Monitoring and modeling of nitrogen conversions in membrane-aerated biofilm reactors: Effects of intermittent aeration

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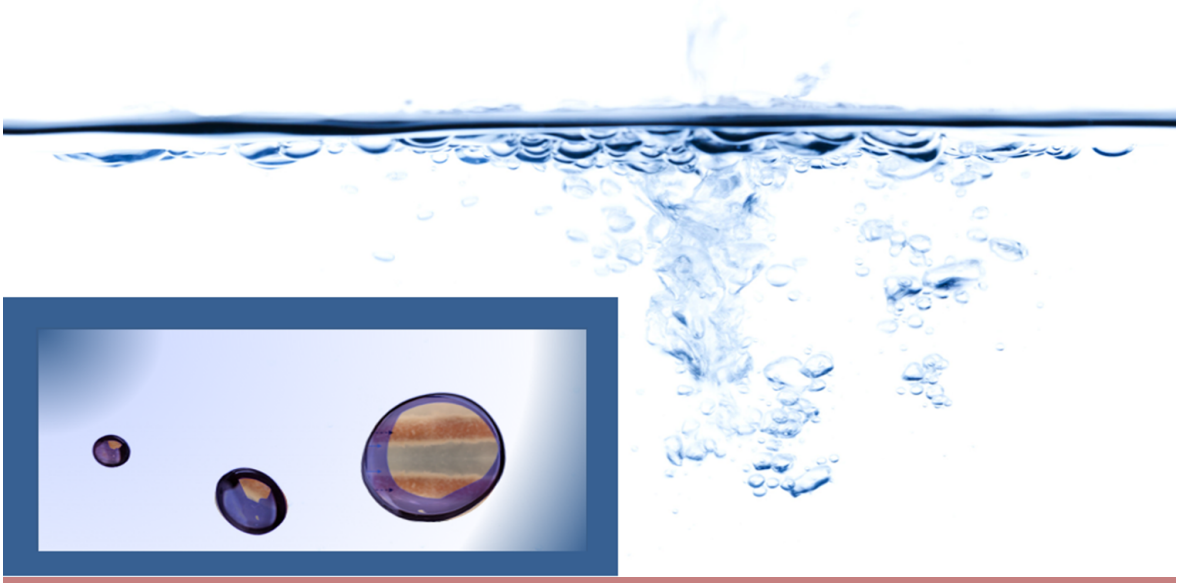

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PhD thesis
January 2018

DTU Environment
Department of Environmental Engineering

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Effects of intermittent aeration

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Preface

This thesis is based on the work carried out at the Technical University of Denmark, Department of Environmental Engineering from October 2014 to December 2017. It is organised in two parts: the first part puts into context the findings of the PhD in an introductive review, and the second part consists of the papers listed below. The research was co-funded by the China Scholarship Council and Technical University of Denmark, and was performed under the main supervision of Professor Barth F. Smets (DTU Environment).

I **Ma, Y.**, Domingo-Félez, C., Plósz, B.G., Smets, B.F., 2017. Intermittent aeration suppresses nitrite-oxidizing bacteria in membrane-aerated biofilms: A model-based explanation. Environ. Sci. Technol. 51, 6146–6155.

II **Ma, Y.**, Piscedda, A., Smets, B.F., Nitrogen conversion in membrane-aerated biofilm reactors affected by intermittent aeration. *Submitted to Water Research*.

III **Ma, Y.**, Valverde-Pérez, B., Picioreanu, C., Smets, B.F., Intermittent aeration can reduce denitrification-related N_2O production in membrane aerated nitrifying biofilms: Results from a modeling study. *Manuscript in preparation*.

In addition, the following co-authored publication, not included in this thesis, was also concluded during this PhD study:

- Blum, J., Su, Q., **Ma, Y.**, Valverde-Pérez, B., Domingo-Félez, C., Jensen, M., Smets, B.F. The pH dependency of N-converting enzymatic processes, pathways and microbes: effect on net-N_2O production. *Manuscript in preparation*.

This PhD study also contributed to international conferences with the following proceeding and conference papers:

- **Ma, Y.**, Piscedda, A., Smets, B.F. Membrane-aerated nitrifying biofilms: Continuous versus intermittent aeration. 10th International Conference on Biofilm Reactors, 2017, Dublin, Ireland.
  Oral Presentation
- **Ma, Y.,** Domingo-Felez, C., Smets, B.F. Nitrous oxide production in membrane-aerated nitrifying biofilms: Experimentation and modelling. Frontiers International Conference on Wastewater Treatment, 2017, Palermo, Italy.
  Flash Oral Presentation
- Valverde-Pérez, B., **Ma, Y.,** Morset, M., Domingo-Félez C., Mauricio-Iglesias M., Smets B.F. Model-based optimization of biofilm based systems performing autotrophic nitrogen removal using the comprehensive NDHA model. 6th IWA/WEF Water Resource Recovery Modelling Seminar, 2018, Lac Beauport, Quebec, Canada.
  Accepted for Poster Presentation
- **Ma, Y.**, Domingo-Félez, C., Plósz, B.G., Smets, B.F. Suppression of nitrite-oxidizing bacteria in intermittently aerated biofilm reactors: a model-based explanation. IWA Specialist Conference of Microbial Ecology in Water Engineering & Biofilms 2016, Copenhagen, Denmark.
  Poster Presentation

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I would like to thank the China Scholarship Council for their financial support and the great organization, and Technical University of Denmark for co-financing my PhD studies. In addition, I acknowledge the support for my research activities by the Danish Free Research Council (Project N2OMANgrant no.DFF-1335-00100 to B.F. Smets). I wish to thank the former and present members of the Metlab research group. It has been a pleasure to work and share this period of time with all of you. I am also grateful for the assistance of the laboratory technicians, especially Flemming Møller, the administration staff and the IT group from this department. A big thank to Marlene Mark Jensen for translating the summary into Danish. Special thanks to my students Andrea Piscedda, Jingyu Wang, Hanchen Ma, and Antía de la Campa Veras for their enthusiasm and their hard work.

Thanks to all my colleagues and friends who have made me enjoy the time not only at DTU, but also in Denmark: Arda, Arnaud, Bas, Borja, Dongah, Dorka, Elena, Gordon, Hailin, Jan, Jane, Kai, Marta, Ravi, Pauly, Sara, Uli, Vaibhav, Waqas, and many others. I also need to thank Carlos and Borja for helping me struggle with all the parameters and equations in the many, many models; and Fabio for helping me correct my Chi-English writing in the thesis; and Palomo for all the help and laughs together; and all my Chinese girls Xiangdan, Jiayi, Nannan, Weijing, Qingxian, Liguan- just talking with you in our language, already makes me feel relaxed.

Finally, I would really like to thank my family and always-being friend Qun, who has been there supporting me, and did not succeed in persuading me to give up every time when I started to complain. Thanks for being with me for happiness and everything in the future. 真的爱你们!

本事

记得当时年纪小，

我爱谈天你爱笑。

有一回并肩坐在桃树下，

风在林梢鸟在叫。

我们不知怎样困觉了，

梦里花儿落多少。

- 卢前 1934

Summary

Nitrogen can be removed from sewage by a variety of physicochemical and biological processes. Due to the high removal efficiency and relatively low costs, biological processes have been widely adopted for treating nitrogen-rich wastewaters. Among the biological technologies, biofilm processes show great advantages as compared to suspended growth processes, allowing for biomass accumulation and retention without the need of external solid separation devices. The decoupling of solids retention from hydraulic retention is especially useful for slow-growing microorganisms, such as nitrifying bacteria, e.g. ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), and anaerobic ammonium-oxidizing bacteria (AnAOB), which are involved in ammonium (NH_4^+) removal process.

Stability of engineered biological processes requires an appropriate balance between activities of the main microbial groups involved in the system. However, finding proper operational conditions is especially challenging in biofilms. On the one hand, the existence of strong spatial chemical gradients within biofilms increases the difficulty to prescribe environmental conditions that favor any desired biological process. On the other hand, the presence of multiple simultaneous chemical gradients complicates the performance optimization. Mathematical modeling offers a way to describe and analyze multiple processes that occur simultaneously in time and space in biofilm systems.

This PhD project investigated NH_4^+ removal process in membrane-aerated biofilm reactors (MABRs), focusing on aeration control, especially the application of intermittent aeration. Compared to conventional biofilms which are characterized by co-diffusion, MABRs display counter-diffusion fluxes of substrates: oxygen is supplied through the membrane, whilst NH_4^+ is provided from the bulk liquid phase. The counter substrate supply not only offers flexible aeration control, but also supports the development of a unique microbial community and spatial structure inside the biofilm. In this study, lab-scale MABRs were operated under two types of aeration control: continuous versus intermittent aeration. Long-term reactor performance was monitored. Based on bulk measurements of NH_4^+, nitrite (NO_2^-) and nitrate (NO_3^-), microbial activities of individual functional guilds were evaluated. I found that NOB suppression occurred under intermittent aeration, but not under continuous aeration. Relative aeration duration and aeration intermittency were two effective operational factors in regulating MABR performance under inter-

mittent aeration. Besides daily bulk monitoring, *in situ* microprofiles of dissolved oxygen (DO), pH and nitrous oxide (N_2O) were performed. The significant temporal fluctuations in local biofilm pH (not DO) during aeration control suggested that pH-related effects drive the changing microbial activities under intermittent aeration, as compared to continuous aeration. Total N_2O emissions were dramatically reduced at the onset of intermittent aeration, due to the development of an anoxic N_2O reduction zone by heterotrophic bacteria (HB).

To further investigate the causal link between NOB suppression and aeration regime change, a 1-dimensional (1-D) multispecies nitrifying biofilm model was developed in Aquasim software, incorporating a pH calculation. Kinetic parameters to be estimated were chosen based on a local sensitivity analysis, and were estimated from *in situ* microprofiles. With the calibrated model, I identified that the periodically varying free ammonia inhibition, which was associated with transient pH variations, was the likely key factor causing NOB suppression in intermittently-aerated nitrifying MABRs.

To further investigate the mechanisms of N_2O mitigation under aeration control, the 1-D biofilm model was extended to a partial nitritation-anammox (PNA) biofilm model, including description of all relevant biological N_2O production pathways. Sensitive kinetic parameters were estimated with long-term bulk performance data. With the calibrated model, roles of HB and AnAOB were discussed and evaluated in mitigating N_2O emissions in autotrophic nitrogen removal MABRs. Moreover, I developed a 1-D biofilm model in Matlab software describing the counter-diffusion PNA process, aiming at an improved model calibration/evaluation for the highly variable N_2O emissions.

Overall, a combination of experimental and modeling efforts were implemented to study nitrogen conversions in MABRs. The results showed that intermittent aeration was an efficient strategy to regulate microbial activities in counter-diffusion biofilms, achieving an energy-efficient NH_4^+ removal process with low N_2O emissions.

Dansk sammenfatning

Kvælstof kan fjernes fra spildevand ved hjælp af en række fysisk-kemiske og biologiske processer. Biologisk spildevandsrensning er udbredt til behandling af spildevand med højt kvælstofindhold på grund af høj fjernelseseffektivitet og de forholdsvis lave omkostninger. Indenfor biologisk rensning er der flere fordele ved biofilmsystemer i forhold til et aktivt slam anlæg, hvor mikroorganismer er opslæmmet i vandfasen. Biofilmsystemer muliggør akkumulering og opbevaring af biomasse uden behov for sekundærbundfældningstanke. Sidstnævnte er særlig nyttig for langsomt voksende mikroorganismer, såsom nitrificerende bakterier, f.eks. Ammonium-oxiderende bakterier (AOB), nitrit-oxiderende bakterier (NOB) og anammox bakterier (AnAOB), som alle er involveret i ammonium (NH_4^+) fjernelse.

Stabiliteten af de biologiske processer kræver en passende balance imellem aktiviteten af de vigtigste mikroorganismer i systemet. Det er imidlertid særligt udfordrende at finde passende driftsbetingelser i biofilmsystemer. Kemiske gradienter i biofilmen vanskeliggør bestemmelsen af de ydre forhold, der favoriserer den ønskede biologiske proces. Ligeledes komplicerer de kemiske gradienter driftsreguleringen. Matematiske modelleringsmetoder giver os en mulighed til at beskrive og analysere flere processer, der forekommer samtidigt i tid og rum i biofilmsystemerne.

Denne ph.d.-afhandling omhandler undersøgelser af NH_4^+ fjernelse processen i membran-aerated biofilm reactors (MABRs) (membran bioreaktor med beluftning igennem membranerne) med fokus på regulering intermitterende beluftning. Sammenlignet med konventionelle biofilm med et diffusionssystem i en retning, viser MABR'er med et diffusionssystem med modsat rettede substrat strømninger, dvs. luft leveres via membranmodulerne (deraf navnet MABR), mens NH_4^+ suppleres igennem væskefasen. Dette modstrømsdiffusionssystem giver muligheden for en fleksibel luftningskontrol samt støtter udviklingen af unikke mikrobielle samfund i biofilmen. I dette studie blev MABR'er i laboratoriet udsat for forskellige beluftningskontrol - konstant kontra intermitterende beluftning. Længerevarende reaktor drift blev undersøgt. Den mikrobielle aktivitet af individuelle funktionelle samfund blev evalueret på baggrund af målinger af NH_4^+, nitrit (NO_2^-) og nitrat (NO_3^-). Vi fandt ud af, at der forekom en undertrykkelse af NOB under intermitterende beluftning modsat konstant beluftning. Udover daglige målinger af kvælstofsalte blev der udført profilmålinger af opløst oxygen (DO), pH og lattergas

(N_2O) i selve biofilmen. De betydelige ændringer af pH (ikke DO) ned igennem biofilmen antydede, at pH-relaterede påvirkninger kan have betydning for de ændrede mikrobielle aktiviteter, når reaktorerne blev udsat for intermitterende beluftning sammenlignet med konstant beluftning. Den samplede frigivelse af lattergas (N_2O) blev betydeligt reduceret i starten af intermitterende beluftning på grund af anaerob N_2O reduktion via heterotrofe bakterier (HB).

For yderligere at kunne undersøge årsagsforbindelsen mellem undertrykkelsen af NOB og regulering af beluftningen, udviklede vi en 1-dimensionel (1-D) multispecies nitrificerende biofilmmodel i Aquasim, hvor der er taget højde for pH-beregninger. De kinetiske parametre blev valgt på baggrund af en lokal følsomhedsanalyse og estimater fra in situ profiler. Med den kalibrerede model fandt jeg at den periodisk ændrede fri ammoniakhæmning, der var associeret med transiente variationer i pH som en sandsynlig nøglefaktor for undertrykkelsen af NOB i intermitterende beluftede nitrificerende MABR'er.

For yderligere at undersøge mekanismerne bag den observerede reduktion i N_2O frigivelse under beluftningskontrol, blev 1-D udvidet med en partiel nitrifikation-anammox (PNA) biofilmmodel, der inkluderede alle biologiske lattergas produktionsveje. De følsomme kinetiske parametre blev estimeret via den langsigtede drift. Med den kalibrerede model blev roller af HB og AnAOB fremhævet med hensyn til formindskelse af N_2O-emissioner i de autotrofe nitrogenfjernelses-MABR'er. Desuden udviklede jeg en 1-D biofilm model i Matlab, der beskriver PNA processen opereret med modsatrettede diffusion, med henblik på en forbedret modelkalibrering / evaluering for de høje og variable N_2O frigivelser.

Samlet set blev en kombination af eksperimentel og model-baseret fokus implementeret for at studere kvælstofomdannelserne i MABR'er. Resultaterne viste, at luftningskontrol med intermitterende beluftning er en effektiv strategi til at regulere kvælstofomsættende mikrobielle aktiviteter i modsat rettede diffusionsbiofilm, hvilket giver en energieffektiv NH_4^+ fjernelsesproces med lave N-emissioner.

Table of contents

Abbreviations

AMO	Ammonia monooxygenase
AnAOB	Anaerobic ammonium-oxidizing bacteria
Anammox	Anaerobic ammonium-oxidizing process
AOB	Ammonium-oxidizing bacteria
ASMN	Activated sludge model of nitrogen
BNR	Biological nitrogen removal
CO_2	Carbon dioxide
COD	Chemical oxygen demand
DO	Dissolved oxygen
FA	Free ammonia
FNA	Free nitrous acid
HAO	Hydroxylamine oxidoreductase
HB	Heterotrophic bacteria
HD	Heterotrophic denitrification
HDH	Hydrazine dehydrogenase
HRT	Hydraulic retention time
HZS	Hydrazine synthase
MABR	Membrane-aerated biofilm reactor
N_2	Dinitrogen gas
N_2O	Nitrous acid
NAR	Nitrate reductase
ND	Nitrifier denitrification
NH_2OH	Hydroxylamine
NH_4^+	Ammonium
NIR	Nitrite reductase
NN	Nitrifier nitrification
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOB	Nitrite-oxidizing bacteria
NOR	Nitric oxide reductase
NOS	Nitrous oxide reductase
PE	Parameter estimation
PN	Partial nitritation
PNA	Partial nitritation-anammox
RMSE	Root mean squared error
SA	Sensitivity analysis
SRT	Solid retention time
WWTP	Wastewater treatment plant

1. Introduction and objectives

Most of the nitrogen (N) in municipal and industrial wastewater occurs in the form of ammonium (NH_4^+). NH_4^+ removal from sewage is an important task of modern biological wastewater treatment in consideration of (1) the overgrowth of plants and algae resulting in oxygen depletion of water bodies (eutrophication), (2) the biotoxicity of NH_4^+, especially its unionized species (free ammonia, FA) and oxidized species (nitrite, NO_2^-), and (3) the need for water-reuse applications (Metcalf and Eddy, 2014; Ahn, 2006). Permitted N concentrations in effluents of wastewater treatment plants (WWTPs) are strictly limited for both NH_4^+ and its oxidation products NO_2^- and nitrate (NO_3^-). The total N concentration of typical municipal wastewaters is between 20 ~ 80 mg-N/L (Henze and Comeau, 2008). The effluent of a municipal treatment plant with more than 100,000 population equivalents must contain not more than 10 or 15 mg-N/L (NH_4^+-N < 5 mg-N/L) according to regulations in the European Union and in China, respectively (Yi *et al.*, 2008).

Typically, biological N removal (BNR) from wastewater is performed through nitrification/denitrification process: NH_4^+ is oxidized to NO_3^-, which is subsequently denitrified to denitrogen gas (N_2). However, short-cut NH_4^+ removal via NO_2^- is more energy-efficient than the traditional removal via NO_3^- due to reduced aeration costs and external electron donor requirements (Hellinga *et al.*, 1998; Regmi *et al.*, 2014). This shortcut process requires full nitritation and zero nitratation, which can be achieved by suppressing activity of nitrite-oxidizing bacteria (NOB) and enhancing activity of ammonium-oxidizing bacteria (AOB). Besides energy savings, NOB suppression can also allow to exploit a more resource efficient N removal process- partial nitritation/anammox (Strous *et al.*, 1998; Kuenen, 2008; Ali and Okabe, 2015).

As a by-product in BNR, emissions of nitrous oxide (N_2O) attract increasing attention in the last decades. N_2O is known as an ozone depleting compound and a greenhouse gas with a high warming potential (IPCC, 2001; Ravishankara *et al.*, 2009; Kampschreur *et al.*, 2009). Even though the emitted N_2O is low compared to the influent N load, it can significantly affect the carbon footprint of WWTPs (Gustavsson and Tumlin, 2013). Moreover, N_2O emissions are extremely variable and depend on many operational parameters in the nitrification process (Béline, 2002; Kampschreur *et al.*, 2009; Law *et al.*, 2011).

In WWTPs, NH_4^+ removal can be accomplished in both suspended growth (activated sludge) and attached growth (biofilm) processes. Compared to the suspended growth systems, biofilm systems can retain and accumulate slow-growing nitrifying bacteria, while still being able to operate at short hydraulic retention times (HRT) (Aybar *et al.*, 2014; Isanta *et al.*, 2015; Jo *et al.*, 2016). Recent studies have proposed a novel biofilm technology, employing membrane-aerated biofilm reactors (MABRs), as an alternative to conventional biofilm systems due to the flexible air supply control and the development of unique biofilm community (Syron and Casey, 2008; Martin and Nerenberg, 2012; Nerenberg, 2016). Nevertheless, an effective strategy to suppress NOB in MABRs has not yet been well documented, which is needed to realize the energy-efficient nitritation process. In addition, a comprehensive understanding of N_2O production dynamics in counter-diffusion biofilm systems is lacking.

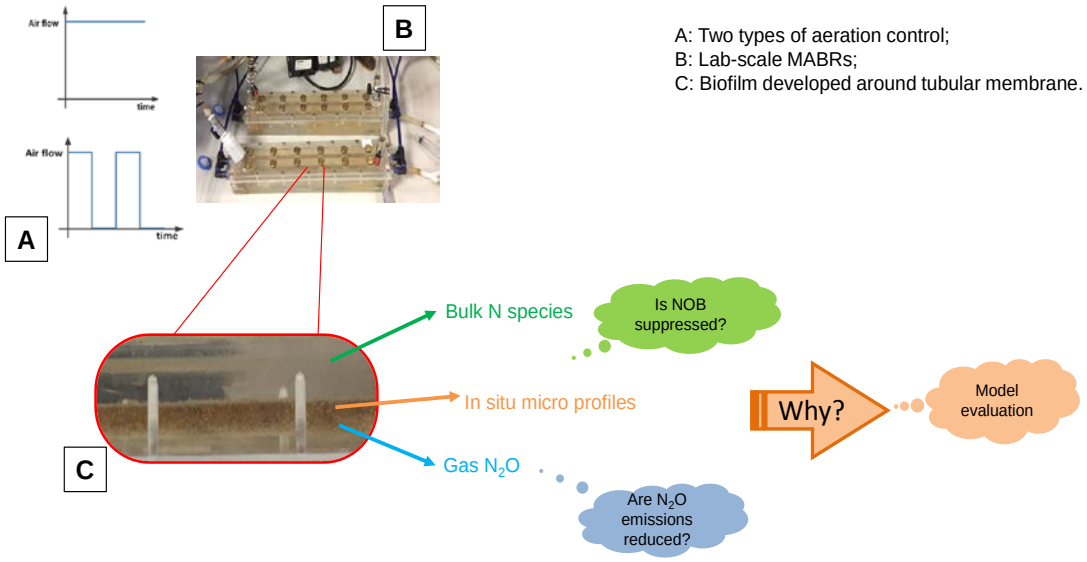


Figure 1.1 Schematic representation of the research conducted in this thesis.

Due to the ability to control air supply without modifying hydrodynamic conditions in the bulk phase, aeration control may provide a strategy to regulate MABR performance. Therefore, the overall aim of this PhD research project was to:

- To test the practicability of aeration control (intermittent aeration) in optimizing MABR performance (**Paper II**);
- To investigate whether NOB can be suppressed via aeration control (**Paper II**) and explore the potential reasons (**Pape I**);

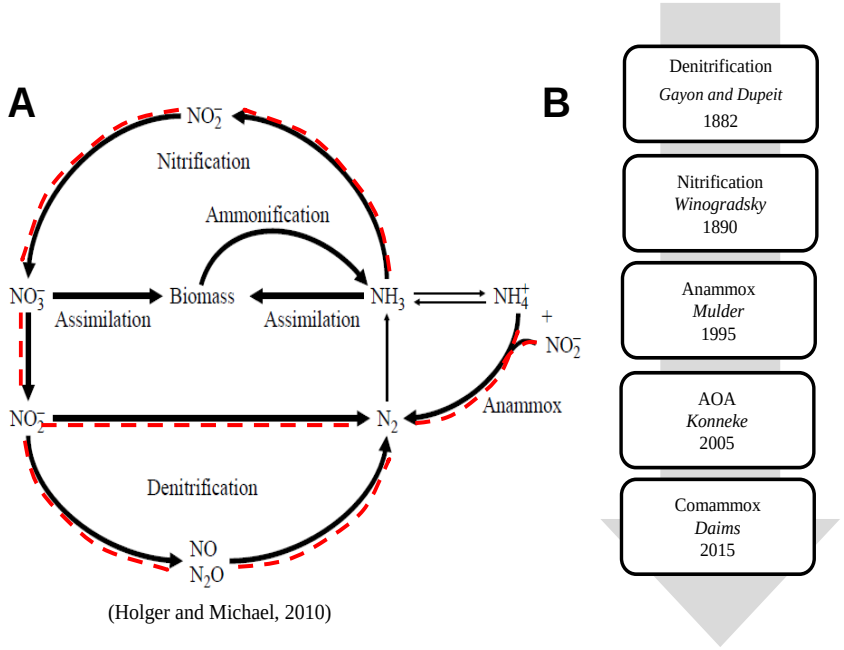
- To investigate whether N_2O emissions can be mitigated by aeration control (**Paper II**) and explore the underlying mechanisms (**Paper III**);
- To develop mathematical models capable of evaluating influencing factors of AOB/NOB competition (**Paper I**), and the contribution of different production pathways to total N_2O emissions (**Paper III**) in counter-diffusion MABRs.

2. Biological ammonium removal

2.1 Microbiology

In 1890, Winogradsky (1890) observed that under aerobic conditions NH_4^+ was converted into NO_3^- in a two-step nitrification process, carried out by ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). The combination of nitrification with denitrification, where oxidized N species produced in the first process are subsequently reduced to nitrogen gas (N_2), has been the conventional approach for N removal from domestic and industrial wastewater (Metcalf and Eddy, 2014). However, this classical N treatment approach is costly, as aeration is needed for the nitrification stage, and organic carbon sources are commonly added to maintain denitrification performance. Two decades ago, microorganisms able to remove NH_4^+ in the absence of oxygen were discovered in a process called anammox (Mulder, 1995; Strous *et al.*, 1998). This process constitutes a 'shortcut' in the biogeochemical N cycle (Figure 2.1A). To date, Anammox-based processes have found full-scale application for N removal from anaerobic dewatering recycle streams, reducing the cost of wastewater treatment plants (WWTPs), but it has not yet been proved reliable for treatment at low or ambient temperature and for diluted NH_4^+ streams. Könneke et al. (2005) further showed that NH_4^+ oxidation is not limited to bacterial organisms by isolating a marine crenarchaeote (ammonium-oxidizing archaea, AOA). Recently, it was reported that a so far known NOB was also able to perform NH_4^+ oxidation, being therefore capable of complete nitrification (comammox) (Daims *et al.*, 2015). Although both AOA and comammox have been detected in WWTPs (Park *et al.*, 2006; Zhang *et al.*, 2011; Daims *et al.*, 2015), their abundance is lower than AOB in these N-rich systems (Wells *et al.*, 2009; Limpiyakorn *et al.*, 2011; Chao *et al.*, 2016). Therefore, we solely focused on AOB as aerobic NH_4^+ oxidizers in the following sections.

Ammonium-oxidizing Bacteria. Aerobic ammonium-oxidizing bacteria (AOB) are chemolithoautotrophs that oxidize free ammonia (NH_3) to NO_2^- in nitritation process. Nitritation is a two-step process catalyzed by two enzymes: the membrane-bound ammonia monooxygenase (AMO) and the periplasmic hydroxylamine oxidoreductase (HAO) (Hooper *et al.*, 1997; Bock and Wagner, 2006):



(Holger and Michael, 2010)

Figure 2.1 (A) Schematic illustration of the biogeochemical nitrogen cycle: Processes that are relevant to nitrogen removal in wastewater treatment plant are marked by bold arrows (Holger and Michael, 2010), and biological processes marked in red dash line are discussed in detail in this study. (B) Historical timeline of discoveries of relevant biological processes.

$$NH_3 + O_2 + 2\,H^+ + 2\,e^- \xrightarrow{Amo} NH_2OH;$$
$$NH_2OH + H_2O \xrightarrow{HAO} HNO_2 + 4\,H^+ + 4\,e^- \qquad (2.1)$$

As the first step is endergonic, the actual energy source for AOB must be the intermediate hydroxylamine (NH_2OH), which is the substrate of the second exergonic step. During the second step, four electrons are released, of which two are redirected to support the first step of NH_3 oxidation and the other two are utilized for carbon dioxide (CO_2) fixation and generation of proton motive forces. The low energy yield of these biochemical reactions (-275 kJ/mol) justifies the low growth rates and yields (0.18 g-COD/g-N, (Hiatt and Grady, 2008)) of AOB. Under low oxygen availability, some AOB are able to obtain energy from the reduction of NO_2^- by performing nitrifier denitrification (ND), which is a process catalyzed by two additional enzymes nitrite reductase (NIR) and nitric oxide reductase (NOR).

$$NO_2^- + 2\,H^+ + e^- \xrightarrow{Nir} NO + H_2O;$$
$$2\,NO + 2\,H^+ + e^- \xrightarrow{Nor} N_2O + H_2O \qquad (2.2)$$

Two electrons from NH_2OH oxidation are used to reduce NO_2^- producing nitric oxide (NO) and nitrous oxide (N_2O) (Schreiber *et al.*, 2012). As a powerful greenhouse gas, N_2O is also produced from the incomplete oxidation of NH_2OH to NO_2^- via NO or its reduced form HNO (Hooper and Terry, 1979).

$$NH_2OH \xrightarrow{Nor} NO + 3\ H^+ + 3\ e^- \quad (2.3)$$

AOB belong to the Proteobacteria phylum, including Betaproteobacteria and Gammaproteobacteria. In wastewater treatment systems, the dominant AOB are the betaproteobacterial members *Nitrosomonas* and *Nitrosospira* (Kowalchuk and Stephen, 2001). From a kinetics perspective, some *Nitrosomonas spp*. display high growth rates in niches with high ammonia availability (r-strategists), while *Nitrosospira spp*. typically have high ammonia affinity (K-strategists) (Metcalf and Eddy, 2014). Thus, *Nitrosospira* are usually outcompeted by *Nitrosomonas* in WWTPs, but they are more frequently found in soils, where NH_4^+ concentrations are lower than in sewage.

Nitrite-oxidizing bacteria. Nitrite-oxidizing bacteria (NOB) are aerobic chemolithoautotrophs that oxidize NO_2^- to NO_3^- in the nitratation process. NO_2^- oxidation is catalyzed by the membrane-bound nitrite oxidoreductase (NXR) (Bock and Wagner, 2006). During this process, two electrons are released and transferred to oxygen via a respiratory chain for generating water.

$$NO_2^- + H_2O \xleftrightarrow{Nxr} NO_3^- + 2\ H^+ + 2\ e^-;$$
$$2\ H^+ + 2\ e^- + 0.5\ O_2 \rightarrow H_2O \quad (2.4)$$

The reaction yields even less energy (-75 kJ/mol) than NH_3 oxidation, which leads to even lower growth rates and cell yields (0.06 g-COD/g-N, (Hiatt and Grady, 2008)) than those of AOB. NOB have been found in several genera distributed among different phylogenetic lineages of bacteria, presenting more heterogeneity than AOB. The best characterized NOB are members of the genus *Nitrobacter* in the class Alphaproteobacteria (Winogradsky, 1890; Daims *et al.*, 2016). Nevertheless, the genus *Nitrospira* is often the most abundant NOB in WWTPs, and constitutes the most diverse group of known NOB (Juretschko *et al.*, 1998; Daims *et al.*, 2001). With respect to growth kinetics, *Nitrospira spp*. have a higher affinity for NO_2^- but a slower growth rate compared to *Nitrobacter spp*. (Kits et al., 2017; Schramm et al., 1999). Hence, *Nitrobacter spp*. (r-strategists) are generally outcompeted by *Nitrospira spp*. under NO_2^--limited conditions, but can grow faster with increasing NO_2^- availability. In relation to oxygen, little is known about the specific affinities of different NOB, thus requiring further investigation.

Anaerobic ammonium-oxidizing bacteria. Anaerobic NH_4^+-oxidizing bacteria (AnAOB) are lithotrophs which obtain energy from oxidizing NH_4^+ to dinitrogen gas (N_2) with NO_2^- as electron acceptor in anammox process. The

existence of AnAOB bacteria was not confirmed until the observation of NH_4^+ loss in an anoxic fluidized bed bioreactor (Mulder, 1995). Based on experimental observations, thermodynamic calculations and genome analysis (van de Graaf *et al.*, 1997; Strous *et al.*, 2006), the anammox reaction mechanism was proposed, as follows.

$$NO_2^- + 2\ H^+ + e^- \xrightarrow{NirS} NO + H_2O;$$

$$NH_4^+ + NO + 2\ H^+ + 3\ e^- \xrightarrow{HZS} N_2H_4 + H_2O;$$

$$N_2H_4 \xrightarrow{HDH} N_2 + 4\ H^+ + 4\ e^- \qquad (2.5)$$

NO_2^- is first reduced to NO by a NO_2^- reductase (NIR). Then, hydrazine synthase (HZS) forms hydrazine (N_2H_4) by reducing NO and oxidizing NH_4^+. Eventually, N_2H_4 is oxidized to N_2 by an HAO-like enzyme, hydrazine dehydrogenase (HDH), and four electrons are released to sustain the first two steps of the pathway (Kartal *et al.*, 2013). Released energy is conserved in a membrane potential for carbon fixation and cell growth. The oxidation of NO_2^- to NO_3^- (catalyzed by the NXR) further supplies extra electrons that are reversely transported to NO_2^- and NO reactions, replenishing the electron-consumption in carbon fixation. This explains why NO_3^- is produced in anammox reaction, besides N_2. AnAOB cells display compartmentalization, i.e. they possess an intracellular compartment called the “anammoxosome”, in which enzymes for catabolism are located (Lindsay *et al.*, 2001). Despite the very energetically favorable pathway above (-357 kJ/mol), AnAOB have extremely low growth rate and a low biomass yield (0.16 g-COD/g-N, (Strous *et al.*, 1998)). Culture-independent molecular methods revealed that AnAOB are members of a deep-branching lineage in the bacterial phylum Planctomycetes (Strous *et al.*, 1999). In total, five genera of AnAOB bacteria have been detected in wastewater treatment systems, sea- and freshwater habitats and sediments. The application of anammox technology in the treatment of NH_4^+-rich wastewaters has a number of advantages over the traditional nitrification-denitrification process in terms of reduced aeration cost, no requirements of addition of external organic carbon source and reduced biosolid production (Lackner *et al.*, 2014; Kuenen, 2008).

Denitrifying (heterotrophic) bacteria. Denitrifying bacteria are normally heterotrophic bacteria (HB) that can use NO_3^- and NO_2^- (as well as the gaseous NO and N_2O) as terminal electron acceptors in their metabolism. Denitrification is a four-step process, during which NO_3^- is reduced sequentially to NO_2^-, NO, N_2O and N_2.

$$NO_3^- \xrightarrow{Nar} NO_2^- \xrightarrow{Nir} NO \xrightarrow{Nor} N_2O \xrightarrow{Nos} N_2 \quad (2.6)$$

Four different enzymes are involved in the reduction steps, namely nitrate reductase (membrane-bound NAR or periplasmic NAP), nitrite reductase (iron-containging or copper-containing NIR), nitric oxide reductase (membrane-bound NOR) and nitric oxide reductase (periplasmic NOS) (Berks *et al.*, 1995; Tavares *et al.*, 2006). Denitrification is widely distributed among prokaryotes, hence presenting a rather high diversity in terms of growth kinetics and physiological features (Daims *et al.*, 2015).

Besides NH_4^+ assimilation in cell synthesis and production via ammonification, HB do not directly contribute to the NH_4^+ removal. However, most known denitrifiers are facultative anaerobes, hence can also respire with O_2 as electron acceptor. The energetics of heterotrophic processes is far more favorable than that of nitrification processes. Therefore, HB is still in the scope of discussion in the current thesis of autotrophic nitrifying MABRs, considering the potential O_2 competition under aerobic conditions. Interestingly, even though it has not been commonly taken into the consideration, aerobic denitrification was observed by Robertson and Kuenen (1984) as NO_3^- and O_2 were consumed simultaneously at DO concentrations up to 90% of air saturation. Additionally, denitrification does not always proceed to complete reduction to N_2. The gaseous intermediates NO and N_2O may accumulate considerably during heteorotrophic denitrification, particularly under low C-to-N conditions (Domingo-Félez *et al.*, 2016; Kampschreur *et al.*, 2009). On the other hand, HB can also be considered as N_2O-sink as they host the specific N_2O-consuming enzyme (NOS).

2.2 Processes

Biological processes for NH_4^+ removal in wastewater treatment can be classified into suspended growth processes and attached growth processes (Metcalf and Eddy, 2014). As to suspended growth treatment, there are three common process configurations:

1) A completely mixed activated sludge system with continuous inflow wastewater stream, where the tank contents are mixed thoroughly and the bulk concentrations are uniform throughout the tank, in which food-to-microorganism ratio (F/M) is low. Despite the simple operability, this condition encourages the growth of filamentous bacteria, which can cause sludge bulking problems.

2) A sequencing batch reactor (SBR) system, where the reactor is operated in a fill-and-draw mode and the subsequent steps of aeration and clarification occur in the same tank. It is cost-effective for small treatment plants, while high skills are required for instruments, monitoring devices and automatic valves.

3) A multi-staged nitrification system, where baffle walls are used to intentionally create a number of completely mixed activated sludge zones operating in series. It provides better treatment than a single completely mixed reactor for the same total reactor volume. However, design and operation of tapered aeration can be complex.

In general, suspended growth processes are simple and their operation is flexible, being suitable for all types of aeration equipment. The most critical parameter for process design and operation is the solid retention time (SRT), as it affects directly the treatment performance, reactor volume, sludge production and oxygen requirements.

In attached growth processes, microorganisms can grow and attach on support packing material, thus developing a biofilm. Contrary to suspended growth processes (where substrate utilization kinetics is related to the dissolved compounds in the bulk liquid), substrates are consumed within a biofilm in attached growth processes. The success of biofilm technologies is related to their ability to decouple two important process parameters, SRT and hydraulic retention time (HRT) (Metcalf and Eddy, 2014). Specifically, these technologies allow achieving high biomass concentrations and long SRTs in relatively small tank volumes. This results in efficient accumulation of slow-growing bacterial groups, such as nitrifiers, and treatment of low-concentration pollutants such as xenobiotic compounds (Ottengraf *et al.*, 1986; Torresi *et al.*, 2017). An important feature of biofilm processes is the fact that the process performance is often limited by substrate mass transfer into the biofilm, which is generally not a problem in suspended growth processes. Nevertheless, diffusion limitation makes it possible to develop redox-stratification within biofilms enriching diverse microbial communities (Lorenzen *et al.*, 1998; Pellicer-Nàcher *et al.*, 2014).

Attached growth processes have typically been classified into five general categories (Table 2.1) (Peters and Alleman, 1982; Metcalf and Eddy, 2014). Moreover, several emerging biofilm processes have been evaluated in lab and pilot testing over recent decades, and they are promising biotechnologies in wastewater treatment, including a use for innovative NH_4^+ removal.

Table 2.1 Classification of attached growth processes in wastewater treatment with respective advantages and limitations, summarized from the study of Metcalf and Eddy (2014).

(1) Non-submerged attached growth process	
Example: trickling filter	
Advantages:	simple and low-energy process, continuous operation mode
Limitations:	relatively high incidence of clogging, low treatment loading, high suspended solids concentration in effluent, low BOD loadings to maintain nitrification process
(2) Partially submerged attached growth process	
Example: rotating biological contactor (RBC)	
Advantages:	no need of pumping or recirculating wastewater
Limitations:	low performance at designed loadings, difficulty in biomass control and excess biomass accumulation
(3) Sequential non-submerged attached growth-activated sludge process	
Example: trickling filter/activated sludge system (TF/AS)	
Advantages:	combined benefits of both processes: energy saving and good effluent quality
Limitations:	uncertainty in oxygen demand in activated sludge process
(4) Submerged attached growth process	
Example: moving bed bioreactor (MBBR)	
Advantages:	small footprint required (one-fifth or one-third of the area needed for activated sludge process), the ability of handle dilute wastewaters and no activated sludge settling concerns
Limitations:	high capital costs (high energy demand to operate at elevated DO levels), proprietary media needed, demand for improved influent wastewater screening, additional hydraulic profile headloss
(5) Activated sludge process with biofilm carriers	
Example: integrated fixed-film activated sludge (IFAS)	
Advantages:	high treatment capacity, high nitrification process stability, low sludge production, no increase in maintenance costs
Limitations:	a higher energy demand to operate at elevated DO levels, proprietary media needed, demand for improved influent wastewater screening, additional hydraulic profile headloss
(6) Emerging biofilm process	
Examples: aerobic granules reactors, biofilm airlift reactors, membrane biofilm reactors	
Advantages:	a high loading capacity with a small footprint, high biomass settling velocities, or a counter-diffusion system with flexibility in aeration control
Limitations:	high maintenance skills and proprietary media needed, difficulty in biomass control, lack of wide plant-scale test

2.3 Membrane-aerated biofilm reactors

2.3.1 Counter-diffusion nitrifying biofilm

As introduced above, biofilm systems are widely applied in biological wastewater treatment due to their long solids retention time and relatively

small footprint (solids production, reactor volume required). Compared to conventional biofilm processes where a co-diffusion of substrates occurs, counter-diffusion biofilm systems are an emerging technology for water and wastewater treatment. For instance, APTwaster, Inc. (Long Beach, CA) has developed the first commercial application of counter-diffusion biofilm system- a hydrogen-based denitrification process for autotrophic reduction of NO_3^-. Moreover, they are uniquely suited for various treatment applications, including the reduction of oxidized contaminants (Martin *et al.*, 2015; Chung *et al.*, 2008; Nerenberg and Rittmann, 2004), and the removal of carbon and nitrogen pollutants (Aybar *et al.*, 2014; Li *et al.*, 2008)(**Paper I**).

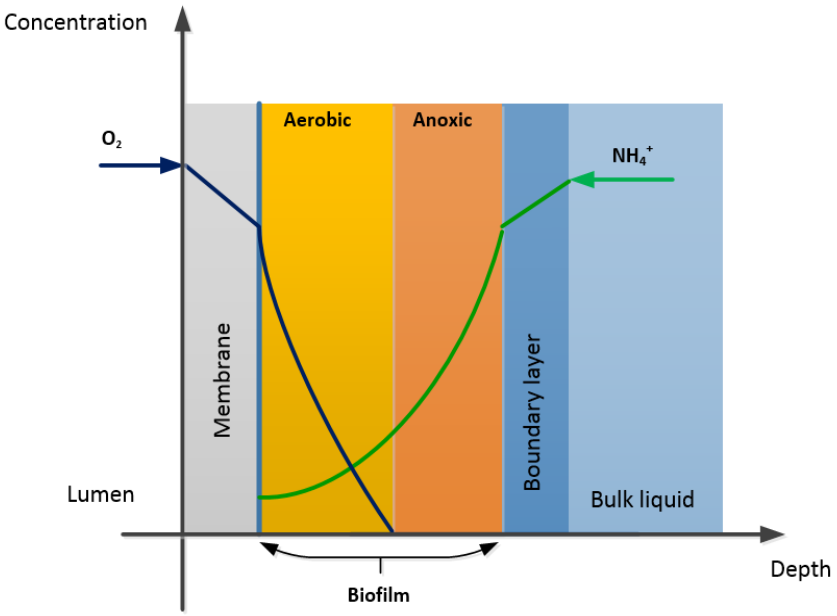


Figure 2.2 Concepts of counter-diffusion in membrane-aerated biofilm reactors with NH_4^+ as the main soluble compound provided via liquid phase.

In counter-diffusion biofilms, the gaseous substrate (e.g., dissolved oxygen) is supplied through the biofilm substratum (supporting membrane) and diffuses into the biofilm from the base, while the dissolved substrate is provided from the bulk liquid and diffuses from the top part of the biofilm. Counter-diffusion leads to unique process features, including (i) gas supply control, (ii) unique microbial community structures, (iii) high sensitivity to biomass accumulation, and (iv) low susceptibility to boundary layer resistance (Martin and Nerenberg, 2012; Syron and Casey, 2008). In this section, counter-diffusion is discussed in the context of NH_4^+ removal processes, hence referring to oxygen (air) as the gaseous substrate. Hereinafter, oxygen-based

counter-diffusion biofilm reactors will be defined as membrane-aerated biofilm reactors (MABRs) (Figure 2.2).

Flexible gas supply control. A major benefit of MABRs is energy cost savings due to high standard aeration efficiencies achieved at very low gas flow rates and low pressure losses (Syron *et al.*, 2015). Gas transfer fluxes can easily be controlled by manipulating the gas supply pressure, which does not modify the hydrodynamic conditions in the bulk liquid. Moreover, the gas flux can perform self-regulation, as oxygen demand from the biofilm can increase the concentration gradients, thereby increasing the driving force for oxygen supply. In MABRs, gas supply can be in two modes: (i) dead-end mode and (ii) flow-through mode. Dead-end mode operation allows oxygen transfer efficiency (OTE) to 100%, but gas back-diffusion into the sealed membrane lumen can significantly dilute the supply gas and consequently compromise the overall transfer ability of the system (Ahmed and Semmens, 1992). An approach of appropriate frequency and duration of gas purges is proposed to minimize the potential impact of gas back-diffusion on reactor performance (Martin and Nerenberg, 2012). In flow-through mode, air is continuously vented maintaining high oxygen concentrations throughout the entire membrane. This mode allows high oxygen transfer rates (OTR, $mg/(m^2d)$), which are calculated as follows:

$$OTR = k_{m,o2} \cdot \left(\frac{S_{o2,g}}{H} - S_{o2,\,mb}\right) \qquad (2.7)$$

where $k_{m,o2}$ is the oxygen mass transfer coefficient of the membrane (m/d), $S_{o2,g\ (mb)}$ is the oxygen concentration in the gas phase (or at the membrane-biofilm interface) (g/m^3), and H is the unitless Henry's law constant for oxygen (31.45, 298 K).

Unique microbial community structure. Differently from conventional co-diffusion nitrifying biofilms, in nitrifying MABRs aerobic microorganisms grow at the biofilm base (Downing and Nerenberg, 2008; Schramm *et al.*, 1999; Terada *et al.*, 2010), or in middle layers, depending on the spatial distributions of electron donor and acceptor (Essila et al., 2000)(**Paper I**). This unique biofilm structure in MABRs can improve the process performance, e.g. by increasing resistance to toxic shocks from inhibitory compounds, which would have lower effects on the inner layers of biofilms (Syron *et al.*, 2009). Nevertheless, the overlying biofilm layers will impede microbe outcompetition if an unwanted microbial group resides at the biofilm base. Additionally, if the biofilm top layers remian anoxic, both nitrification and anam-

mox processes can talk place within the same biofilm (Gilmore *et al.*, 2013; Pellicer-Nàcher *et al.*, 2014).

High sensitivity to biomass accumulation. In counter-diffusion MABRs, excessive biofilm thickness limits the substrate fluxes to both inner and outer biofilm layers, which might push the microbial activities to take place in the middle biofilm layers where both substrates exist but with relatively low concentrations. On the other hand, thin biofilm also leads to low fluxes due to biomass limitation. As concluded, biomass accumulation is important for biofilm performances (Nerenberg, 2016; Matsumoto *et al.*, 2007).

Low susceptibility to boundary layer resistance. Although the dissolved substrate provided from the liquid phase still needs to overcome the boundary layer resistance (similarly to conventional biofilms), the gaseous substrate flux into the biofilm is not influenced by liquid boundary layers. The boundary layer can further represent a barrier to the loss of the internal substrate to the bulk phase (Nerenberg, 2016), especially for gases with relatively low solubility in water, e.g. methane.

2.3.2 Challenges in nitrifying MABRs

In the previous sections, I have briefly addressed the microbiology of microbes involved in NH_4^+ removal and the relevant biological removal systems, with focus on MABRs. As this broad topic embraces bacteriology and biochemistry, as well as physics of mass transfer and aspects of process engineering, an encompassing exploration is not possible. In the next sections, I will focus on challenges in context of regulation and optimization of NH_4^+ removal in MABRs.

Competition between AOB and NOB. Short-cut NH_4^+ removal (via NO_2^-) is more energy-efficient than traditional NH_4^+ removal via NO_3^-, due to reduced aeration and external electron donor requirements (Hellinga *et al.*, 1998; Jenicek *et al.*, 2004). Achieving short-cut removal depends on NOB suppression. However, the microbial interactions between AOB and NOB are complex: (i) AOB and NOB compete for O_2 as the terminal electron acceptor; (ii) AOB and NOB are mutualistic symbionts, as AOB produce the NO_2^- substrate needed by NOB, which in turn prevent accumulation of NO_2^- to toxic concentrations (Stein and Arp, 1998); (iii) AOB and NOB may also be involved in the exchange of other important growth factors (Holger and Michael, 2010). It is even more complicate to speculate interactions in the complete autotrophic N removal, i.e. partial nitritation-anammox (PNA) pro-

cess. In PNA systems, NOB compete with AOB for O_2 and with AnAOB for NO_2^-. These multiple interactions require precise control of system operation to outcompete NOB, in order to get a stable and long-term coexistence of AOB and AnAOB. Even though NOB suppression has been widely studied and successfully tested under various conditions in suspended systems (Sliekers *et al.*, 2005; Vadivelu *et al.*, 2006b), there are few studies with biofilm systemss (Fux *et al.*, 2004), especially counter-diffusion biofilm systems as examined in this study.

Growth of heterotrophic bacteria. Nitrifiers (both AOB and NOB) interact with other microorganisms: nitrifiers reduce inorganic carbon to form organic carbon in cell synthesis, while producing and releasing soluble microbial products (SMP) from substrate metabolism and biomass decay (Rittmann *et al.*, 1994). Therefore, HB interact through the exchange of organic matter. Kindaichi et al. (2004) observed that the long sludge retention time in biofilm systems produces a large amount of SMP, supporting the growth of HB in autotrophic nitrifying biofilms, and HB can consitute up to 50% of the total microbial community. However, our knowledge regarding SMP of nitrifiers is far from complete, and more work is required to fully understand their contribution to HB growth. How does a biofilm community function as a biological unit? Which pathways are used by a biofilm community to maximize utilization of the metabolites of nitrifiers? From the perspective of biofilm performance, it needs to be assessed whether the organic carbon utilization by HB in autotrophic nitrifying biofilms would affect the nitrifier activities or the intermediate accumulation, such as N_2O, to significant levels.

Control of N_2O emissions. As introduced earlier, N_2O is a by-product of AOB reactions (NN pathway and ND pathway) and an obligate intermediate of denitrification process (HD pathway) (Richardson *et al.*, 2009; Domingo-Félez *et al.*, 2016). N_2O is a stratospheric ozone depleter and an important greenhouse gas 300-fold stronger than carbon dioxide (CO_2) (IPCC, 2001). Even low N_2O emissions significantly affect the carbon footprint of WWTPs (Gustavsson and Tumlin, 2013). Therefore, reducing N_2O emissions is beneficial for wastewater treatment processes. The underlying mechanisms and regulation of N_2O production have been studied in suspended systems and conventional biofilms (Pocquet *et al.*, 2016; Rodriguez-Caballero *et al.*, 2015; Bollon *et al.*, 2016; Schreiber *et al.*, 2009). However, many unknowns remain in counter-diffusion biofilms (Kinh *et al.*, 2017): what are the N_2O production pathways under COD limited conditions in an autotrophic nitrifying biofilm? what are the key operation factors driving N_2O emissions?

Other challenges in MABRs. Besides the specific challenges discussed above in the NH_4^+ removal process, additional investigations are needed, with respect to: (1) Physicochemical characterization of counter-diffusion biofilms. The density stratification in counter-diffusion biofilms has been hypothesized to differ from conventional co-diffusion biofilms (Pellicer-Nàcher and Smets, 2014). (2) Membrane selection. The choice of membrane directly affects gas transfer properties, membrane mechanical strength, chemical resistance and the process cost (Casey *et al.*, 1999; Terada *et al.*, 2006a). (3) Membrane module designs. A suitable module design ensures maximal mass transfer without requiring large amounts of mixing energy (Ho *et al.*, 2002; Ahmadi Motlagh *et al.*, 2008). (4) Biofilm thickness control. Biofilm thickness affects substrate fluxes, which have been identified as crucial in pilot scale studies (Semmens *et al.*, 2003; Lackner *et al.*, 2008; Martin and Nerenberg, 2012). Overall, successful optimization of MABRs relies on a better understanding of the interrelationship between the various factors governing their performance.

3 Control of the nitrifying process

AOB and NOB are the main microbial groups in autotrophic nitrifying biofilms, even though HB can grow on organic carbon released via biomass decay and AnAOB can grow on residual NH_4^+ and accumulated NO_2^- (Kindaichi *et al.*, 2004)(**Paper II**). Energy-efficient nitritation requires the suppression of NOB. Various strategies have been successfully tested for NOB suppression in suspended systems. However, maintaining long-term nitritation in biofilm-based reactors is still challenging, especially in counter-diffusion biofilms (Fux *et al.*, 2004)(**Paper I & II**). Furthermore, N_2O production remains one of the main challenges (**Paper II & III**). As a by-product of nitrification and an intermediate of denitrification, N_2O is produced in any NH_4^+ removal process, and its emission is influenced by multiple operational conditions. The common knowledge suggestes that nitritation produces more N_2O than complete nitrification (Rathnayake *et al.*, 2013). Therefore, the increased N_2O production can compromise the benefit of energy savings in nitritation, since carbon footprint of wastewater treatment plants is very sensitive to total N_2O emissions (Gustavsson and Tumlin, 2013). In this section, I summarize potential influencing factors of nitrifying activities and operational parameters leading to N_2O emission in NH_4^+ removal process. Lastly, I highlight the importance of mathematical models in evaluating process behaviors, testing potential effects, and exploring underlying microbial reaction mechanisms in a complex biofilm reactor system.

3.1 Influencing factors

Nitrifying microbial activities and N_2O emissions are known to be affected by various factors. Table 3.1 and 3.2 provides a list of the main factors and a description of the respective effects on nitrifying activity and N2O emissions, respectively.

Table 3.1 Operational factors influencing AOB/NOB activities

Factors	Effects
(1) Temperature, T	The relation between T and maximum growth rate is different between AOB and NOB. Generally, AOB have higher growth rates than NOB at evaluated temperature (> 20 ^{o}C) (Hellinga *et al.*, 1998; Randall and Buth, 1984; Bougard *et al.*, 2006). However, T impacts on biofilm nitrification rates are considered less significant than on suspended systems (Zhu and Chen, 2002).
(2) pH	pH affects nitrifying activities directly by changing the enzyme reaction mechanism or increasing the demand for maintenance energy (Siegrist and Gujer, 1987; Van Hulle *et al.*, 2007). A bell-shaped pH dependence of nitrifying enzyme kinetics is proposed with the optimum pH at 8.2±0.3 for AOB and at 7.9±0.4 for NOB (Park *et al.*, 2007)
(3) Oxygen concentration, DO	AOB are considered to have higher affinity for oxygen than NOB (Schramm *et al.*, 1998; Sliekers *et al.*, 2005; Downing and Nerenberg, 2008).
(4) Free ammonia, FA	NOB are more sensitive to FA inhibition than AOB (Anthonisen *et al.*, 1976; Kim *et al.*, 2006; Ma *et al.*, 2017).
(5) Free nitrous acid, FNA	NOB are more sensitive to FNA inhibition than AOB (Anthonisen *et al.*, 1976; Vadivelu *et al.*, 2006a, 2006b).
(6) Substrate limitation	FA and FNA are the true substrate for AOB and NOB growth, respectively. Their concentration depend on pH, total NH_4^+ and total NO_2^- concentration, respectively (Hiatt and Grady, 2008).
(7) Inorganic carbon, IC	AOB and NOB have different carbon fixation pathways (Palomo *et al.*, 2016). NOB show more resilience than AOB (or AnAOB) to IC limitation (Ma *et al.*, 2015; Chen *et al.*, 2012).
(8) Other inhibitors	Volatile fatty acid (VFA) (Takai et al., 1997), nitric oxide (Courtens *et al.*, 2015) and salinity (Sudarno *et al.*, 2011) also show different effects on AOB and NOB activities.
(9) Inoculum composition	AOB and NOB species composition of the inoculum can affect community composition at steady state (Terada *et al.*, 2010).
(10) Aeration control	Aeration control can operate the system at controlled aerobic SRTs which retain AOB but out-select NOB (Regmi *et al.*, 2014), and cause lag phase of NOB response via introducing transient air-off disturbances (Kornaros *et al.*, 2010; Gilbert *et al.*, 2014).

Table 3.2 Operational factors influencing N_2O emissions

Factors	Effects
(1) Temperature, T	T changes can cause variations of N_2O emissions as microbial activities vary with T (Hu *et al.*, 2013)
(2) pH	As nitrifying activities are affected by pH levels, pH influences N_2O production (Hanaki *et al.*, 1992; Law *et al.*, 2011), and pH decrease from 7.5 to 7.2 enhances N_2O production by AOB pathways (Frame *et al.*, 2017).
(3) Oxygen concentration, DO	DO concentration strongly impacts the level of N_2O emission (Kampschreur *et al.*, 2008; Wunderlin *et al.*, 2012). Tallec et al. (2006) showed that the highest N_2O emission occurred at DO of 1 mg/L, and a decrease emission happened both at higher and lower oxygenation.
(4) Ammonium	Increased NH_4^+ loads can result in higher N_2O emissions (Lotito *et al.*, 2012; Kampschreur *et al.*, 2008). Under aerobic conditions, AOB-driven N_2O production is positively correlated with extant NH_4^+ oxidation (Domingo-Félez *et al.*, 2014).
(5) Nitrite	Increased NO_2^- concentrations can increase N_2O emission both during nitrification (Okabe *et al.*, 2011) and denitrification (Park *et al.*, 2000).
(6) Inorganic carbon, IC	Both excess and limiting IC availability can increase overall N_2O emissions, due to the imbalance between the anabolic process of carbon fixation and the catabolic oxidation of NH_3 to NO_2^-. The imbalance causes the overflow of electrons into the respiratory chain and into N_2O production from NO_2^- reduction (Jiang *et al.*, 2015; Ma *et al.*, 2015; Mellbye *et al.*, 2016)
(7) COD/N	Under low COD/N conditions, N_2O is significantly produced by heterotrophic pathway (Itokawa *et al.*, 2001; Schalk-Otte *et al.*, 2000; Domingo-Félez *et al.*, 2016).
(8) Internal storage compounds	Denitrification by glycogen accumulating organisms can increase N_2O emission (Zeng *et al.*, 2003; Lemaire *et al.*, 2006).
(9) Rapid operation changes	Dynamic process conditions (DO, NO_2^-, NH_4^+ concentrations) lead to a dramatic rise in N_2O emission, due to the imbalance in the involved gene expressions (Yu *et al.*, 2010; Kampschreur *et al.*, 2008)
(10) Aeration control	With intermittent aeration, most of the N_2O is emitted during the air-on periods, and an optimum combination of air-on and air-off conditions or aeration intermittency are very important to control N_2O emission (Kimochi *et al.*, 1998; Domingo-Félez *et al.*, 2014; Rodriguez-Caballero *et al.*, 2015).

3.2 Aeration control

Among all factors influencing nitrifying activity (Table 3.1), aeration control has proven to be effective to suppress NOB in various suspended growth processes. Aeration is controlled by terminating aeration upon the completion of NH_4^+ oxidation while accumulated NO_2^- still remains (Yang *et al.*, 2007; Blackburne *et al.*, 2008; Guo *et al.*, 2009). NOB are kinetically to be washed out, because (1) the transient anoxic periods (air-off) in aeration control regulate aerobic SRT in the system, at which AOB can be retained while NOB are out-selected; (2) transient anoxia introduces a slower NOB response to aera-

tion transition from air-off to air-on conditions, as compared to AOB response, either due to NO_2^- limitation at the aeration onset (Lemaire *et al.*, 2008) or a metabolic delay (Gilbert *et al.*, 2014). Despite an infinite SRT in the biofilm system, nitrifying activity is also observed to respond to aeration control (Kong *et al.*, 2013; Zekker *et al.*, 2012). For instance, Pellicer-Nàcher et al. (2010) achieved high N removal rates and decreased NOB activity under intermittent aeration in MABRs, while no N removal occurred and NO_3^- (not NO_2^-) was produced under continuous aeration. Regarding to NOB suppression in biofilms with aeration control, the underlying reasons need to be investigated: Is NOB suppressed because of the slow NOB responses after anoxia conditions as observed in suspended systems, or other potential influencing factors (Table 3.1)? To answer this question, the causal link between aeration control and N regulations, focusing on NOB suppression in MABRs, was explored (**Paper I & II**).

Aeration control also regulates N_2O emissions in activated sludge systems (Park *et al.*, 2000; Béline, 2002). In the treatment of pig slurry, Béline (2002) studied N transformation during the NH_4^+ removal process, and observed decreased N_2O emissions under intermittent aeration, as compared to continuous aeration. His study suggested that N_2O was consumed by heterotrophic denitrification under anoxic conditions, thus reducing the total emissions. While N_2O was also significantly reduced via operating intermittent aeration in a one-stage PNA biofilm system, (Gilmore *et al.*, 2013; Pellicer-Nàcher *et al.*, 2014), the underlying mechanisms remained unclear. In this thesis, the impact of aeration control on N_2O productions in counter-diffusion nitrifying MABRs was studied (**Paper II & III**).

3.3 Mathematical modeling

Mathematical models allow us to describe multiple processes that occur simultaneously in time or space. They provide an effective approach to study complex systems (Carrera *et al.*, 2004; Martin *et al.*, 2015; Sabba *et al.*, 2015). A multi-species nitrifying model was earlier developed to study the competition between AOB and NOB in conventional biofilms, and effects of DO, pH, FA and FNA on growth kinetics were incorporated in a spatially explicit way (Park *et al.*, 2015, 2010; Shanahan and Semmens, 2015). Therefore, influencing factors of NOB suppression can be evaluated individually, and underlying mechanisms of nitritation success can be identified. With different N_2O reaction pathways incorporated, models can be further utilized to

quantify the contribution of each N_2O production and consumption pathway to the total N_2O pool. Therefore, N_2O mitigation strategies in N-related wastewater treatment can be developed and evaluated (Ni *et al.*, 2011, 2014, 2015; Domingo-Félez and Smets, 2016; Domingo-Félez *et al.*, 2017).

Aiming at a deep exploration of examining MABR performance, a one-dimension biofilm model was developed to study:

(1) NOB suppression in MABRs, especially under intermittent aeration control (**Paper I**);

Based on the counter-diffusion nitrifying biofilm model of Terada et al. (2007), I developed an extended nitrifying biofilm model in Aquasim (Reichert, 1998) incorporating explicit pH calculation.

(2) N_2O production in MABRs, especially under dynamic aeration control (**Paper III**).

Based on the N_2O model of Domingo-Félez and Smets (2016) and the biofilm model of Vangsgaard et al. (2013), I developed a counter-diffusion biofilm in Aquasim and Matlab 2016b (MathWork, Natick, MA), respectively.

4 MABR performance under aeration control

Two laboratory-scale MABRs ($MABR_1$ and $MABR_2$) were operated under continuous aeration and different degrees of intermittent aeration, in order to study the impact of aeration on long-term bioiflm performance. The MABRs consisted of two tubular gas filled PDMS membranes (3100506, Labmarket, Germany), both fixed in parallel to their longer dimension (Figure 4.1). The system had a liquid volume of 0.8 L (reactors: 31.5×5×3.5 cm) and was inoculated with enriched nitrifying biomass obtained from the Mølleåværket WWTP (Lundtofte, Denmark). To start up the system, the reactor was first run in batch mode with an initial NH_4^+ concentration at 300 mg-N/L and continuous aeration. The onset of NH_4^+ consumption without oxygen accumulation in the bulk suggested biomass attachment around the membranes. Subsequently, MABRs were operated in continuous-flow mode under continuous or intermittent aeration. Synthetic wastewater was fed continuously with influent NH_4^+ concentration at 75 mg-N/L and without external organic carbon for approximately 400 days. The influent N-loading was 9.1 g-N/(m^2·day). The bulk phase was completely mixed using internal recirculation, and dissolved oxygen (DO) and pH were measured with electrodes in the recirculation line (CellOX 325 and Sentix 41, WTW, Germany). Bulk temperature and pH were not controlled, and varied from 24 to 30°C and 6.8 to 7.1, respectively. Adequate buffer was provided from influent with a molar ratio of bicarbonate (HCO_3^-) to NH_4^+ at 1.8. During the operation, reactor temperature was occasionally above the ambient temperature, due to the unintentional heat added by the recirculation pump.

Duplicate MABRs were operated under identical conditions, with the only exception of aeration control. $MABR_1$ was operated under either continuous or intermittent aeration, while $MABR_2$ was operated under continuous aeration throughout the whole period (Table 4.1). Intermittent aeration cycles consisted of an air-on period (100% air) followed by an air-off period (100% N_2) which were controlled by solenoid valves.

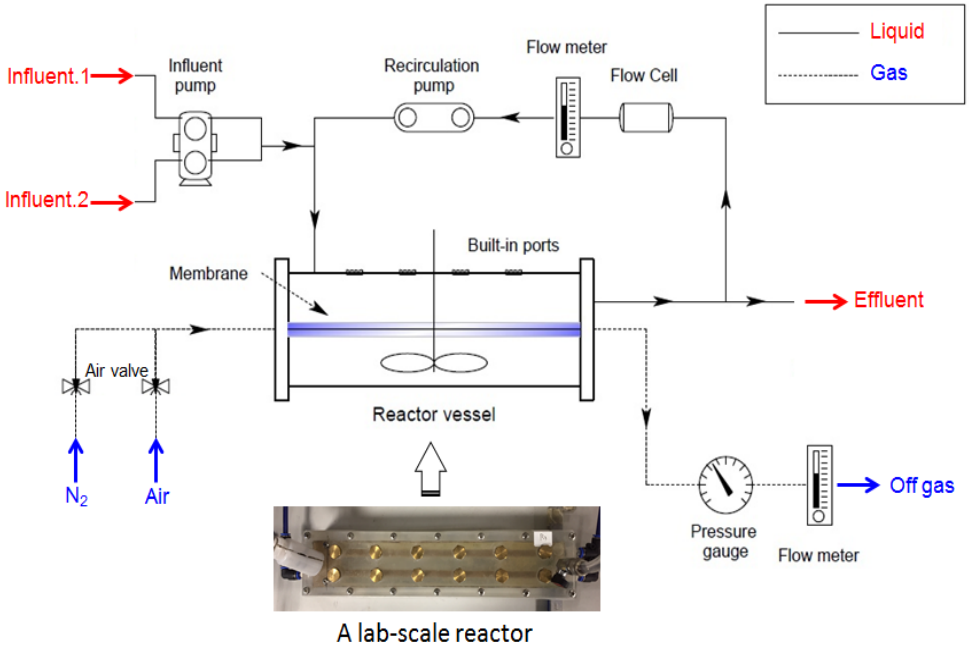


Figure 4.1 Schematic diagram of laboratory-scale membrane-aerated biofilm reactors. Influent.1 provided NH_4^+ and bicarbonate source, and influent.2 provided mineral source for microbial growth.

Table 4.1 Timeline of imposed aeration conditions in $MABR_1$ and $MABR_2$

Time (day) / Aeration control		1-67	68-94	95-143	144-196	197-255	256-301	302-368	369-430
$MABR_1$		Cont	Int_{6+6}	Cont*	Int_{6+6}*	Int_{11+1}	Int_{9+3}	Int_{6+2}	Int_{1+1}
	R_{on}	1	0.5	1	0.5	0.9	0.75	0.75	0.5
	f_{int}	1	2	1	2	2	2	3	12
$MABR_2$		Cont							

Cont: continuous aeration; Int_{A+B}: intermittent aeration with a cycle comprised of A-hour air-on and B-hour air-off.

Different controls of intermittent aeration were described by the relative aeration duration (R_{on}) and the aeration intermittency (f_{int}, 1/d):

$$R_{on} = t_{AirOn}/(t_{AirOn} + t_{AirOff});$$

$$f_{int} = 24/(t_{AirOn} + t_{AirOff}) \quad (4.1)$$

where t_{AirOn} and t_{AirOff} are air-on and -off duration in intermittent aeration cycles (h). Bulk N measurements showed that under continuous aeration a nitrifying biofilm developed in $MABR_2$. $MABR_1$ showed similar nitrifying performance during start-up period, as NO_3^- was the main N species produced, but bulk N concentrations changed with aeration control **(Paper II)**. In the following sections, results will be presented on how microbial activities evolved with aeration control based on daily bulk measurements, and how

reactor performance changed accordingly. Lastly, the effects of aeration control on local concentrations of pH, DO and N_2O in biofilm are discussed based on experimentally measured in-situ microprofiles.

4.1 NOB suppression and AnAOB activation

Individual N consumption rates by different microbial guilds (AOB, NOB, AnAOB and HB) were calculated based on mass balance analysis of N species (**Paper II**). This analysis revealed changes in microbial activities with aeration control (Figure 4.2). Two ratios ($R_{NH4^+,AOB}/R_{NO2^-,NOB}$ and $R_{NO2^-,AnAOB}/R_{NO2^-,NOB}$) were calculated to show how relative activities of different groups evolved with the aeration control. Low ratio values at the beginning of reactor operation suggested high activities of both AOB and NOB, but low initial AnAOB activities. The considerable increase of $R_{NH4^+,AOB}/R_{NO2^-,NOB}$ and $R_{NO2^-,AnAOB}/R_{NO2^-,NOB}$ under intermittent aeration (Int_{6+6}) indicated NOB suppression and AnAOB activation. NOB suppression via intermittent aeration has also been observed in other studies (Pollice, 2002; Pellicer-Nàcher *et al.*, 2010; Yang *et al.*, 2015). However, potential reasons for NOB suppression due to aeration control were not yet comprehensively examined in a biofilm system. In MABRs, we observed that the pH-related effect of free ammonia (FA) inhibition- was the crucial factor in NOB suppression (**Paper I and II**). Neither DO limitation effect nor the limited oxygen supply into the system contributed significantly to NOB suppression in the counter-diffusion system. This finding differs from observations by Downing and Nerenberg (2008) who suggested that controlling DO concentrations in biofilm sufficed to maintain nitritation. In suspended growth systems, Gilbert et al. (2014) studied the response of nitrifying bacteria to anoxia in batch experiments, and attributed delayed NO_3^- production after anoxia to a slow response in NOB metabolism. The latter study further suggested that NOB might be successfully suppressed by intermittent aeration if air-on duration is shorter than the lag phases of NOB response (5-15 minutes). However, intermittent aeration in $MABR_1$ had air-on periods far longer than the reported lag phases. Our study revealed major differences in mechanisms of NOB suppression under intermittent aeration between biofilms and suspended systems.

As a result of NOB suppression, NO_2^- produced at the biofilm base was available for AnAOB growth at the top anoxic biofilm zone (Zekker *et al.*, 2012). Intermittent aeration was efficient in regulating N microbial activities in MABRs. Moreover, the regulation mechanism was reversible, as these two

ratios ($R_{NH4}^{+}{}_{,AOB}/R_{NO2}^{-}{}_{,NOB}$ and $R_{NO2}^{-}{}_{,AnAOB}/R_{NO2}^{-}{}_{,NOB}$) increased repeatedly under the intermittent aeration of Int_{6+6}*. Clearly, aeration control, not the time of operation, affected the microbial activities (Figure 4.2B).

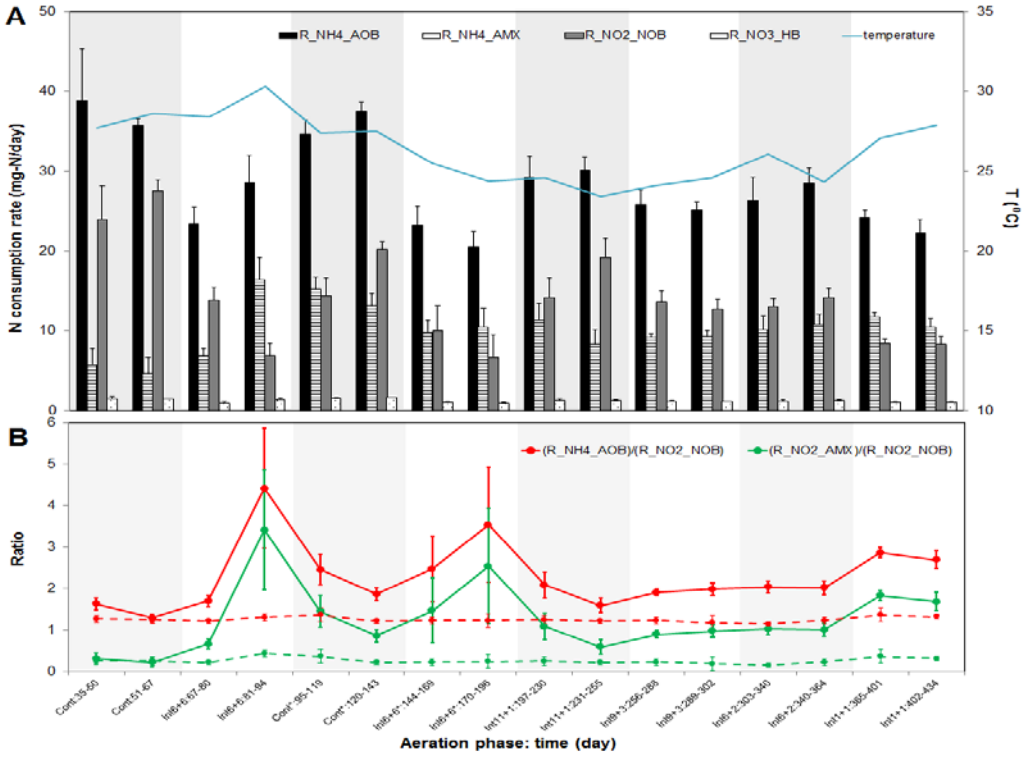


Figure 4.2 (A) Calculated nitrogen consumption rates of each microbial group and working temperature under different aeration controls in $MABR_1$: $R_{NH4}^{+}{}_{,AOB}$, $R_{NO2}^{-}{}_{,NOB}$, $R_{NH4}^{+}{}_{,AnAOB}$ and $R_{NO3}^{-}{}_{,HB}$ represented NH_4^+ consumption rate by AOB, NO_2^- consumption rate by NOB, NH_4^+ consumption rate by AnAOB, and NO_3^- consumption rate by HB in denitrification (mg-N/day); (B) Variations of relative microbial activities in $MABR_1$: $R_{NH4}^{+}{}_{,AOB}/R_{NO2}^{-}{}_{,NOB}$ represents the competition between AOB and NOB for oxygen and $R_{NO2}^{-}{}_{,AnAOB}/R_{NO2}^{-}{}_{,NOB}$ represents the competition between NOB and AnAOB for NO_2^- (dash lines represent the reference ratios in $MABR_2$ which operated under continuous aeration all the time).

4.2 Reactor performance evaluation

Reactor performance was expressed as NH_4^+ removal efficiency (($NH_4^+{}_{inf}$ - $NH_4^+{}_{eff}$)/$NH_4^+{}_{inf}$, %) and the degree of NOB suppression ($R_{NH4}^{+}{}_{,AOB}/R_{NO2}^{-}{}_{,NOB}$). Different performance was observed during the aeration control. Overall, NH_4^+ removal efficiency varied in the range of 29.6-48.2%, and the degree of NOB suppression was in the range of 1.3-4.4. Effects of aeration control on the performance were evaluated with two operational factors of intermittent aeration, i.e. relative aeration duration (R_{on}) and aeration intermittency (f_{int}).

NH_4^+ removal increased with relative aeration duration due to increased oxygen supply (Figure 4.3A). However, the degree of NOB suppression decreased with the relative duration (Figure 4.3B). A tradeoff was observed between NOB suppression and NH_4^+ removal. Consistent with the study of Mota et al. (2005), it indicates that low NOB abundance is achieved in an intermittently aerated reactor with long anoxic durations when high NH_4^+ effluent concentrations were produced. Residual NH_4^+ concentrations were highlighted in NOB suppression, which supported our conclusion that FA inhibition was the key factor in achieving nitritation in MABRs. Therefore, the maximum aeration duration should be set and a suitable ratio of air-on to air-off duration should be chosen critically (Regmi *et al.*, 2014; Yang *et al.*, 2015; Kornaros *et al.*, 2008) (**Paper I**). Effects of aeration intermittency on biofilm performance should be evaluated under specific conditions: (i) NOB suppression could be favored or impeded by increased aeration intermittency (Figure 4.3B); (ii) if NOB suppression was favored, NH_4^+ removal efficiency would be enhanced as more oxygen source was available for AOB reactions (**Paper II**). Our study indicates that both R_{on} and f_{int} are effective in manipulating the performance of an intermittently aerated MABR.

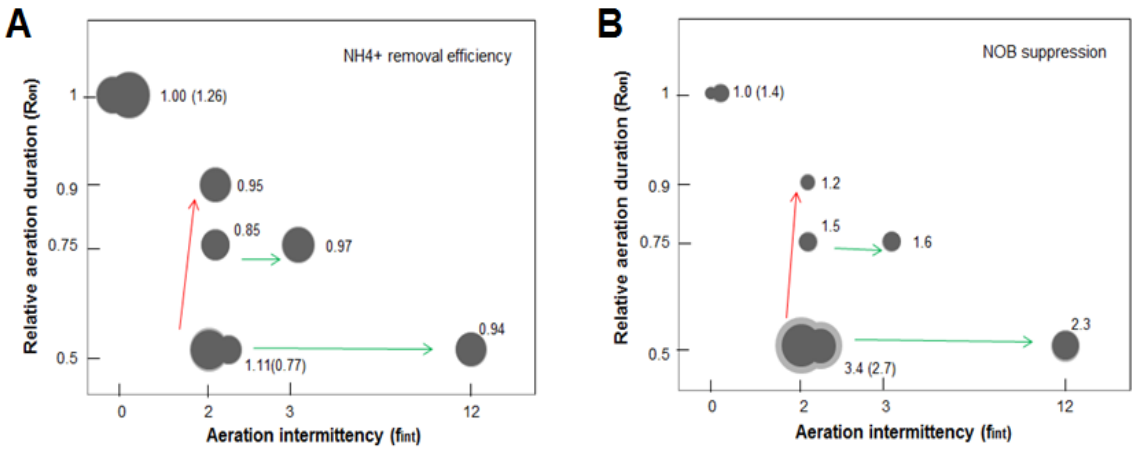
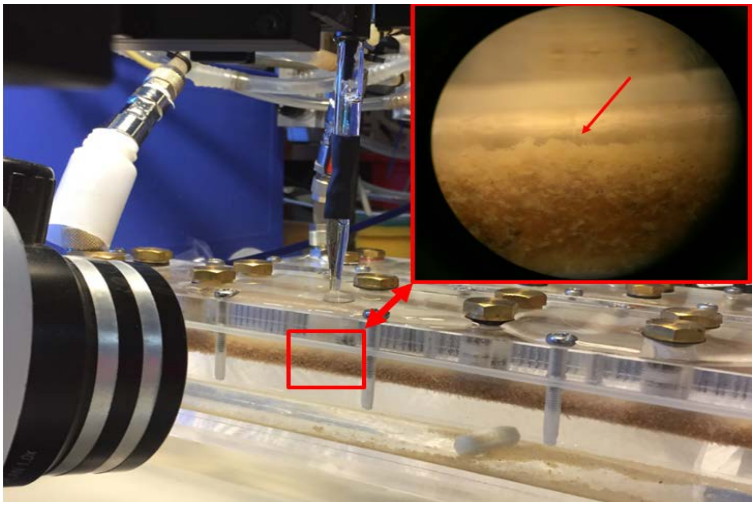


Figure 4.3 $MABR_1$ performance during aeration control. (A) NH_4^+ removal efficiency. (B) The degree of NOB suppression. Diameter of dark dots represents the removal efficiencies or the degree of suppression normalized by the performance in the first continuous aeration phase (Phase Cont: $f_{int} = 0$, $R_{on} = 1$). Diameter of grey circles represents the relevant standard deviations (the standard deviations of removal efficiencies were too small to see clearly).

4.3 In-situ micro profiling measurements

4.3.1 Changes of local DO and pH

Both DO and pH affect the activities of AOB and NOB, considering DO limitation effect, direct pH effect on enzymes, and indirect pH effect on substrate speciation (Park *et al.*, 2010)(**Paper I**). To study local variations of DO and pH in biofilm during aeration control, in-situ microprofiles were measured and compared in $MABR_1$ under continuous versus intermittent aeration (Figure 4.4). Averaged microprofiles (> 3) were considered. A two-tailed student's t-test was employed at a 95% confidence interval to verify significant differences. DO microprofiles were compared in terms of oxygen penetration depth (oxic zone, µm) and DO concentrations (mg/L) at the biofilm base, i.e. the membrane-biofilm interface. No significant differences were observed (Table 4.2). pH microprofiles were compared in terms of bulk pH and pH at the biofilm base. Significant differences were observed: (1) pH decreased with biofilm depth when air was on because of proton production in nitritation, while it increased with depth until 7.52 (± 0.03) at the biofilm base during air-off periods under intermittent aeration due to continuous CO_2 stripping from the biofilm base to the membrane lumen (Ma *et al.*, 2017); (2) Bulk pH was significantly different between continuous and intermittent aeration ($p \ll 0.001$), despite that there was no significant difference between pH at the biofilm base during air-on periods; (3) Upon air-on switches under intermittent aeration, pH in biofilm decreased slowly until reaching steady state after 30 minutes (transition time in Table 4.2), showing pH stabilization lagged behind DO stabilization. Through comparison of local pH and DO concentrations between different aeration controls, variations of relevant effects on AOB/NOB growth could be evaluated: changed microbial activities under intermittent aeration probably resulted from pH-related effects, rather than DO effects.



Figrue 4.4 In-situ microprofiling measurements in MABRs

Table 4.2 Comparison of DO and pH microprofiles in $MABR_1$ between continuous and intermittent aeration

	Aeration control		p-value	Intermittent aeration	
	Cont	$Inte_{AirOn}$		$Inte_{AirOff}$	[e]transition time
DO					
[a]oxic zone (μm)	[c]63 ± 23 (n=10)	67 ± 28 (n=13)	0.62	[d]NA	1 min
DO at the base (mg/L)	2.95 ± 0.83 (n=10)	3.14 ± 0.91 (n=13)	0.72	NA	
pH					
[b]bulk pH	6.82 ± 0.08 (n=28)	7.03 ± 0.08 (n=14)	<0.001	7.02 ± 0.07 (n=10)	30 mins
pH at the base	6.20 ± 0.15 (n=5)	6.25 ± 0.13 (n=5)	0.56	7.52 ± 0.03 (n=4)	

[a]Depth of oxic zone was defined as the distance from the biofilm base to the biofilm layer where DO concentration was 0.01 mg/L. 0.01 mg/L was the detection limit of DO microsensors. [b]Bulk pH was measured by pH electrodes daily (Sentix 41, WTW Germany). [c]Averaged values of n measurements (± standard deviations). [d]No measurements. [e]Transition time under intermittent aeration was the time duration from air-on switch to the time point when biofilm pH or DO reached steady state.

4.3.2 Mitigation of N_2O emissions

Total N_2O emission was measured in $MABR_1$ during the aeration control, including both liquid and off-gas phases. Measurements showed that N_2O had the highest emission during the first continuous aeration (2.35% of influent N load), and emissions in the off-gas phase were similar in magnitude as those in the liquid phase. However, the relatively high initial emission in a nitrify-

ing biofilm was comparable to emissions in partial nitritation systems (0.8% ~ 5.6% of N load) (Rathnayake *et al.*, 2013; Kampschreur *et al.*, 2008; Ishii *et al.*, 2014). This finding is in disagreement with common knowledge that conventional nitrification processes produce less N_2O than PN processes (Rathnayake *et al.*, 2013). Total emissions decreased dramatically after intermittent aeration and remained low during the following aeration controls (< 0.35% of N load). In addition, low but highly dynamic N_2O concentrations were detected in off-gas upon air on/off switches under intermittent aeration. This variations suggests considerable formation of N_2O when the N-cycling community undergoes perturbationn, such as aeration changes (Schreiber *et al.*, 2009).

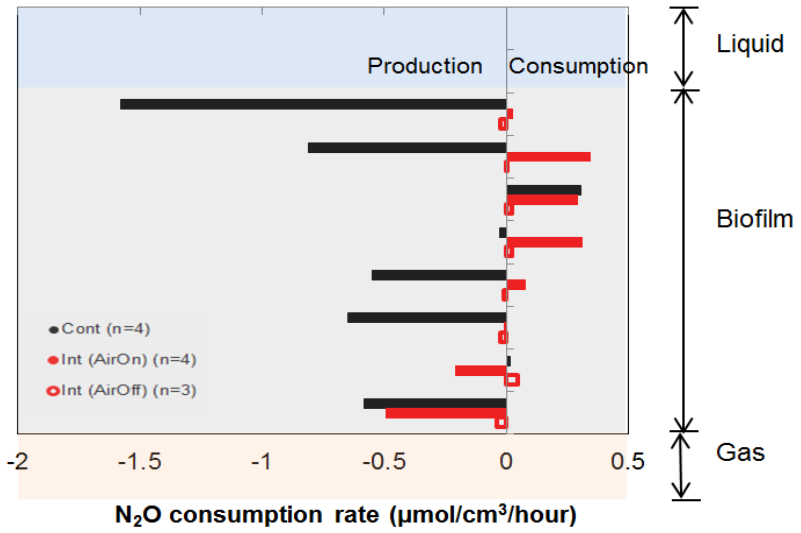


Figure 4.5 Spatial distribution of net volumetric N_2O production/consumption rates in biofilm during the first continuous aeration (Cont) and the following intermittent aeration (Int_{6+6}: air-on and air-off periods).

In-situ N_2O microprofiles were measured. Based on average profiles, net volumetric N_2O reaction rates were estimated using Fick's second law of diffusion (Lorenzen *et al.*, 1998). N_2O production dynamics in MABRs were studied and compared before and after intermittent aeration (Figure 4.5). During the first continuous aeration, N_2O was produced throughout the whole biofilm, with especially high production rates at the anoxic zone most likely by heterotrophs utilizing organic carbon produced via biomass degradation (Kindaichi *et al.*, 2004; Okabe *et al.*, 2011). Under intermittent aeration, however, N_2O was mostly produced at the biofilm base (oxic zone) and consumed at the biofilm top (anoxic zone). AOB became the main N_2O producers, while HB established a N_2O-reduction zone outside anoxic biofilm minimizing N_2O diffusion into the liquid phase (Pellicer-Nàcher *et al.*, 2010;

Kinh *et al.*, 2017). During aeration control, HB significantly affected N_2O emissions in MABRs. Additionally, decreased N_2O production under intermittent aeration was associated to the disappearance of bulk NO_2^- (**Paper II**). NO_2^- was completely consumed by the activated AnAOB in anoxic biofilm zones and the liquid phase, which might reduce N_2O production from denitrifying pathways (Gilmore *et al.*, 2013)(**Paper II and III**).

Taken together, these observations suggest that intermittent aeration can favor NOB suppression and AMX activation in nitrifying MABRs. Influences of two operational factors (relative aeration duration and aeration intermittency) were evaluated considering NH_4^+ removal efficiency and the degree of NOB suppression. A mass-balance based approach was proposed to analyze activity changes of different microbial groups. Transient variations of biofilm pH and DO under aeration control were documented for the first time in this study. Moreover, a significant decrease in biofilm N_2O was detected at the onset of intermittent aeration. Overall, aeration control is an efficient strategy to regulate nitrogen microbial activities in counter-diffusion MABRs, aiming at high nitritation efficiency and low N_2O emissions (**Paper II)**.

5 Modeling study 1: NOB suppression in nitrifying MABRs

Even though in suspended growth systems, various conditions have been successfully tested to suppress NOB over AOB activity or wash-out NOB over AOB biomass to attain nitritation, finding operational conditions and confirming mechanisms that suppress NOB in biofilms remains a challenge (Fux *et al.*, 2004; Ma *et al.*, 2015; Park *et al.*, 2010, 2015). Therefore, I developed a mathematical modeling to describe and examine nitrifying biofilms, where simultaneously occurring processes are modeled in time and space (Carrera *et al.*, 2004; Martin *et al.*, 2015) (**Paper I and III**). Park et al. (2015) developed a multi-species nitrifying biofilm model to study the competition between AOB and NOB in co-diffusion biofilms, and effects of DO, pH, FA and FNA on growth kinetics were incorporated in a spatially explicit way to evaluate operational conditions. Here we improve a counter-diffusion nitrifying biofilm model in the study of Terada et al. (2007) by incorporating pH calculation. The model was calibrated with long-term experimental data from a nitritation MABR system with intermittent aeration (**Paper I)**. Using the calibrated model, we systematically evaluated potential causes for NOB suppression associated with the aeration control, and proposed a suitable operational window for an effective nitritation process in counter-diffusion systems.

5.1 Model description

5.1.1 1-D nitrifying biofilm model with pH calculation

The counter-diffusion nitrifying biofilm model is a one-dimensional (1-D) model based on Terada et al. (2007), incorporating additional explicit pH calculation (**Paper I**). The model was implemented in Aquasim 2.1 with a completely mixed gas compartment and a biofilm compartment including bulk liquid phase (Reichert, 1998). In the counter-diffusion regime, a physical diffusive link connecting the gas compartment to the biofilm base was defined. The model includes three active microbial groups (AOB, NOB, HB) and inerts accumulated during decay processes. For the two-step nitrification process, FA and FNA are considered as true substrates for growth and as inhibitors (Hiatt and Grady, 2008). NO_2^- and NO_3^- are modeled as separate electron acceptors in denitrification. To avoid unnecessary complexity and focus on AOB/NOB competition, the intermediates, such as hydroxylamine, NO, N_2O,

are not considered. The growth rate expressions for AOB and NOB consider DO and pH effects. DO limitation effect is defined with a Monod-type expression. Two pH effects are included:

(1) direct pH-enzyme effect (Sötemann *et al.*, 2006; Van Hulle *et al.*, 2007), as defined by a Gaussian bell-shaped dependency of the maximum growth rates on pH conditions (Park *et al.*, 2007):

$$\mu = \frac{\mu_{max}}{2}\{1 + \cos[\frac{\pi}{\omega}\cdot(pH - pH_{opt})]\} \qquad |pH - pH_{opt}| < \omega \qquad (5.1)$$

(2) and indirect pH effect on substrate-speciation of FA and FNA, and the FA/FNA inhibition are defined with the Andrews equation or a non-competitive inhibition equation (Hiatt and Grady, 2008; Hellinga *et al.*, 1999):

$$\text{AOB: } \mu_{AOB}\cdot X_{AOB}\cdot\frac{S_{O2}}{K_{O2}^{AOB}+S_{O2}}\cdot\frac{S_{FA}}{K_{FA}^{AOB}+S_{FA}+S_{FA}\cdot S_{FA}/K_{I,FA}^{AOB}}\cdot\frac{K_{I,FNA}^{AOB}}{K_{I,FNA}^{AOB}+S_{FNA}} \qquad (5.2)$$

$$\text{NOB: } \mu_{NOB}\cdot X_{NOB}\cdot\frac{S_{O2}}{K_{O2}^{NOB}+S_{O2}}\cdot\frac{S_{FNA}}{K_{FNA}^{NOB}+S_{FNA}+S_{FNA}\cdot S_{FNA}/K_{I,FNA}^{NOB}}\cdot\frac{K_{I,FA}^{NOB}}{K_{I,FA}^{NOB}+S_{FA}} \qquad (5.3)$$

where μ and μ_{max} is the specific growth rate and its maximum value at the optimal pH- pH_{opt}; ω is the pH range within which μ is larger than $0.5\cdot\mu_{max}$; S, K and K_I are substrate concentration, half-saturation coefficient and inhibition coefficient (mg/L). Additionally, FA/FNA speciation between ionized/unionized species is calculated based on instantaneous equilibrium and the relevant dissociation equilibrium constants (Musvoto *et al.*, 2000).

The 1-D model can simulate local pH changes along the biofilm depth based on the proton production via nitrification and consumption via denitrification, equilibrium reaction with the bicarbonate system, and carbon dioxide (CO_2) stripping to the membrane lumen. The acid-base reactions with bicarbonate buffer are assumed to occur significantly faster than biological processes (Sötemann *et al.*, 2006), as described

$$H^+ + HCO_3^- \leftrightarrow H_2CO_3\ (CO_2)$$

$$\text{rate expressions: } \left(\frac{S_{HCO3}-S_H}{K_{a,HCO3}} - S_{CO2}\right)\cdot 10^7\ (1/\text{day}) \qquad (5.4)$$

where S_H, S_{HCO3-} and $S_{H2CO3(CO2)}$ are concentrations of proton, bicarbonate and the sum of carbonic acid and dissolved carbon dioxide, respectively (μmol/L); $K_{a,HCO3}$ is the dissociation equilibrium constant of carbonic acid (0.574 μmol/L, 33 ℃, 1 atm), and 10^7 is the specific rate coefficient (1/day).

5.1.2 Sensitivity analysis and parameter estimation

To investigate the most influencing parameters on reactor performance, a sensitivity analysis was performed. Initial values of kinetic parameters were taken from literature (the activated sludge model for nitrogen of Hiatt and Grady (2008)), and information on optimal pH ranges for microbial growth were taken from Park et al. (2007). The model was first run under continuous aeration with default values to achieve a nitrifying biofilm. A local sensitivity analysis was then performed after switching from continuous aeration to intermittent aeration, introducing a ±100% change for each individual parameter while all others remained constant (Reichert, 1998). Reactor performance was evaluated in terms of bulk nitrogen species. Sensitivity of biokinetic and stoichiometric parameters was ranked (Figure 5.1): Maximum growth rate of AOB (μ_{max}^{AOB}) was the most determinant parameter among all kinetic parameters governing nitrogen conversions. This is in agreement with sensitivity analysis in Wang et al. (2009), who ran a similar counter-diffusion biofilm model ranking kinetic parameters in terms of nitritation performance and biofilm development in nitrifying biofilms. The higher sensitivity of model outputs (NH_4^+ removal efficiency and nitritation efficiency) in the biofilm versus the bulk phase (**Paper I**) suggests that in-situ microprofiling data can be more informative in model calibration than bulk measurements, which have been typically used (Brockmann *et al.*, 2008; Downing and Nerenberg, 2008).

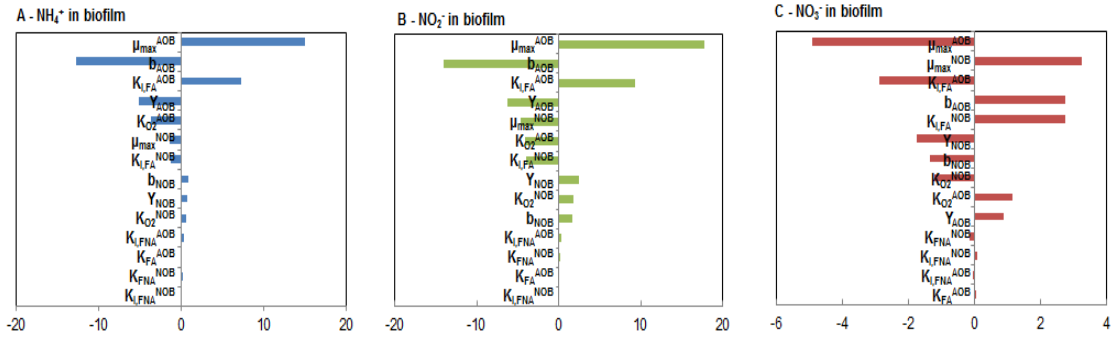


Figure 5.1 Sensitivity ranking of kinetic parameters with default values, considering the individual reactor performance in terms of ammonium (A), nitrite (B) and nitrate (C) in biofilm. As the sensitivity regarding performance within the biofilm was higher than the bulk performance, sensitivity ranking considering the individual reactor performance in bulk phase was not shown.

Based on sensitivity analysis results, microprofiles of NH_4^+, NO_2^-, NO_3^- and DO were used to estimate sensitive parameter(s). Parameter estimation was carried out by trial and error through adjusting parameter values one by one

to minimize the fitting error. Root mean squared error was used to assess the quality of model-data fit as the objective function,

$$RMSE = \sqrt{average(\sum_j \sum_i (\frac{y_{model,i,j} - y_{meas,i,j}}{y_{meas,j,average}})^2)} \qquad (5.5)$$

where *j* is the targeted variable measured or estimated (NH_4^+, NO_2^-, NO_3^- and DO), *i* is a sample point along biofilm depth (*i* = 20). Parameter estimation continued until model predicted consistent profiles in different scenario validations, including microprofiling measurements at steady state (N species and DO), bulk performance in a batch test and at steady state (N species, DO and pH). The calibrated parameters (μ_{max}^{AOB} and μ_{max}^{NOB}) **(Paper I)** were within the reported ranges in the study of Vannecke and Volcke (2015).

5.2 NOB suppression under intermittent aeration

NOB suppression is the consequence of indirect and direct (competitive) interactions between AOB and NOB. Net microbial competitiveness is captured in the specific growth rates, meaning that microbial types with the highest specific growth rate outcompete those slow growers. To investigate underlying reasons of NOB suppression associated with intermittent aeration, the calibrated model was used to simulate a fully nitrifying biofilm which was then subject to intermittent aeration (Int_{6+6} in this study). Profiles of specific growth rates of AOB and NOB during an aeration cycle (6 hours) showed μ variations with space and time (Figure 5.2A). Microbial growth processes only occurred in the first 100 μm at the biofilm base, i.e. the effective DO penetration depth. Both growth rates were relatively low with the onset of aeration, and then increased gradually, suggesting lag phases of AOB/NOB activities after anoxic conditions. Moreover, the average ratio of μ_{AOB} to μ_{NOB} was higher under intermittent aeration compared to continuous aeration, indicating NOB suppression.

Individual influence of DO and pH-related effect on AOB/NOB activities was calculated (Figure 5.2B). Lag phases of microbial activities were caused by strong FA inhibition, especially for NOB, which are more sensitive to FA inhibition than AOB (Anthonisen *et al.*, 1976; Vadivelu *et al.*, 2007). DO limitation effect, pH-enzyme effect and FNA inhibition were insignificant in differentiating kinetically between AOB and NOB. The extremely high FA inhibition at the onset of aeration was due to transient pH upshifts at the biofilm base in the previous air-off phase (Figure 5.3A). When N_2 gas flowed through the membrane lumen, CO_2 continuously diffused from the biofilm

base to the gas phase, resulting in alkalinity accumulation and pH increase (**Paper I**). As aeration continued, pH decreased. Biofilm pH transitions upon air on/off switches were predicted by model simulations (**Paper I**), and also in-situ experimentally detected and recorded (Figure 5.3B) (**Paper II**).

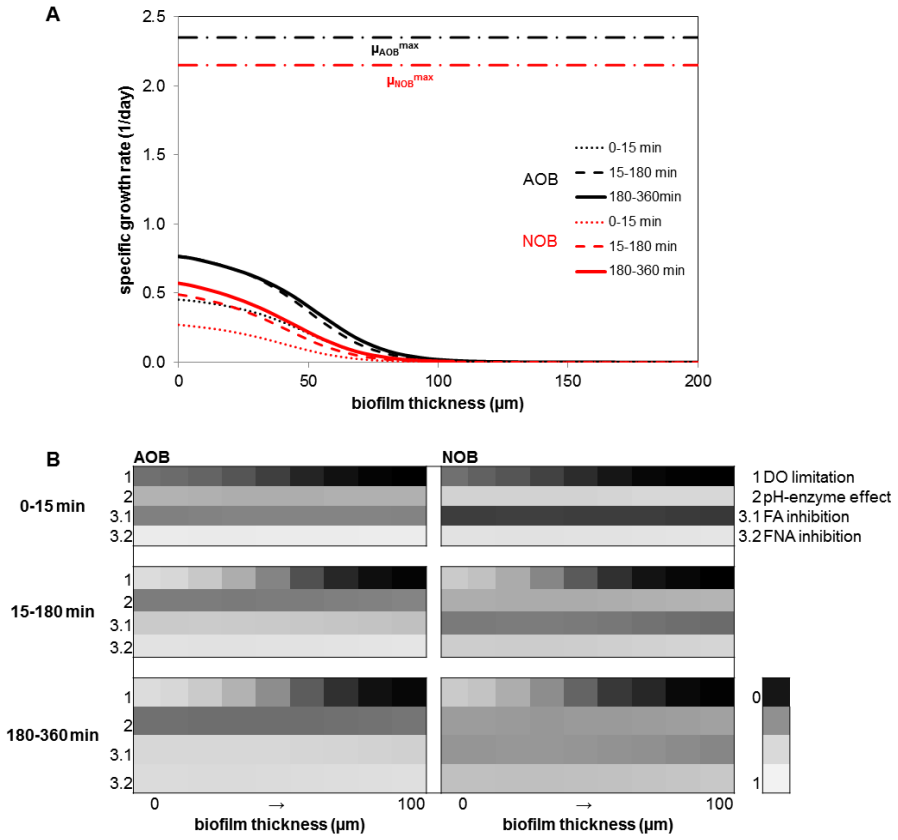


Figure 5.2 (A) Predicted specific growth rates of AOB and NOB within the biofilm during the air-on period (AOB- black, NOB- red); (B) Individual effect on AOB and NOB within the 100μm-aerated biofilm base in during air-on periods. (0- strong limitation/inhibition effect, 1- no limitation/inhibition effect)

Aiming at an effective nitritation control in counter-diffusion systems via aeration control, model simulations further proposed aeration duration and aeration intermittency as main controlling factors for reactor performance (**Paper I**). Specifically, longer aeration duration ensured a higher NH_4^+ removal efficiency, yet impeded NOB suppression. Higher aeration intermittency presented unchanged NH_4^+ removal performance, while its effect on NOB suppression was evaluated under specific conditions. Following this model-based analysis, experimental validation of model predictions was carried out (**Paper II**).

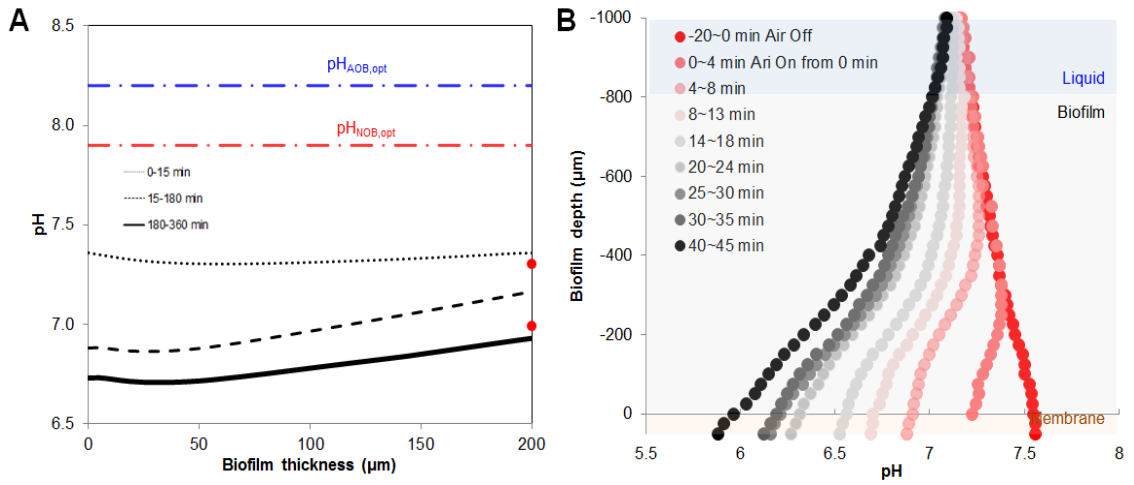


Figure 5.3 (A) Predicted dynamic changes of pH in biofilm at different time intervals during air-on phases, and red dots represent concentrations in the bulk (Paper I). (B) In-situ measurements of dynamic pH profiles in biofilm at different time points during the aeration switch from air off to air on phase (Paper II).

5.3 Counter- versus Co- diffusion biofilms

Counter- and co-diffusion biofilms have different spatial structures and population distributions (Nerenberg, 2016; Wang *et al.*, 2009). Therefore, NOB suppression in these two different biofilm-type systems presents different mechanisms and challenges (Lackner and Smets, 2012) (**Paper I**). First, the inherent system geometry of counter-diffusion biofilms complicates NOB washout. Different from conventional biofilms, active bacteria thrive at the base of a counter-diffusion nitrifying biofilm, where they utilize oxygen supplied from the membrane lumen. Growth of bacteria at the biofilm base limits the chance for outcompetition, and the overlying biofilm layers protect NOB from being washed out of the system. Secondly, the inherent chemical distribution in counter-diffusion biofilms challenges NOB inhibition. In established counter-diffusion biofilms, both S_{O2}/K_{O2} and S_{FNA}/K_{FNA} have the highest values at the biofilm base, which is a theoretically optimal habitat for NOB growth. However, the biofilm base is not optimal for AOB growth, as S_{O2}/K_{O2} and S_{FA}/K_{FA} may not be at their maximum at the same spatial position. Conversely, NOB share optimal habitats with AOB at the biofilm top near the biofilm/liquid interphase in co-diffusion biofilms, making NOB outcompeting relatively easier than in counter-diffusion systems. These considerations might explain the observations of Wang et al. (2009) that NOB survived better in counter- versus co- diffusion biofilm reactors, even when operated under constant oxygen limited (DO < 0.1 mg/L) and high pH (8.0-8.3) conditions in the bulk.

As to aeration control, e.g. intermittent aeration, it exerts a significant effect on NOB dynamics in counter-diffusion biofilms due to periodic pH variations at the biofilm base. However, such pH variations are not expected in co-diffusion biofilms. In co-diffusion systems, there is no continuous CO_2 stripping into the gas phases but sufficient buffer capacity in the liquid phase.

DO limitation effect on NOB suppression in counter-diffusion biofilms appears not as substantial as reported for co-diffusion biofilms (**Paper I and Paper II**). In counter-diffusion systems, Pellicer-Nàcher et al. (2010) found that nitritation could not be achieved by solely reducing air pressure in the membrane lumen, and Lackner and Smets (2012) concluded that nitritation efficiencies could not be predicted by DO limitation effects in N-rich wastewater treatment. However, DO limitation is considered as the main mechanism for NOB suppression in co-diffusion biofilm reactors, and it is strongly required for maintaining long-term nitritation performance (Park *et al.*, 2010; Chung *et al.*, 2007; Park *et al.*, 2015; Brockmann and Morgenroth, 2010).

In conclusion, a pH-explicit 1-D biofilm model was developed to describe the counter-diffusion nitrifying process in MABRs. The model predicted strong periodic shifts in the spatial gradients of DO, pH, FA and FNA, associated with air-on and air-off periods under intermittent aeration. Moreover, upon aeration switches, the stabilization of transient pH in biofilm was in a similar but much slower pattern than DO stabilization. Therefore, NOB suppression under intermittent aeration was mostly explained by the periodic FA inhibition. In counter-diffusion biofilm, pH effects are more important than DO limitation effect on nitritation process (**Paper I**).

6 Modeling study 2: N_2O dynamics in PNA MABRs

As introduced above, AOB can produce N_2O via two pathways: one is associated with nitrifier nititrification (NN pathway) and the other is associated with nitrifier denitrification (ND pathway) (Kozlowski *et al.*, 2016; Wunderlin *et al.*, 2012), and HB produce N_2O via incomplete denitrification (HD pathway) (Itokawa *et al.*, 2001; Hiatt and Grady, 2008). N_2O production is extremely variable within WWTPs and depends on many operational parameters, such as DO and NO_2^- concentrations (Béline, 2002; Kampschreur *et al.*, 2008), pH (Law *et al.*, 2011), carbon availability (Itokawa *et al.*, 2001) and rapidly changing process conditions (Schreiber *et al.*, 2009; Kampschreur *et al.*, 2008). It remains a challenge to identify the key factors that regulate N_2O production for any given process and to infer mitigation options. Among the operational strategies, proper aeration control has proven to be able to reduce total N_2O emissions from a full-scale activated sludge SBR system (Rodriguez-Caballero *et al.*, 2015), or a one-stage autotrophic N removal biofilm (Pellicer-Nàcher *et al.*, 2010). Therefore, MABR seems to be a potential technology for N_2O mitigation, due to the flexible air supply (Syron *et al.*, 2015; Nerenberg, 2016), Additionally, counter substrate supply itself can minimize N_2O production in MABRs due to the unique microbial stratification in biofilms **(Paper II).** For instance, Kinh et al. (2017) compared N_2O emissions from simultaneous nitrification and denitrification process between MABRs and conventional biofilm reactors, and found zones for N_2O production and consumption were adjacent only in MABR biofilms. Therefore, N_2O emissions from counter-diffusion biofilm systems deserve in-depth investigations. Here a 1-dimentional biofilm model was developed, based on the biological N_2O models of Domingo-Félez and Smets (2016) and Hiatt and Grady (2008). The model was used to investigate the possible mechanisms of N_2O production in counter-diffusion biofilm, and the potential reasons of observed N_2O emission mitigation under intermittent aeration (**Paper II)**. Additionally, the roles of HB and AnAOB in N_2O productions in NH_4^+-removing MABRs were analyzed.

6.1 Model description

6.1.1 1-D partial PNA biofilm model with N_2O processes

The developed multiple species nitrifying biofilm model of Ma et al. (2017) was extended to describe biological N_2O dynamics. The N_2O biofilm model included six particulates: four active microbial groups (X_{AOB}, X_{NOB}, X_{AnAOB} and X_{HB}), slow biodegradable organic matter (X_S) and inerts (X_I), and twelve soluble compounds (S) (**Paper III)**. A physical diffusion link was defined to describe the gas transport between gas compartment and the biofilm substratum for five gaseous compounds (S_{NO}, S_{N2O}, S_{CO2}, S_{N2} and S_{O2}). Membrane transfer rate of oxygen ($k_{m,o2}$) was measured at 2.5 m/day in a clean water test following the method of Pellicer-Nàcher et al. (2013). Transfer rates of other gases were approximated based on the gas diffusivity ratios (Spérandio and Paul, 1997).

To describe AOB-driven N_2O productions (NN and ND pathways), the AOB growth was modeled as a two-step process, as suggested by Domingo-Félez and Smets (2016). Anoxic growth of HB was described as a 4-step denitrification process (Hiatt and Grady, 2008), where N_2O can be either produced or consumed via heterotrophic denitrification (HD pathway). Local pH within the biofilm was calculated based on proton consumption and production (Ma *et al.*, 2017). Protons are produced in the oxidation of NH_4^+ to NO_2^- and the assimilation of NH_3 by biomass (Henze *et al.*, 2000; Hiatt and Grady, 2008). A net production of 2 protons occurs in NH_4^+ oxidation: we assume one proton is released during NH_3 speciation from NH_4^+ forming the true substrate, and the other one is released when produced HNO_2 is deionized forming NO_2^- as the main species of total nitrite nitrogen in the system. Protons are consumed in denitrification process, the ammonification of soluble organic nitrogen and the acid-base reactions with bicarbonate buffer, respectively (**Paper III)**. Proton consumption during denitrification is assigned to the reduction of NO_2^- to NO (Hiatt and Grady, 2008). Two pH effects were considered: (1) Direct pH effect: pH can affect microbial activity directly by changing enzyme reaction mechanisms (Van Hulle *et al.*, 2007; Henze *et al.*, 2000). (2) Indirect pH effect: pH determines FA/FNA speciation from total NH_4^+/NO_2^-, and FA/FNA is modeled as true substrate and inhibitor in nitrifier growth processes. The speciation between ionized/un-ionized species was assumed at instantaneous equilibrium, and calculated with dissociation equilibrium constants of NH_4^+ and HNO_2 (**Paper I)**.

6.1.2 Parameter estimation and model evaluation

A local sensitivity analysis was performed to identify the most sensitive parameters for extimation. Default parameter values were taken from ASMN (Hiatt and Grady, 2008), with AOB-related N_2O parameter values from Domingo-Félez et al. (2017), FA and FNA inhibition constants for AOB/NOB growth from Park et al. (2010), and AnAOB-related parameters from Strous et al. (1999). The model was run in continuous aeration for 500 days to achieve a stable nitrifying biofilm. Sensitivity of reactor performance was evaluated at different time points (day 50, 100 and 500). The averaged values were considered in the sensitivity rankings, in terms of bulk N species (NH_4^+, NO_2^-, NO_3^- and N_2O), and biofilm DO and N_2O at the membrane/biofilm interface (**Paper III)**. Regarding N conversions (not consider N_2O emissions), the most sensitive kinetic parameters were maximum growth rates for AOB ,NOB and AnAOB, consistent with sensitivity analysis in other studies of nitrifying biofilms (Wang *et al.*, 2009)**(Paper I)**. However, kinetic parameters of HB (μ_{HB}^{NOS}, $K_{S,HB}^{NOS}$, μ_{HB}^{NIR} and $K_{S,HB}^{NIR}$) significantly affected N_2O productions.

Parameters were estimated using the simplex algorithm (Nelder and Mead, 1965), with a two-step estimation procedure (Domingo-Félez *et al.*, 2016). Bulk performance of NH_4^+, NO_2^- and NO_3^- was fitted first; then, bulk N_2O was fitted by estimating extra sensitive parameters. The most sensitive parameter was preliminary selected, and the size of the parameter subset to be calibrated was increased one by one until the fitting error could not be further minimized (**Paper III**). All the calibrated parameters were bounded in the reported uncertainty ranges (Domingo-Félez *et al.*, 2017; Boiocchi *et al.*, 2017).

The calibrated model could predict the bulk N changes from continuous to intermittent aeration (Figure 6.1): NH_4^+ increased, while NO_3^- and NO_2^- decreased; NO_2^- did not accumulate in the bulk liquid phase after NO_2^- addition (5 mg-N/L) into the influent from day 95. Predicted bulk N_2O decreased at the onset of intermittent aeration, in agreement with experimental observations. Predicted biofilm N_2O was overall a bit lower than measured values under continuous aeration, while slightly higher under intermittent aeration, when the N_2O kinetic parameters were bounded in the reported ranges (Domingo-Félez *et al.*, 2017; Boiocchi *et al.*, 2017). Errors in the biofilm N_2O fitting might result from the limitation of a local sensitivity performed in Aquasim, which the sensitivity rankings depend on the parameter values and

do not capture parameter interactions. Therefore, a 1-D biofilm model was developed in the Matlab-Simulink environment (The MathWorks, Natick, MA) describing the counter-diffusion PNA process (**Appendix I**), enabling an improved model calibration/evaluation for the highly variable N_2O emissions. Nevertheless, simulated biofilm N_2O profiles followed the observed varying patterns: it decreased from continuous to intermittent aeration; under continuous aeration N_2O displayed higher concentration at the biofilm top, compared to the biofilm base; under intermittent aeration, N_2O was mainly produced during air-on periods, rather than air-off periods.

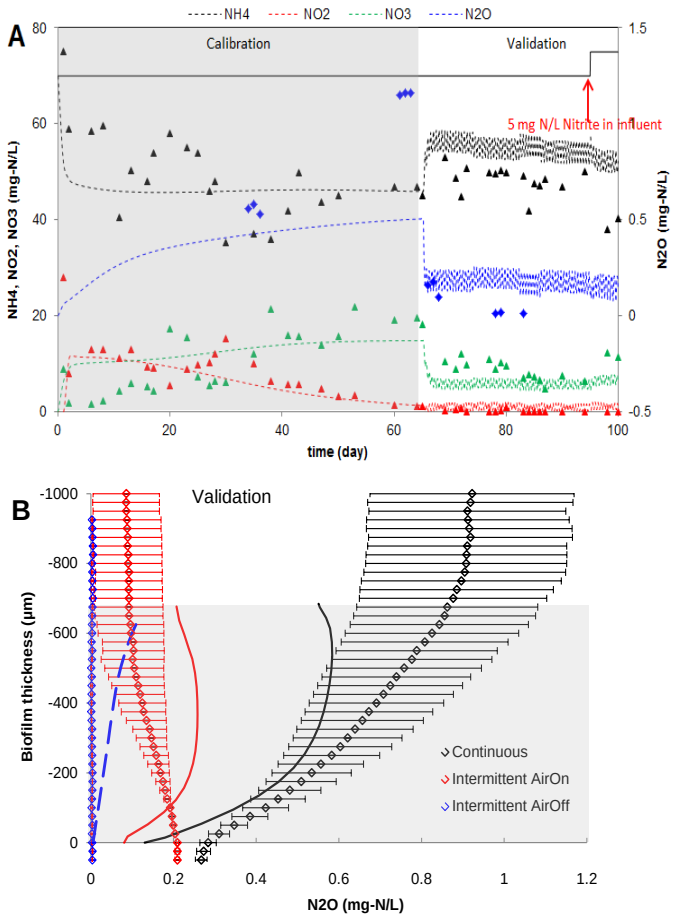


Figure 6.1 Experimental data (discrete symbols) and predicted (line) concentrations in the MABR: (A) bulk N species of NH_4^+, NO_2^-, NO_3^- and N_2O during model calibration (continuous aeration: from day 0 to 65) and model validation (intermittent aeration: from day 65 to 100). From day 95 on, 5 mg-N/L of NO_2^- was added in the influent. (B) Model validation: predicted N_2O profiles in the biofilm under continuous aeration (day 60), and intermittent aeration (air-on period: day 80.1and air off period: day 80.4). Averaged microprofiles under each aeration model were shown.

6.2 N_2O production: continuous versus intermittent aeration

To evaluate the contribution of each production pathway to total N_2O emissions, the volumetric N_2O reaction rates were plotted within the biofilm (Figure 6.2). The following were observed: (1) under continuous aeration, N_2O was produced throughout the whole biofilm, mainly via the ND pathway (ND> HD> NN); (2) under intermittent aeration (air-on periods), N_2O production via denitrifying processes decreased from both AOB- and HD-driven pathways, while the production rates via NN pathways was not significantly changed; (3) under intermittent aeration (air-off periods), N_2O was consumed in HD pathways, and there was zero production from AOB-driven pathways.

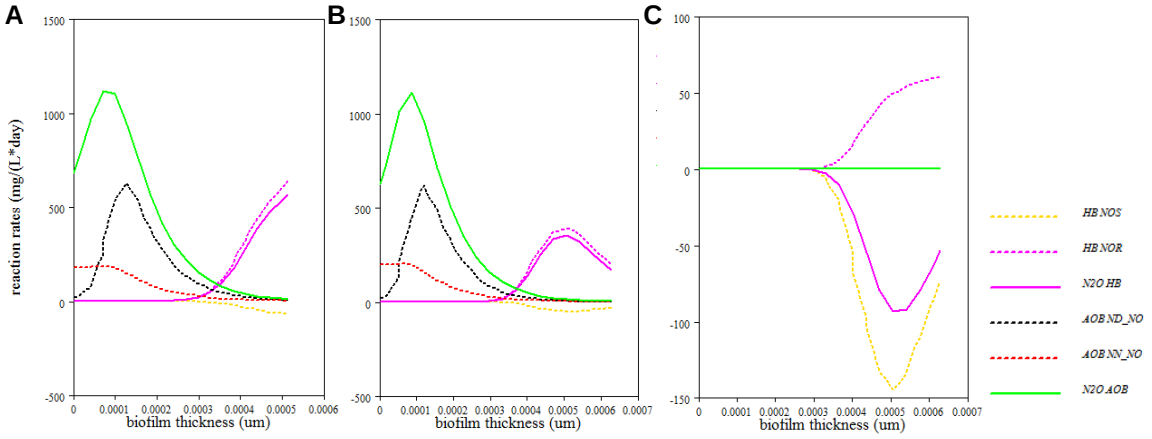


Figure 6.2 N_2O production rates (g N_2O_N/(m^3·day) from each pathway: (A) at day 50- continuous aeration, (B) at day 80.1- air on period of intermittent aeration, and (C) at day 80.4- air off period of intermittent aeration.

Overall, N_2O emission was reduced under intermittent aeration in MABRs. Pellicer-Nàcher et al. (2010) also observed that via intermittent aeration, 100-fold lower N_2O was emitted in a one-stage PNA MABR, compared to other autotrophic N removal systems. Simulation results suggested that N_2O reduction was mainly due to the decreased production in ND and HD pathways and increased consumption in HD pathway under air-off periods, as the substrate (NO_2^-) of denitrifying process was prior consumed by AnAOB which was activated under intermittent aeration (**Paper II)**. Aeration control has been utilized to reduce N_2O emissions in lab-scale and full-scale N removal systems (Béline, 2002; Kimochi et al., 1998; Rodriguez-Caballero et al., 2015, Domingo-Félez et al., 2014). Béline (2002) attributed the emissions minimization to the consumption of N_2O in heterotrophic denitrification during an-

oxic conditions. Our study additionally shows that aeration control further reduced production from nitrifier denitrification.

6.3 Model-based exploration

6.3.1 HB affects N_2O emissions

Sensitivity analysis showed that HB had a minor contribution to the overall NH_4^+ or total nitrogen removal in nitrifying MABRs, in agreement with the results based on a mass-balance approach **(Paper II)**. Nevertheless, HB had a significant impact on N_2O emissions in the biofilm system. HB can develop N_2O production hotspots at anoxic biofilm area, both in autotrophic N removal systems (Okabe *et al.*, 2011) or simultaneous nitrification and denitrification systems (Kinh *et al.*, 2017). Furthermore, HB can establish a potential N_2O-reduction zone to mitigate total emissions **(Paper II&III)**. Kindaichi et al. (2004) reported heterotrophs composed 50% of the total bacteria in autotrophic nitrifying biofilms. Therefore, N_2O models without HB considered might provide misleading conclusions (Domingo-Félez et al., 2016).

6.3.2 AnAOB affects N_2O emissions

Intermittent aeration is not only an efficient way to suppress NOB to achieve nitritation (Ma *et al.*, 2017; Pellicer-Nàcher *et al.*, 2010; Katsogiannis *et al.*, 2003), but also a strategy to promote AnAOB activity (Yang *et al.*, 2015; Zekker *et al.*, 2012)**(Paper II).** AnAOB consumed NO_2^- which otherwise would accumulate due to NOB suppression under intermittent aeration, thus reducing N_2O production via denitrifying-related pathways. The role of AnAOB in N_2O dynamics was also highlighted in an autotrophic N removal system of Gilmore et al. (2013) that the total emissions were reduced from 10% of the removed N load to almost zero after the proliferation of AnAOB in the system.

As to the AnAOB activity in MABRs, different effects on N_2O emissions can be distinguished associated to the NH_4^+ and NO_2^-consumptions during AnAOB growth. (1) NH_4^+ is consumed at the biofilm top where AnAOB are active, resulting in the decreased NH_4^+ flux towards the biofilm base where AOB grow. Therefore, N_2O production via AOB-driven pathways can be affected, as N_2O production rates were observed to increase with the increasing NH_4^+ oxidation rates in a AOB enriched culture (Law *et al.*, 2011). (2) NO_2^-

is also consumed by AnAOB, scavenging the residual NO_2^- at the anoxic biofilm area and in the bulk liquid phase. Since total N_2O emissions directly relate to NO_2^- concentrations (Okabe *et al.*, 2011; Kampschreur *et al.*, 2008), N_2O production via denitrifying pathways might be reduced as a result of NO_2^- consumption by AnAOB.

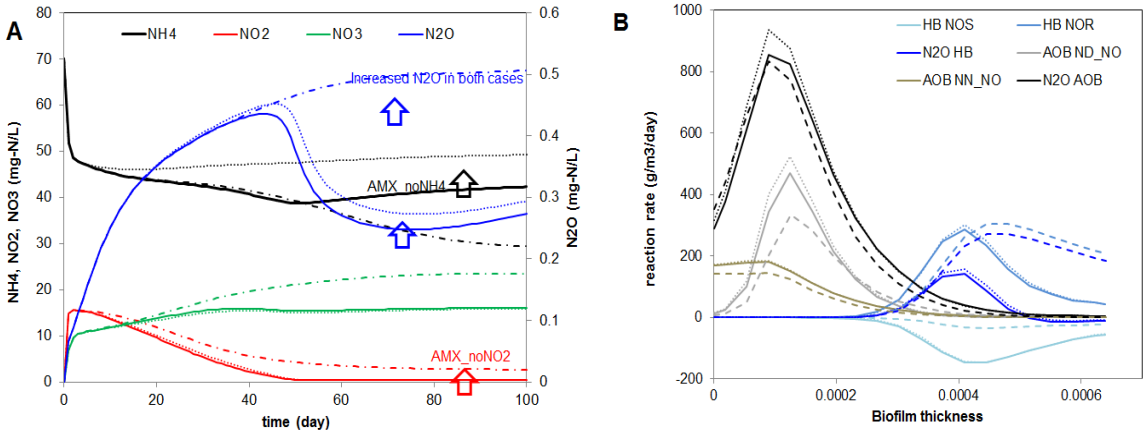


Figure 6.3 Simulation results of different stoichiometry in AnAOB reactions: (A) bulk NH_4^+, NO_2^-, NO_3^- and N_2O, (B) production rates of each N_2O pathway (g N_2O_N/(m^3·day) at day 100. After an increase of AnAOB composition for the model initialization, simulations were run in parallel, with the only difference in the stoichiometry of AnAOB reactions: conventional AnAOB (solid line), AnAOB reaction without NH_4^+ consumption- $AnAOB_{nonh4}$ (dotted line), AnAOB reaction without NO_2^- consumption- $AnAOB_{nono2}$ (dashed line).

Finally, the model was run with different stoichiometry of AnAOB reactions, to test and evaluate the effects of reduced NH_4^+ and NO_2^- fluxes on N_2O dynamics (Figure 6.3). First, the initial AnAOB composition during model simulations was increased to initializae a PNA biofilm system. Then the model was run in parallel but with different AnAOB reactions: (a) conventional stoichiometry, (b) AnAOB without NH_4^+ consumption- $AnAOB_{nonh4}$, (c) AnAOB without NO_2^- consumption- $AnAOB_{nono2}$. Both simulations of $AnAOB_{nonh4}$ and $AnAOB_{nono2}$ showed higher N_2O emissions, compared to the simulation of conventional AnAOB. $AnAOB_{nonh4}$ simulation showed increased N_2O production via AOB-driven pathways, indicating that high AOB activity might increase N_2O production (Blum *et al.*, 2018). $AnAOB_{nono2}$ simulation showed increased N_2O production in HD pathway resulting from the accumulated NO_2^- (Wunderlin *et al.*, 2012). But AOB-driven N_2O productions slightly decreased, which was probably due to the decreased AOB activity under low NH_4^+ conditions. Taken together, AnAOB had no direct but

important effects on N_2O emissions in counter-diffusion MABRs **(Paper II&III)**.

In conclusion, a 1-D multispecies partial nitritation/anammox biofilm model was developed to study the dynamics of N_2O production in counter-diffusion biofilms **(Paper III)**. The calibrated model suggested that denitrifying pathways were the main contributors to N_2O production in the NH_4^+-removing MABRs. Intermittent aeration could significantly reduce N_2O production via denitrifying pathways, while had a minor effect on production via nitrifier nitrification pathway. Moreover, the roles of AnAOB and HB in N_2O dynamic were evaluated: AnAOB played a central role in competing for the NO_2^- substrate of denitrifying processes, thus contributing to a reduction in heterotrophic, and overall, N_2O production; HB could develop N_2O-production hotspots or establish an anoxic N_2O-consumption sink in autotrophic nitrogen removal biofilm systems.

7 Conclusions

This PhD project has examined how N conversions in counter-diffusion MABRs can be affected by the aeration mode. The main findings are summarized below:

- Lab-scale MABRs were successfully operated under continuous aeration versus different degrees of intermittent aeration. MABRs were inoculated with enriched nitrifying biomass, and fed with NH_4^+ as the sole nitrogen source without external organic carbon. Under continuous aeration, a nitrifying biofilm developed in MABRs. Under intermittent aeration, NOB activity was suppressed, and nitritation was observed. NOB suppression enabled NO_2^- availability for potential AnAOB growth, thus improving NH_4^+ removal performance.
- Activities of individual microbial groups (AOB, NOB, AnAOB and HB) were successfully assessed from a mass-balance approach.
- MABR performance was assessed with NH_4^+ removal efficiency and NOB suppression, as a function of aeration mode. Under continuous aeration, high NH_4^+ removal efficiency but no NOB suppression was achieved. As to intermittent aeration, both the relative aeration duration and the aeration intermittency were effective operational factors in controlling the performance: NH_4^+ removal efficiency was favored with long aeration duration and high aeration intermittency, while the degree of NOB suppression increased with short aeration duration.
- NOB suppression, associated with intermittent aeration, was likely governed by periodic FA inhibition as a consequence of transient pH upshifts during air-off periods. These pH upshifts were experimentally confirmed and can be explained by alkalinity increases due to CO_2 stripping to the membrane lumen together with the cessation of proton production of nitritation. Upon the aeration switches under intermittent aeration, stabilization of pH within the biofilm lagged behind DO stabilization. This is the first experimental observation of transient pH variations in MABRs.
- Modeling results suggest that DO limitation (evaluated by Monod-type kinetics) or oxygen supply limitation was not responsible for NOB suppression in the studied MABRs.
- Total N_2O emissions from the MABRs were significantly reduced by switching from continuous to intermittent aeration. This is likely due to the establishment of the anoxic N_2O-reduction zone by HB. Under inter-

mittent aeration, NO_2^-, a substrate of denitrifying N_2O production process, did not accumulate in anoxic biofilm or the liquid phase, but was consumed by activated AnAOB. Both experimental observation and model-based evaluation suggested that AnAOB played a central role in competing for the substrate of denitrifying HB to prevent N_2O production in autotrophic nitrogen removal biofilms.

- Model-based evaluations suggest that denitrifying pathways were the main contributor to N_2O production in the MABRs in the presence of residual bulk NO_2^-. Intermittent aeration reduced N_2O production via denitrifying pathways significantly, but had minor effect on production from nitrifier nitrification pathway. In relation to N_2O production, even in an autotrophic nitrogen removal biofilm, the role of HB cannot be neglected- denitrifying bacteria could develop N_2O production hotspots or establish anoxic N_2O-reduction zones in biofilms.
- A simple method of pH calculation was developed and incorporated in a biofilm model, which predicted the spatial and temporal biofilm pH variations under intermittent aeration. Effects of pH and DO influencing factors on AOB/NOB competition were comprehensively evaluated.
- A mathematical model, extended with all known biological N_2O production pathways, was developed and evaluated against experimental N_2O measurements. Dynamic N_2O productions in MABRs under aeration control were analyzed, and contributions of each production pathway (NN, ND and HD) to total N_2O emission were evaluated. Additionally, 1-D biofilm model in Matlab software was developed, enabling an improved model calibration/evaluation for the high variable N_2O emissions.
- The counter-diffusion biofilm models develop in this study contribute to the further optimization of MABR technology for environmental applications.
- Aeration control is an efficient strategy to regulate nitrogen microbial activities in counter-diffusion MABRs: by imposing intermittent aeration, high nitritation efficiencies with low N_2O emissions can be achieved.

8 Future perspectives of N removal in MABRs

In this study, I utilized a mass-balance based approach to evaluate the activities of different microbial guilds, including AOB, NOB, AnAOB and HB. We found a decreased in the amount of NO_2^- oxidized to NO_3^- under intermittent aeration, and attributed it to the suppression of NOB activity. However, the overall decrease of NO_2^- oxidation could be caused by the suppression of a certain NOB species, or due to a changes in the NOB composition, e.g. from Nitrobacter spp. to Nitrospira spp. Therefore, **abundance quantification of different microbial species** throughout the whole regulation process would provide more detailed information. If a certain NOB species is robust throughout the operation of aeration control, we can make a critical choice of the inoculum biomass, as it can affect the community composition and nitritation performance of a nitrifying biofilm from the beginning (Terada *et al.*, 2006b).

N_2O emission is known to be extremely variable and dependent on many operational conditions. Mathematical modeling offers a simple way to describe and evaluate N_2O productions, but still requires a large number of parameters (72 in the studied model here) for a partial nitritation/anammox biofilm system. Currently, N_2O modeling efforts focus on evaluating the capability of model structure to describe N_2O production with best-fit simulations (Ni *et al.*, 2013; Pocquet *et al.*, 2016) **(paper III)**. However, **a rigorous calibration framework** will be needed and to be followed to achieve an improved quality of N_2O calibration results. For instance, the approach to properly select the subset of parameters for model calibration can play a crucial role on simulation results. With increasing computational power, a global sensitivity analysis is recommended to capture the interactions between parameters with respect to model outputs. A counter-diffusion biofilm model built in the Matlab-simulink environment will make the improved model calibration feasible (**Appendix I**).

We observed that HB in autotrophic nitrifying biofilms have minor influence on the removal performance of NH_4^+ or total N, or in the competition between AOB and NOB. However, HB significantly affect N_2O dynamics. Thus, further investigation on **the organic carbon flux in a biofilm community** would be desirable, especially in relation to the autotrophic biofilm pro-

cesses, as low C-to-N conditions stimulates N_2O productions (Domingo-Félez *et al.*, 2016) **(paper II)**.

Transient accumulation of N_2O occurs upon perturbations of surrounding environment (Schreiber *et al.*, 2009; Kampschreur *et al.*, 2008), such as the aeration switches between air-on and air-off periods in this study. Extra dynamic parameters will be needed in N_2O production processed, to mimic the peak increase in N_2O emissions upon the transient aeration (Schreiber *et al.*, 2009; Zheng and Doskey, 2015).

As an operation factor, pH significantly affects the growth kinetics of nitrifying microorganisms (Park and Bae, 2009) **(paper I&II)**, and N_2O emissions in nitrification or denitrification stages (Law *et al.*, 2011). Therefore, an in-depth understanding of **pH effects on enzymes, pathways and organisms** involved in the N-cycle in water engineering applications deserves further investigation.

Finally, as an emerging biofilm technology, MABRs are approaching maturity and are now available at the commercial scale (Nerenberg, 2016). The strategy of aeration control proposed in this study is ready to be tested and verified in **pilot-scale implementations** of autotrophic NH_4^+ removal treatment.

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10 Papers

I **Ma, Y.**, Domingo-Félez, C., Plósz, B.G., Smets, B.F., 2017. Intermittent Aeration Suppresses Nitrite-Oxidizing Bacteria in Membrane-Aerated Biofilms: A Model-Based Explanation. Environ. Sci. Technol. 51, 6146–6155.

II **Ma, Y.**, Piscedda, A., Smets, B.F., Nitrogen conversion in membrane-aerated biofilm reactors affected by intermittent aeration. *Submitted to Water Research*.

III **Ma, Y.**, Valverde-Pérez, B., Picioreanu, C., Smets, B.F., Intermittent aeration can reduce denitrification-related N_2O production in membrane aerated nitrifying biofilms: Results from a modeling study. *Manuscript in preparation*.

11 Appendices

I A N_2O partial nitritation/anammox biofilm model for MABR (Matlab model)

The Department of Environmental Engineering (DTU Environment) conducts science based engineering research within six sections: Water Resources Engineering, Water Technology, Urban Water Systems, Residual Resource Engineering, Environmental Chemistry and Atmospheric Environment.

The department dates back to 1865, when Ludvig August Colding, the founder of the department, gave the first lecture on sanitary engineering as response to the cholera epidemics in Copenhagen in the late 1800s.

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