]Capital cost	100000 €
Depreciation time	15 years (5% interest)
Benefits and products	
[9]Extra biogas	5 – 15 %
	27-82 € d^{-1}
[10]$(NH_4)_2CO_3$	143 kg d^{-1}
	11 € d^{-1}
[11]K_2CO_3	411 kg d^{-1}
	329 € d^{-1}
[12]H_2	5.9 kg d^{-1}
	40 € d^{-1}

[1] Assumed 300 L biogas per kg of COD.

[2] Assumed CH_4 content of 60%, 10.1 kWh Nm^{-3}, 40% electrical efficiency and 0.15 € kWh^{-1} (including certificates for green energy).

[3] Based on results obtained in this study (optimisation feasible at higher current densities).

[4] Increasing cell voltage required with increasing current density. Cell voltage required for higher current densities than 30 A m^{-2} extrapolated from results obtained in this study.

[5] http://205.254.135.7/emeu/international/elecprii.html (09/04/2013).

[6] Personal communication, to date this is a realistic assumption.

[7] Taken from Siegrist (1996). The authors calculated a required aeration energy of 7.3 kWh kg^{-1} N, however, as in this study a clean stripping solution without solids was obtained the required energy was assumed to be 5 kWh kg^{-1} N.

[8] Personal communication.

[9] Personal communication with OWS (Belgium), 5% extra biogas is certainly feasible. Angelidaki and Ahring (1993) observed a 25% lower methane yield at 4 g N L^{-1}

[10] Assumed 50% current efficiency and 80 € per tonne $(NH_4)_2CO_3$. Price taken from www.alibaba.com (09/04/2013).

[11] Assumed 50% current efficiency and 800 € per tonne K_2CO_3. Price taken from www.alibaba.com (09/04/2013).

[12] Joined with the biogas to increase the caloric value. Assumed 10.0 kWh Nm^{-3}, 40% electrical efficiency and 0.15 € kWh^{-1} (including certificates for green energy).

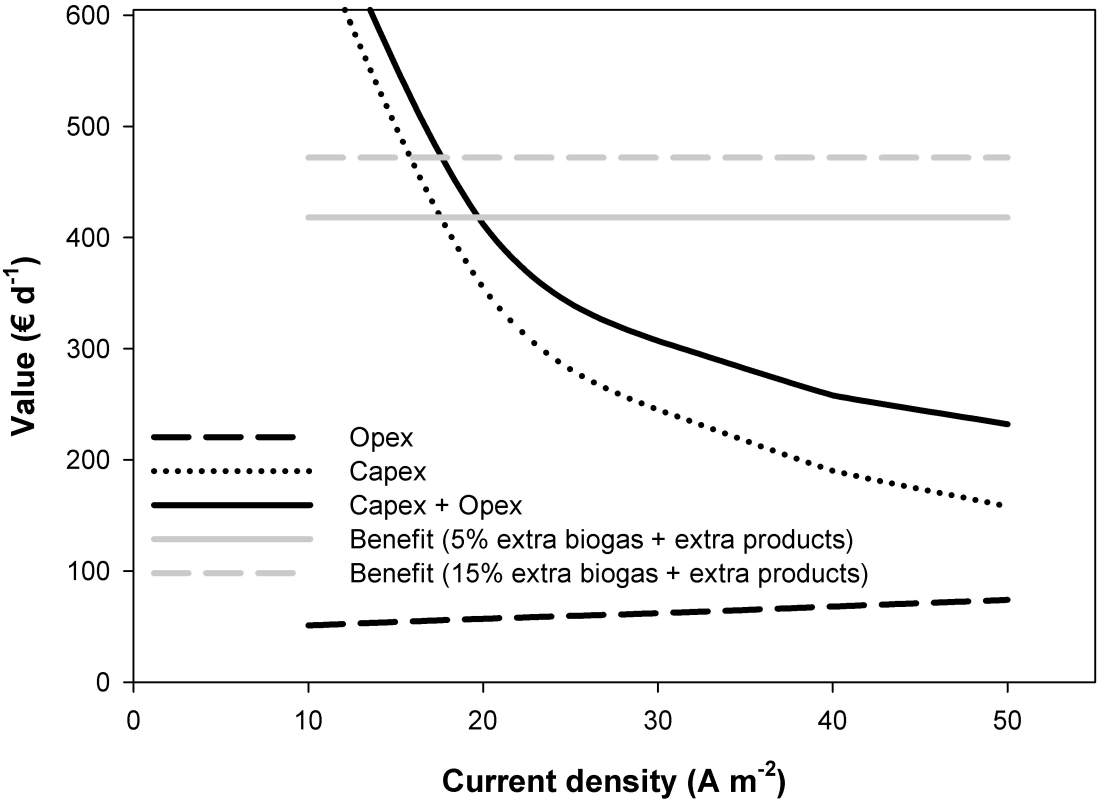

Figure 7.1 Economical balance for an electrochemical system with stripping and absorption unit for N/K recovery, and which lowers the nitrogen level in a 1000 m^3 digester from 3.0 to 2.5 g N L^{-1}.

5 Conclusions

Biological nitrogen removal is a well-established technology within the wastewater treatment industry. At the beginning of this research, the amount of N_2O emitted from these systems was largely unknown, as well as the responsible processes and mechanisms involved in N_2O production. Furthermore, mitigation strategies were lacking.
This work has significantly contributed to this research area, thereby covering 3 main sub-topics: quantification, understanding and mitigation of N_2O emissions.

- Quantification (Chapter 2): N_2O emission from BNR has a minor contribution to the overall anthropogenic N_2O emission (±3%). However, they can represent a very significant fraction (60-90%) on the CO_2 footprint of a WWTP when the N_2O emission represents up to several percentages of the nitrogen load.

- Understanding (Chapter 2 and 3): nitritation by AOB is generally the main responsible process for N_2O production during BNR. The presence of NH_4^+ has a strong physiological role on AOB activity and the N_2O production pathways.

- Mitigation:
    - Preventive through process optimisation (result from Chapter 1, 2 and 3): an overview of preventive mitigation strategies was provided.

    - Curative (Chapter 4 and 5): two curative mitigation technologies were proposed based on autotrophic (Chapter 4) and heterotrophic (Chapter 5) N_2O consumption.

    - Preventive through nutrient recovery (Chapter 6): an electrochemical nutrient recovery process was demonstrated, thereby allowing direct recovery of nitrogen as a product instead of destruction in a BNR plant with concomitant N_2O emission.

Abstract

Biological nitrogen removal (BNR) is a well-established technology enabling to convert reactive nitrogen compounds present in wastewater into harmless dinitrogen gas (N_2), which is subsequently released to atmosphere. However, next to N_2, also nitrous oxide (N_2O) has been reported to be one of the gaseous products during BNR (Kampschreur et al., 2009b). This compound, generally known as laughing gas, has a 300 times stronger global warming potential (GWP) compared to CO_2 (Solomon et al., 2007), and is depicted as one of the most important threats to the ozone layer in the 21st century (Ravishankara et al., 2009).

At the beginning of this research, the magnitude of the N_2O emission from BNR systems was largely unknown as accurate measurements were lacking. Furthermore, there was quite some debate regarding the responsible microbial processes, as well as the N_2O production pathways involved. Finally, no mitigation strategies have been developed yet, aiming at proper nitrogen removal with minimal N_2O emission. Therefore, the goal of this research was to contribute to each of these three abovementioned research areas, that is, quantification (**Chapter 2**), understanding (**Chapter 2 and 3**) and mitigation (**Chapter 4, 5 and 6**) of N_2O emission from BNR systems.

In a first research chapter (**Chapter 2**), the N_2O emission from a full-scale industrial BNR plant was accurately quantified with an on-line measurement device. The 4-stage treatment plant comprised a batch-fed partial nitritation reactor followed by an anammox stage, and subsequently a denitrification and nitrification compartment for effluent polishing. Overall, a nitrogen removal efficiency of 95% was obtained, however, about 5.1 to 6.6% of the nitrogen load was released as N_2O to the atmosphere. The latter was entirely derived from the partial nitritation reactor as no N_2O emission could be observed from the anammox, denitrification and nitrification compartments. Remarkably, although the N_2O emission from BNR only represents a minor fraction of the anthropogenic N_2O emissions (±3%), the N_2O emission represented up to 80% of the operational CO_2 footprint of this BNR plant. This strongly marks the possible impact of N_2O to the greenhouse gas (GHG) balance of a wastewater treatment plant (WWTP).

Initially, denitrification was considered as the main responsible process for N_2O production during BNR since this compound is the final intermediate in the denitrification pathway (Zumft, 1997). However, recent studies as well as Chapter 2 demonstrated that in most cases nitrification is the main contributor to the overall N_2O production. More specifically nitritation, the first step of nitrification, conducted by ammonia-oxidising bacteria (AOB) can result in the production of N_2O through distinct pathways. Therefore, in the next chapter (**Chapter 3**), the effect of hydroxylamine (NH_2OH), an important metabolic intermediate during nitritation, on the nitrite and N_2O production was investigated on an enriched ammonia-oxidising culture. Interestingly, when NH_2OH was provided as the sole electron donor, the NO_2^- and N_2O production rate were 10-fold and 7-fold lower, respectively. In contrast to the case where NH_4^+ was present, increase in dissolved oxygen (DO) or NO_2^- concentration could not promote the NO_2^- production rate and the N_2O production rate did not decrease with increasing DO or NO_2^- concentration. We can conclude from the results that the presence or absence of NH_4^+ plays has an important effect on the AOB activity, as well as on the N_2O production pathways.

In the following research chapters, both curative (**Chapter 4 and 5**) and preventive (**Chapter 6**) N_2O mitigation strategies were investigated.

A first curative strategy was the development of a bioelectrochemical system (BES) with an N_2O reducing biocathode (**Chapter 4**). Until now, only physicochemical technologies have been applied to mitigate point sources of N_2O, and no biological treatment technology has been developed so far. In this chapter high N_2O removal rates were obtained ranging between 0.76 and 1.83 kg N m^{-3} Net Cathodic Compartment (NCC) d^{-1}. These rates were proportional to the current production, resulting in cathodic coulombic efficiencies near 100%. Furthermore, the results demonstrated the active involvement of microorganisms as the catalyst for the reduction of N_2O to N_2, and the optimal cathode potential ranged from -200 to 0 mV vs Standard Hydrogen Electrode (SHE) in order to obtain high conversion rates.

Secondly, the N_2O reduction process was investigated from a more fundamental perspective (**Chapter 5**). Interestingly, the microbial consumption of N_2O, as part of the denitrification process, is the only known biochemical pathway able to convert N_2O into harmless N_2. Furthermore, the enzyme catalysing N_2O reduction is very oxygen sensitive, thereby often impeding this pathway to act as an N_2O sink. Moreover, there is on-going debate regarding an alternative pathway, namely reduction of N_2O to NH_4^+, or assimilatory N_2O consumption. In this chapter, enrichment of activated sludge rendered a mixed culture capable of anoxic and

oxic N_2O consumption, and a collection of *Pseudomonas stutzeri* strains as dominant N_2O consumers in both anaerobic and aerobic enrichments was identified. A detailed isotope tracing experiment with a *Pseudomonas stutzeri* isolate showed that consumption of N_2O via assimilatory reduction to NH_4^+ was absent. Conversely, respiratory N_2O reduction was directly coupled to N_2 fixation. The obtained enrichment shows potential for curative N_2O mitigation through application as inoculum for biofilters or as a bio-augmentative strategy to improve aerobic denitrification.

Thirdly, a preventive mitigation strategy was considered through direct recovery of nitrogen by the use of an electrochemical system (ES) (**Chapter 6**). First of all, direct nitrogen recovery from waste streams could potentially provide a significant amount (±50%, calculated from Sutton et al. (2013)) of the reactive nitrogen demand currently provided by the Haber-Bosch process. Several agricultural and industrial liquid waste streams contain nitrogen concentrations up to several grams per liter. For these streams, nitrogen recovery could be a more sustainable and economical attractive technology compared to biological nitrogen removal (BNR), as the latter becomes very energy-intensive. More important in the context of this research, this approach significantly decreases the amount of nitrogen that has to pass through a BNR system and concomitantly results in a much lower overall N_2O emission from BNR processes. In the ES developed in this chapter, NH_4^+ fluxes to the recovery (cathode) compartment of 100-150 g N m^{-2} d^{-1} (2.5-3.8 kg N m^{-3} d^{-1}) at a current density of 10 A m^{-2} could be obtained with municipal solid waste (MSW) digestate; resulting in an electrical energy input of 13 kWh kg^{-1} N removed and 41% current efficiency. A more efficient NH_4^+ transfer could be established by maintaining a high concentration of other cations such as sodium in the cathode compartment. Also potassium fluxes of up to 241 g K^+ m^{-2} d^{-1} (6 kg K^+ m^{-3} d^{-1}) could be obtained at 23% current efficiency. The latter is a very important and valuable nutrient in the agricultural sector. As the cathode can be operated at high pH without the need for chemical addition, stripping and absorption of dissolved ammonia in the recovery compartment could reach 100% efficiency.

In the general discussion (**Chapter 7**), the actual impact of N_2O was considered from a global to a more local scale. Whilst N_2O from BNR has a minor impact at a global scale, it certainly can have a significant impact at the level of WWTP, and not only the GHG potential but also the ozone depletion potential of N_2O plays a major role in an impact assessment study.

Furthermore, findings from the literature review (**Chapter 1**), Chapter 2 and 3 were translated into a general overview of possible N_2O mitigation strategies through process optimisation of

BNR systems. These strategies imply minimisation of aerobic N_2O production and emission, and maximasation of anoxic N_2O consumption.

Finally, the practical applicability of novel proposed mitigation technologies was considered. Curative mitigation technologies show potential from a technological perspective, but lack an economical driving force. In contrast, preventive mitigation through direct electrochemical nitrogen recovery seems attractive from both a technological, sustainability and economical point of view.

Samenvatting

Biologische stikstofverwijdering (BNR) is een volwaardige technologie die toelaat om reactieve stikstofcomponenten in afvalwater om te zetten in onschadelijk stikstofgas (N_2), dat vervolgens wordt vrijgelaten in de atmosfeer. Recent onderzoek heeft echter aangetoond dat naast stikstofgas ook distikstofoxide (N_2O) als een bijproduct kan gevormd worden gedurende dit proces (Kampschreur et al., 2009b). Deze component, beter gekend als lachgas, is een 300 maal sterker broeikasgas in vergelijking met koolstofdioxide (CO_2), en is ook gerapporteerd als één van de grootste bedreigingen van de ozonlaag in de 21ste eeuw.

Bij de start van dit onderzoek was de omvang van deze N_2O emissie grotendeels ongekend en accurate metingen waren niet voorhanden. Daarnaast was er heel wat debat omtrent de microbiële processen die verantwoordelijk zijn voor N_2O productie, alsook de mogelijke N_2O productieroutes. Als laatste waren er ook geen mitigatiestrategieën voorhanden met als doel stikstof te verwijderen met minimale N_2O emissie. Het doel van dit onderzoek was dan ook een bijdrage te leveren aan elk van de drie hierboven vernoemde onderzoeksthema's: kwantificatie (**Hoofdstuk 2**), begrijpen (**Hoofdstukken 2 en 3**) en mitigatie (**Hoofdstukken 4, 5 en 6**) van de N_2O emissie afkomstig van biologische stikstofverwijderingsprocessen.

In een eerste onderzoekshoofdstuk (**Hoofdstuk 2**) werd de N_2O emissie van een volle schaal BNR installatie accuraat bepaald met behulp van een online meettoestel. De installatie omvatte een behandelingstrein van 4 compartimenten, respectievelijk bestaande uit een fed-batch partiële nitritatie- en anammoxreactor gevolgd door een denitrificatie- en nitrificatiereactor voor nazuivering. Globaal werd een stikstofverwijderingsefficiëntie van 95% behaald. Echter, 5.1 tot 6.6% van de stikstofvracht werd omgezet en uitgestoten onder de vorm van N_2O. Daarenboven was deze emissie volledig afkomstig van de partiële nitritatiereactor. Een belangrijke conclusie in dit onderzoek was dat, niettegenstaande de N_2O emissie afkomstig uit BNR systemen geschat wordt op slechts 3% van de antropogene N_2O uitstoot, deze emissie tot 80% van de operationele CO_2-voetafdruk van deze installatie inneemt. Dit gegeven toont heel sterk aan dat de N_2O

emissie van een BNR installatie een hele sterke invloed kan hebben op de broeikasgasbalans van een afvalwaterzuiveringsinstallatie (WZI).

Initieel werd aangenomen dat denitrificatie het belangrijkste N_2O-productieproces was gezien deze component het laatste intermediair is van de denitrificatiepathway (Zumft, 1997). Echter, recente studies alsook Hoofdstuk 2 tonen aan dat in de meeste gevallen nitrificatie de grootste bijdrage levert aan de totale N_2O productie tijdens BNR. Meer bepaald nitritratie door ammonium oxiderende bacteriën (AOB), de eerste stap van het nitrificatieproces, kan leiden tot de productie van N_2O via verschillende productiemechanismen. Daarvoor werd in een volgend hoofdstuk (**Hoofdstuk 3**) het effect nageaan van hydroxylamine (NH_2OH), een belangrijk metabolisch intermediair van de nitritatiereactie, op de nitriet- en N_2O-productie van een aangerijkte AOB cultuur. Een interessante observatie was dat respectievelijk de nitriet en N_2O-productiesnelheid 10 en 7 maal lager was in het geval dat NH_2OH als enige elektrondonor gedoseerd werd ten opzichte van een gelijkaardig experiment waarbij ook ammonium aanwezig was. Verder, ook in tegenstelling tot een experiment waar wel ammonium aanwezig was, werd er ook geen stijging van de NO_2- en N_2O-productiesnelheid waargenomen wanneer de opgeloste zuurstofconcentratie (DO) of NO_2^- concentratie werd verhoogd. Uit de resultaten kan worden afgeleid dat de aan- of afwezigheid van ammonium een sterke rol speelt in de activiteit van AOB, alsook een sterkte invloed heeft op de N_2O productiemechanismen.

In de volgende onderzoekshoofdstukken werden zowel curatieve (**Hoofdstuk 4 en 5**) als preventieve (**Hoofdstuk 6**) N_2O-mitigatiestrategieën onderzocht.
Een eerste curatieve strategie bestond uit de ontwikkeling van een bio-elektrochemisch systeem met een N_2O-reducerende biokathode (**Hoofdstuk 4**). Tot dusver werden enkel fysico-chemische technologieën toegepast om puntbronnen van N_2O te mitigeren, en er is momenteel nog geen biologisch alternatief ontwikkeld. In dit hoofdstuk werden hoge N_2O verwijderingssnelheden bekomen tussen 0.76 en 1.83 kg N m^{-3} Netto Kathodecompartiment (NCC) d^{-1}. Deze snelheden waren proportioneel met de stroomproductie van de cel en resulteerde in kathodische coulombische efficiëntie's van bijna 100%. De resultaten toonden bovendien aan dat micro-organismen de katalysator waren voor de reductie van N_2O naar N_2, en de optimale kathodepotentiaal was -200 tot 0 mV vs Standard Waterstofelektrode (SHE) om hoge omzettingssnelheden te bereiken.

Vervolgens werd het N_2O reductieproces onderzocht vanuit een iets fundamenteler perspectief (**Hoofdstuk 5**). De microbiële consumptie van N_2O, als deel van het denitrificatieproces, is het de enige gekende biochemische route die in staat is om N_2O om te zetten in het onschadelijke N_2. Het is ook zo dat het enzyme dat de eigenlijke reductiereactie katalyseert zeer zuurstofgevoelig is, waardoor N_2O dikwijls kan optreden als het eindproduct is van het denitrificatieproces. Verder heerst er ook een discussie omtrent het bestaan van een alternatieve pathway, namelijk reductie van N_2O naar NH_4^+, oftewel assimilatorische N_2O consumptie. In dit hoofdstuk werd na aanrijking van actief slib een mengcultuur bekomen die in staat is om zowel onder anoxische als oxische omstandigheden N_2O te reduceren. Uit deze aanrijking werd een collectie van *Pseudomonas stutzeri* isolaten geïdentificeerd als de dominante species in zowel de anaerobe als aerobe aanrijkingen. Een gedetailleerd isotoop-tracing experiment met een *Pseudomonas stutzeri* isolaat toonde aan dat consumptie van N_2O via assimilatorische reductie tot NH_4^+ afwezig was. Echter, respiratorische N_2O-reductie was gekoppeld aan N_2-fixatie. De bekomen microbiële aanrijking heeft aangetoond potentieel te hebben als toepassing voor curatieve mitigatie van N_2O, bij voorbeeld als inoculum voor biofilters, of als een bio-augmentatiestrategie voor verbeterde aerobe denitrificatie tijdens BNR.

Als laatste werd een preventieve mitigatiestrategie onderzocht door directe herwinning van stikstof met behulp van een elektrochemische cel (ES) (**Hoofdstuk 6**). Het is belangrijk te onderstrepen dat de directe herwinning van stikstof uit afvalwaterstromen reeds een significante hoeveelheid (±50%, berekend uit Sutton et al. (2013)) van de huidige reactieve stikstofvraag kan aanleveren die momenteel onderhouden wordt door het energie-intensieve Haber-Bosch proces. Verschillende vloeibare afvalwaterstromen uit de agricultuur en industrie kunnen stikstofconcentraties bevatten die oplopen tot een aantal gram stikstof per liter. Voor deze stromen zou directe herwinning van stikstof een duurzamer en economischer alternatief kunnen bieden ten opzichte van de huidige biologische stikstofverwijdering tot N_2 gezien dit laatste proces dan wel heel energie-intensief wordt. Belangrijker in de context van dit onderzoek is dat deze recuperatiestrategie de hoeveelheid stikstof die dient behandeld te worden in een BNR systeem gevoelig verlaagt en als gevolg hiervan zal ook de totale N_2O productie van BNR systemen verlagen. In het ES dat ontwikkeld werd in dit hoofdstuk werden NH_4^+ fluxen naar het recuperatie (kathode) compartiment bereikt van 100-150 g N m^{-2} d^{-1} (2.5-3.8 kg N m^{-3} d^{-1}) aan een stroomdensiteit van 10 A m^{-2} en met digestaat komende uit een vaste stof vergister. Dit resulteerde in een een energie-input van 13 kWh kg^{-1} N verwijderd en

41% stroomefficiëntie. Verder kon een efficiëntere NH_4^+ transfer bekomen worden door een hoge concentratie aan te houden van andere kationen zoals natrium in het kathodecompartiment. Daarenboven werden ook kalium fluxen bekomen tot 241 g K^+ m^{-2} d^{-1} (6 kg K^+ m^{-3} d^{-1}) aan een stroomefficiëntie van 23%. Dit laatste is een zeer belangrijk en vooral kostbaar nutriënt in de agricultuur. Een werd een strip- en absorptie-efficiëntie van ammonium in het recuperatie (kathode) compartiment van 100% efficiëntie bekomen gezien het recuperatie (kathode) compartiment kan bedreven worden aan een hoge pH zonder de nood aan extra toevoeging van chemicaliën.

In de algemene discussie (**Hoofdstuk 7**) werd de eigenlijke impact van N_2O beschouwd van een globale naar meer lokale schaal. Niettegenstaande de totale N_2O emissie uit BNR systemen slechts een relatief kleine impact heeft op globale schaal kan het wel een significante impact hebben op de schaal van de afvalwaterzuiveringsinstallatie zelf. Hierbij is niet enkel het broeikasgaspotentieel van N_2O een belangrijke factor, maar ook het ozon-depletie potentieel heeft een belangrijke invloed in een impact assessment studie.
Verder werden resultaten uit een literatuuronderzoek (Hoofdstuk 1), alsook Hoofdstukken 2 en 3 vertaald in een algemeen overzicht van mogelijke N_2O mitigatiestrategieën met nadruk op procesoptimalisatie van de huidige BNR systemen. Deze strategieën omvatten minimalisatie van aerobe N_2O productie en emissie, en maximalisatie van anoxische N_2O consumptie.
Finaal werd de praktische toepasbaarheid van de voorgesteld mitigatiestrategieën beschouwd (Hoofdstukken 4, 5 en 6). Curatieve mitigatietechnologieën tonen potentieel vanuit een technologisch perspectief, maar deze ontbreken echter een economische drijfkracht. Echter, preventieve mitigatie door directe elektrochemische stikstofherwinning lijkt attractief vanuit zowel een technologische, duurzame als economische invalshoek.

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1998-2004 Sint-Bernarduscollege, Oudenaarde (Belgium):
Science-Mathematics

International peer reviewed publications (A1)

Clauwaert, P., **Desloover, J.**, Shea, C., Nerenberg, R., Boon, N. & Verstraete, W. (2009). Enhanced nitrogen removal in bio-electrochemical systems. *Biotechnology Letters*, 31, 1537-1543.

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Conferences and symposia

Oral presentations

Desloover, J., De Clippeleir, H., Boeckx, P., Du Laing, G., Colsen, J., Verstraete, W. & Vlaeminck, S.E. Performance and greenhouse gas emissions from an industrial nitrogen removal plant: Trade-off between water and air quality? 16th Symposium on Applied Biological Sciences. Ghent, 20 December 2010.

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Desloover, J., Abate, A., Sotres Fernandez, A., Verstraete, W., Boon, N. & Rabaey, K. Electrochemical resource recovery from digestate coupled to prevention of ammonia toxicity during anaerobic digestion. 1st European International Society for Microbial Electrochemistry and Technology (EU-ISMET) meeting: From extracellular electron transfer to innovative process development. Ghent, 27-28 September 2012.

Poster presentations

Desloover, J., Puig, S., Verstraete, W. & Boon, N. Nitrous oxide removal in bioelectrochemical systems. 16th Symposium on Applied Biological Sciences. Ghent, 20 December 2010.

Colsen, J., **Desloover, J.**, De Clippeleir, H., Boeckx, P., Du Laing, G., Verstraete, W. & Vlaeminck, S.E. A retrofitted activated sludge plant with sequential nitritation and anammox obtains dischargeable effluent. 12th IWA specialist conference on anaerobic digestion. Guadalajara, October 31th-November 4th 2010.

Desloover, J., De Clippeleir, H., Boeckx, P., Du Laing, G., Colsen, J., Verstraete, W. & Vlaeminck, S.E. Floc-based sequential partial nitritation and anammox at full-scale with contrasting N_2O emissions. 1st international symposium on microbial resource management in biotechnology: concepts and applications. Ghent, June 30th - July 1st 2011.

Awards

- Winner of the B-IWA Research Award 2013 for the best publication in an IWA journal: Floc-based sequential partial nitritation and anammox at full scale with contrasting N_2O emissions. *Water Research*, 45, 2811-2821.

Professional activities during PhD research

- Teaching assistant of the computer exercises of the course "Biotechnological processes"

- Tutor of the master thesis research of 5 thesis students

- Member of the local organizing committee for the 1st European International Society for Microbial Electrochemistry and Technology meeting: From extracellular electron transfer to innovative process development. 27-28 September 2012, Ghent, Belgium.

- Researcher on projects commissioned by wastewater treatment companies:

Colsen BV (Hulst, The Netherlands): reference: ir. Joop Colsen.

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