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Department of Environmental Engineering

PhD Thesis
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Preface

This thesis is based on the PhD project carried out at the Department of Environmental Engineering of the Technical University of Denmark from January 2011 to December 2014. The project was conducted under the supervision of Barth F. Smets (DTU Environment), co-supervised by Gürkan Sin (DTU Chemical and Biochemical Engineering). The research was supported financially by the Danish Strategic Research Council through the Centre for Design of Microbial Communities in Membrane Bioreactors (EcoDesign-MBR).

The thesis is organized in two parts: the first part puts into context the findings of the PhD in an introductive review; the second part consists of the papers listed below. These will be referred to in the text by their paper number written with the Roman numerals **I-IV**.

I **Mutlu, A. G.**, Vangsgaard, A. K., Sin, G., Smets, B. F. (2013). An operational protocol for facilitating start-up of single-stage autotrophic nitrogen-removing reactors based on process stoichiometry. *Water Science and Technology*. **(68)** 3: 514-521.

II **Mutlu, A. G.**, Vangsgaard, A. K., Sin, G., Smets, B. F. (2015). Spatial and temporal differentiation of microbial composition and activity during long term performance enhancement in two single-stage nitritation/anammox sequencing batch reactors. *Manuscript in preparation.*

III **Mutlu, A. G.**, Lv, C., Domingo-Félez, C., Gülay, A., Smets, B. F. (2015). Impact of aeration regimes on activity, community composition and biomass architecture in sequencing batch reactors performing single-stage nitritation/anammox. *Manuscript in preparation.*

IV Domingo-Félez, C., **Mutlu, A. G.**, Jensen, M. M., Smets, B. F. (2014). Aeration strategies to mitigate nitrous oxide emissions from single-stage nitritation/anammox reactors. *Environmental Science and Technology*. **(48)** 15: 8679-8687.

In this online version of the thesis, the papers are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from DTU Environment, Technical University of Denmark, Miljøvej Building 113, 2800 Kgs. Lyngby, Denmark, reception@env.dtu.dk.

The following authored and co-authored publications closely related to the topic of the thesis were also conducted during this PhD study, but are not explicitly considered here:

Journal articles:

Vangsgaard, A. K., **Mutlu, A. G.**, Gernaey, K., Smets, B. F., & Sin, G. (2013). Calibration and validation of a model describing complete autotrophic nitrogen removal in a granular SBR system. Journal of Chemical Technology and Biotechnology, 88(11), 2007-2015. 10.1002/jctb.4060

Pellicer i Nàcher, C., Domingo Felez, C., **Mutlu, A. G.**, & Smets, B. F. (2013). Critical assessment of extracellular polymeric substances extraction methods from mixed culture biomass. Water Research, 47(15), 5564-5574. 10.1016/j.watres.2013.06.026

Popular science:

Vangsgaard, A. K., Gernaey, K., Sin, G., **Mutlu, A. G.**, & Smets, B. F. (2012). Energibesparende biologisk proces til kvælstoffjernelse i spildevand. Dansk Kemi, 93(10), 16-18.

Presentations at international conferences:

Mutlu, A. G., Domingo Felez, C., Vangsgaard, A. K., & Smets, B. F. (2013). Nitrous oxide and nitric oxide emissions from single-stage nitritation/anammox reactors under varying aeration regimes. *Oral presentation.* 86th Annual Water Environment Federation Technical Exhibition and Conference (WEFTEC), Chicago, USA.

Mutlu, A. G., Vangsgaard, A. K., Chen, L., Domingo Felez, C., Sin, G., & Smets, B. F. (2013). Driving towards stratified aggregation in single-stage nitritation/anammox reactors by varying aeration regimes. *Poster presentation (Best Poster Award).* IWA 9th international Conference on Biofilm Reactors, Paris, France.

Gülay, A., Pellicer i Nàcher, C., **Mutlu, A. G.**, Jensen, M. M., Vlaeminck, S., Lackner, S., Terada, A., Sørensen, S. J., Hansen, L., Al-Soud, W. & Smets, B. F. (2013). Diversity of total and functional microbiome of anammox reactors fed with complex and synthetic nitrogen-rich wastewaters. *Oral presentation.* ICON3: 3rd international conference on Nitrification, Tokyo, Japan.

Vangsgaard, A. K., Mauricio Iglesias, M., **Mutlu, A. G.**, Gernaey, K., Smets, B. F., & Sin, G. (2013). Performance of an autotrophic nitrogen removing reactor: Diagnosis through fuzzy logic. *Poster presentation.* 11th IWA conference on instrumentation control and automation, Narbonne, France.

Vangsgaard, A. K., **Mutlu, A. G.**, Gernaey, K., Smets, B. F., & Sin, G. (2012). Calibration and validation of model describing complete autotrophic nitrogen removal in granular sludge *Oral presentation*. IWA Nutrient Removal and Recovery 2012, Harbin, China

Mutlu, A. G., Vangsgaard, A. K., Sin, G., & Smets, B. F. (2012). An operation protocol for facilitating start-up of single-stage autotrophic nitrogen removing reactors based on process stoichiometry. *Oral presentation.* IWA World Water Congress and Exhibition, Busan, Korea.

Mutlu, A. G., Vangsgaard, A. K., Jensen, M. M., & Smets, B. F. (2012). Architecture evolution of biomass aggregates in single stage nitritation/anammox reactors. *Poster presentation.* 14th International Symposium on Microbial Ecology (ISME), Copenhagen, Denmark.

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December 31st, 2014, Kongens Lyngby

Ayten Gizem Mutlu

Summary

Completely autotrophic nitrogen removal from nitrogen-rich wastewaters through the nitritation -plus- anaerobic ammonium oxidation processes can greatly reduce operational energy costs compared to traditional nitrogen removal processes. The footprint can be further reduced by process intensification in single-stage reactors. Single-stage reactors require biofilms or bioaggregates to provide the complementary redox niches for the aerobic and anaerobic bacteria that are required for nitritation and anaerobic ammonium oxidation (anammox), respectively. The nitritation/anammox process might not only reduce aeration and carbon requirements but also reduce emissions of the greenhouse gas nitrous oxide. Successful performance of the intense energy-efficient nitritation/anammox process requires a rather narrow operational window. Outside of this window, disproportionate activities of the involved functional guilds and emergence of undesired guilds can rapidly deteriorate the performance, which will offset the reduced footprint and stability. Hence, robust operational strategies that incorporate microbial process understanding are necessary.

In this work, aeration strategies were systematically evaluated as an approach to manipulate the microbial community structure, to reach efficient nitrogen removal performance, and to reduce nitrous oxide emissions from single-stage nitritation/anammox reactors. First, an iterative protocol was developed to diagnose reactor performance based on process stoichiometry and to propose actions to enhance performance based on discretized aeration parameters, restricted by an overall ratio of oxygen to ammonium loading. The protocol was successfully applied on two bioaggregate-based single-stage sequencing batch reactors during start-up; while recovering from major disturbances such as nitrite accumulation, nitrite oxidizer proliferation, ammonium starvation, and oxygen overloading; and during nitrogen loading increases. Different mitigation methods were validated or falsified ultimately improving the proposed protocol. Differences in performance and, especially, of time resolved nitrogen species dynamics, of the two parallel systems under similar aeration regimes indicated that the aggregate size distribution and microbial community architectures profoundly affected the optimal oxygen to ammonium loadings. Size-segregated aggregates consisting of exclusively aerobic or exclusively anaerobic ammonium oxidizing guilds, could achieve removal efficiencies comparable to stratified aggregates (containing both aerobic and anaerobic ammonium oxidizing guilds), at sufficiently low

oxygen to ammonium loadings. However, transient nitrite accumulation and susceptibility of anaerobic ammonium oxidizing bacteria in systems with size-segregated aggregates were considered to weaken the system robustness.

Further assessment of the interaction between aeration regime and architectural evolution of the nitritation/anammox aggregates was carried out on the two systems once they achieved steady state overall performance. With settling time, volumetric exchange ratio, sludge retention time and influent characteristics kept constant, the aeration regime, itself, caused changes in aggregate architecture and aggregate size distribution. By increasing aeration frequency, the originally size-segregated community became more redox-stratified with larger aggregates. Increasing the duration of aeration, on the other hand, did not significantly alter the original redox-stratified architecture, but allowed proliferation of unwanted nitrite oxidizing bacteria. The decrease in aeration intensity concomitant with increased duration also decreased the aggregate size. Aggregate morphology and settleability were also altered with aeration regime: increased frequencies led to compact but hollow aggregates that transiently accumulated nitrogen gas. Based on the experimental observations, a conceptual scheme was proposed to describe aggregation and architectural evolution in nitritation/anammox reactors, incorporating the possible influences of intermediates formed with intermittent aeration. Community analysis revealed an abundant fraction of heterotrophic types despite the absence of organic carbon in the feed. The aerobic and anaerobic ammonia oxidizing guilds were dominated by fast-growing *Nitrosomonas* spp. and *Ca. Brocadia* spp., while the nitrite oxidizing guild was dominated by high affinity *Nitrospira* spp.

Emission of nitrous oxide (N_2O) was evaluated from both reactors under dynamic aeration regimes. Contrary to the widely held notion that dynamic operation at low dissolved oxygen concentrations would increase nitrous oxide emissions, increasing the aeration frequencies reduced N_2O production and emission. N_2O production was observed primarily at the onset of aeration after anoxia. Nitric oxide and not free nitrous acid or nitrite correlated to production rates. The measured aerobic ammonia oxidation potential correlated to the nitrous oxide production rates. Shortening the duration of single aerated periods was an efficient way of preventing the exponential increase in N_2O production rates. Correspondingly, operating nitritation/anammox reactors under limited aerobic and excess anaerobic ammonia oxidation is recommended to minimize N_2O production and emission.

Aeration impacts the nitritation/anammox process in multiple dimensions. This study focused on the different oxygen delivery schemes, and some of the collateral impacts could be isolated, increasing process understanding. It was demonstrated that aeration strategy can be used as a powerful tool to manipulate the microbial community composition, its architecture and reactor performance. We suggest operation via intermittent aeration with short aerated periods to minimize nitrous oxide emission rates and sufficiently long non-aerated periods to suppress nitrite oxidizing bacteria. Under these conditions, redox-stratified aggregates can be established maintaining simultaneously aerobic and anaerobic autotrophic ammonium oxidation in an intensified single-stage reactor.

Nitritation/anammox processes have already been successfully applied to treat side stream reject waters, landfill leachates and industrial wastewater streams; now this process is being examined to replace or upgrade conventional treatment trains to treat domestic wastewaters under low temperatures in the presence of residual organic carbon. This work, by examining the interplay between macro- and micro-scale phenomena and processes, contributes to establishment of strategies that can be adopted in practice to operate the single-stage nitritation/anammox systems.

Dansk sammenfatning

Fuldstændig autotrof fjernelse af kvælstof (der består af delvis nitrifikation koblet med anaerob ammonium oxidation) fra spildevand med et højt kvælstof indhold kan i høj grad minimere omkostninger i form af energibesparelse sammenlignet med den konventionelle behandling af spildevand. Dette forbrug kan reduceres yderligere ved en intensivering af processen, hvor begge processer kører i samme reaktor. Dette kan lade sig gøre i systemer med biofilmdannelse eller bioaggregater, der skaber de nødvendige redox nicher for iltforbrugende og iltfølsomme mikrober, der udfører aerob og anaerob ammonium oxidation (anammox). Fuldstændig autotrof kvælstoffjernelse vil ikke kun reducere beluftningsbehovet, men også behovet for tilførsel af en ekstern kulstofkilde samt frigivelse af drivhusgassen lattergas. En god præstation af nitritation-anammox processen er betinget af et relativt snævert styringsvindue. Hvis styringsparametrene ligger uden for styringsvinduet, vil denne spildevandsbehandlingsproces forringes fordi det forårsager et disproportioneret aktivitetsniveau blandt de involverede mikroorganismer samt vækst af uønskede bakteriegrupper. Dette vil medføre en forringelse af ovennævnte fordele samt forringe stabiliteten af systemet. Der er således et behov for robuste driftstrategier, der inkluderer en forståelse for de involverede mikrobielle processer.

I denne afhandling blev forskellige beluftningsstrategier systematisk evalueret som et muligt værktøj til at manipulere den mikrobielle samfundsstruktur og til at opnå effektiv kvælstoffjernelse samt reducere lattergasfrigivelse fra en bioreaktor, hvor nitritation og anammox foregår samtidig. Som det første udviklede vi en protokol til at vurdereereaktorens status, hvilken blev baseret på processtøkiometri. Denne protokol blev brugttil at foreslå forskellige tiltag til forbedringer af processen i form af diskretiseret beluftningsparametre – dog begrænset af forholdet mellem ilt- og ammoniumtilførsel. Protokollen blev med stor succes anvendt under opstarten af to bioreaktorer (sequencing batch reactor, SBR) med biomassegranulater. Under opstart af reaktorerne var genopbygning af processen nødvendig pga. akkumulering af nitrit, en øget vækst af nitrit oxiderende bakterier, begrænsning af substrat (ammonium) samt en for høj tilførsel af ilt. Protokollen blev desuden anvendt under øgning af ammoniumtilførslen. Forskellige metoder blev valideret for at forbedre protokollen. Forskelle i hvordan de to reaktorer præsterede og især ændringer i koncentrationer af kvælstofforbindelser over tid under ensartede beluftningsforhold i de to parallelle systemer indikerede at fordelingen i aggregat-

størrelse og opbygningen af det mikrobielle samfund havde stor effekt på den optimale ilt- og ammoniumtilførsel. Ved tilstrækkelig lave ilt- til ammoniumtilførsler var størrelsessegregerede aggregater, bestående af udelukkende aerobe ammonium oxiderende mikrober eller udelukkende anaerobe ammonium oxiderende bakterier, næsten ligeså effektive i fjernelsen af kvælstof som stratificerede aggregater, som indeholdt både aerobe og anaerobe ammonium oxiderende mikrober. Både kortvarig akkumulering af nitrit og følsomheden af de anaerobe ammonium oxiderende bakterier syntes imidlertid at svække systemets robusthed.

En videre vurdering af interaktionen imellem beluftningsregime og udviklingen af aggregater med aerobe og anaerobe ammonium oxiderende bakterier i de to systemer blev udført under steady state. Ændringer i beluftningsregimet forårsagede ændringer i opbygning og størrelsesfordeling af aggregater under forhold med konstant bundfældningstid, tømningsgrad, slamalder og ammoniumtilførsel. Under en ændring i beluftningsfrekvensen ændrede det oprindeligt størrelsessegrerede samfund sig til at være redox-stratificeret med større aggregater. På den anden side førte en stigning i længden af sammenhængende beluftning ikke til nogen væsentlig ændring i den oprindelige redox-stratificering, men tillod i stedet en hurtig vækst af nitrit oxiderende bakterier. Et fald i beluftningshastighed samtidig med en stigning i længden af beluftning medførte en formindskelse af størrelsen af aggregaterne. Ligeledes skete der en forandring i morfologi og bundfældning af aggregater under denne ændring i beluftningsregimeEn øgning i beluftningsfrekvens førte til kompakte, men hule aggregater som midlertidigt akkumulerede kvælstof gas. Baseret på de eksperimentelle observationer udviklede vi en konceptuel plan til at beskrive aggregering samt arkitektonisk udvikling i bioreaktorerne, der inkorporerer de mulige påvirkninger af intermediater dannet under intermitterende beluftning. Analyse af det mikrobielle samfund afslørede en stor heterotrof fraktion til trods for at der ikke var noget organisk stof i mediet reaktorerne blev fodret med. Hurtigt-voksende *Nitrosomonas spp.* and *Ca. Brocadia spp.* var de dominerende indenfor de aerobe og anaerobe ammonium oxiderende grupper, mens *Nitrospira spp* med høj affinitet dominerede den nitrit oxiderende gruppe.

Produktionen og frigivelsen af lattergas (N_2O) blev også målt og vurderet under dynamiske beluftningsforhold i begge reaktorer. Modsat den gængse opfattelse at dynamisk drift af processen under lave iltkoncentrationer medfører en øget lattergasfrigivelse, formindskede en øget frekvens i beluftningen lattergasproduktionen og –frigivelsen. Produktionen af lattergas skete pri-

mært i begyndelsen af den oxiske fase. Kvælstofoxid korrelerede med produktionsrater, mens frit kvælstof eller nitrit ikke korrelerede med produktionsrater. Det målte potentiale for aerob ammonium oxidation korrelerede med lattergas produktionsrater. Det viste sig, at forkortelse af længden af en beluftningsperiode kan forhindre den eksponentielle stigning i lattergas produktionsraterne. Tilsvarende anbefales at man kører nitritation/anammox reaktorer med begrænset aerob ammonium oxidation og overskydende anaerob ammonium oxidation for at minimere lattergasproduktion og –frigivelse.

Beluftning påvirker nitritaion/anammox processen i adskillige dimensioner. Denne afhandling fokuserer på forskellige beluftningsstrategier. Nogle af de samtidige påvirkninger kunne isoleres, hvilketbidrog til en bedre forståelse af processen. Vi har vist at beluftningsstrategier kan bruges som et nyttigt og stærkt redskab til at manipulere sammensætningen af det mikrobielle samfund, dets arkitektur og hvordan reaktoren præseterer. For at minimere lattergasfrigivelse, foreslår vi en driftsstrategi, der omfatter intermitterende beluftning med korte beluftningsperioder og for at hæmme nitrit oxiderende bakterier, bør man overveje tilstrækkelig lange perioder, hvor der ingen beluftning er i systemet.

Fuldstændig autotrof kvækstoffjernelse bliver allerede benyttet til behandling af rejektvand fra slambehandling, perkolat fra affaldsdeponering og industrielt spildevand. Lige for øjeblikket undersøges det om processen kan erstatte eller opgradere konventionel behandling af husspildevand med et højere indhold af organisk kulstof end rejektvand ved lave temperaturer. Ved at undersøge samspillet imellem fænomener og processer i makro- og mikro-skala bidrager denne afhandling til etableringen af styringsprocedurer, der i praksis kan indføres til at køre nitritation/anammox processen i et 1-reaktor-system.

Table of contents

Abbreviations and symbols

ΔG^0	Standard change in Gibbs free energy
ΔN_2O	Nitrous oxide accumulated over Δt
ΔNH_4^+	Cyclic ammonium consumed/removed
ΔNO_2^-	Cyclic nitrite produced
ΔNO_3^-	Cyclic nitrate produced
Δt	Time interval (between 3 microsensor measurement points)
ΔTN	Total nitrogen removal/removed
μ	Specific growth rate
μ_{max}	Maximum specific growth rate
AHL	*N*-Acyl homoserine lactones
AMO	Ammonia monooxygenase
amoA	Ammonia monooxygenase subunit A encoding gene
anammox	Anaerobic ammonium oxidation
AnAOB	Anaerobic ammonium oxidizing bacteria
AOA	Anaerobic ammonium oxidizing archaea
AOB	Aerobic ammonia oxidizing bacteria
BAP	Biomass-associated products
BNR	Biological nitrogen removal
C	Carbon
Ca^{2+}	Divalent calcium cation
CANON	Completely autotrophic nitrogen removal over nitrite
CANR	Completely autotrophic nitrogen removal
c-di-GMP	Cyclic diguanylate
CO_2	Carbondioxide
CSH	Cell surface hydrophobicity
CSTR	Continuous flow stirred-tank reactor
Cy3	Cyanine dye conjuagate 3
Cy5	Cyanine dye conjuagate 5
DEMON	Deammonification
DLVO	Derjaguin, Landau, Verwey, Overbeek
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen

d_p	Particle size/diameter
eDNA	Extracellulalar deoxyribonucleic acid
EPS	Extracellulalar polymeric substances
ER	(Volumetric) exchange ratio
FA	Free ammonia
Fe	Iron
FISH	Fluorescence in situ hybridization
FLUOS	5(6)-carboxyfluorescin N-hydroxysuccinimide ester
FNA	Free nitrous oxide
f_{redox}	Number of aerated or redox cycling periods within a react phase
GHG	Greenhouse gas
HAO	Hydroxylamine oxidoreductase
HB	Heterotrophic bacteria
HRT	Hydraulic residence time
HZO	Hydrazine dehydrogenase
hzoA	Hydrazine dehydrogenase subunit A encoding gene
HZS	Hydrazine synthase
hzs	Hydrazine synthase encoding gene
IFAS	Integrated fixed film activated sludge
IPCC	Intergovernmental panel on climate change
k_La_{N2O}	Volumetric mass transfer coefficient for nitrous oxide
k_La_{O2}	Volumetric mass transfer coefficient for oxygen
K_{NH3}	Half-saturation concentration for ammonia
K_{NO2}	Half-saturation concentration for nitrite
K_{O2}	Half-saturation concentration for oxygen
K_s	Substrate half-saturation constant
L_{NH4}	Volumetric ammonium loading rate per cycle
L_{O2}	Volumetric oxygen loading rate per cycle
L_{O2}/L_{NH4+}	Oxygen to ammonium loading rate per cycle
MABR	Membrane aerated biofilm reactor
MAR	Microautoradioactivity
MBBR	Moving bed biofilm reactor
MLE	Modified Ludzack-Ettinger
N	Nitrogen

N_2	Dinitrogen gas
N_2H_4	Hydrazine
N_2O	Nitrous oxide
N_2O_i	Instantaneous bulk liquid nitrous oxide concentration measured in reactor (at time "i")
NAR	Nitrate reductase
NH_2OH	Hydroxylamine
NH_4^+	Ammonium
NH_{4inf}	Influent ammonium concentration
NIR	Nitrite reductase
nirK	Copper-containing nitrite reductase encoding gene
nirS	Cytochrome cd1-containing nitrite reductase encoding gene
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOB	Nitrite oxidizing bacteria
NOR	Nitric oxide reductase
NOS	Nitrous oxide reductase
N_xO	Nitrogen oxide
NXR	Nitrite oxidoreductase
nxrA	Nitrite oxidoreductase alpha subunit encoding gene
nxrB	Nitrite oxidoreductase beta subunit encoding gene
O_2	Oxygen
OLAND	Oxygen limited autotrophic nitrification-denitrification
ORP	Oxidation-reduction potential
OTU	Operational taxonomic units
P	Phosphorous
PSD	Particle size distribution
Q_{air}	Air flow rate
qPCR	Quantitative polymerase chain reaction
R_{AmmTot}	Ammonium consumed per total nitrogen removed
RBC	Rotating biological disc
$r_{N2O,i}$	Instantaneous net N_2O production rate (at time "i")
R_{NatTot}	Nitrate produced per ammonium removed

R_{NitAmm}	Nitrite produced per total nitrogen removed
R_{on}	Total aerated fraction of a cycle
rRNA	Ribosomal ribonucleic acid
S	Substrate concentration
SBR	Sequencing batch reactor
SHARON	Single reactor high activity ammonia removal over nitrite
SMP	Soluble microbial products
SNAP	Single-stage nitrogen removal using anammox and partial nitritation
S_{O2}	Oxygen concentration in the reactor liquid
$S_{O2,sat}$	Oxygen saturation concentration at $30^{o}C$
SRT	Solids retention time
SVI	Sludge volume index
SVI_{10}	10 minute sludge volume index
SVI_5	5 minute sludge volume index
t_{cycle}	Total cycle duration
TN	Total nitrogen
t_{off}	Non-aerated period duration
t_{on}	Aerated period duration
t_{react}	React phase duration
UAP	Utilization-associated products
UASB	Upflow anaerobic sludge blanket
WWTP	Wastewater treatment plant
Y	Yield coefficient

1 Introduction

1.1 Biological nitrogen removal

Water and nitrogen are both essential for life. Increasing population and poor management have resulted in anthropogenic exploitation of both resources beyond the sustainable planetary boundaries (Rockström et al., 2009). The anthropogenic pollution of water translates in a lack of potable water. Diseases are acute warnings but increased hypoxia in freshwater resources is also a major environmental impact. Hypoxia is primarily caused by eutrophication through release of nutrients; nitrogen and phosphorous, above their natural assimilation level. Reactive nitrogen in water is mainly present in reduced form as ammonium-N and in oxidized form as nitrite-N or nitrate-N. While excess ammonium causes oxygen depletion; high concentrations of nitrite and nitrate in waters are toxic for oxygen respiring animals.

Yearly, European cities discharge 2.3 Tg reactive N to surface waters through wastewater and emit 0.015 Tg N to air as ammonia and nitrous oxide from wastewater and solid waste treatment (Svirejeva-Hopkins and Reis, 2011). Although on global scale, the release of nitrogenous compounds is considered as a slow variable affecting the resilience of coastal and freshwater systems towards the sustainability threshold, gaseous nitrous oxide stands out. Nitrous oxide is currently the leading ozone-depleting substance and has global warming potential 300 times of CO_2, hence even emitted in low amounts it is a systematic driver of global sustainability boundaries (Ravishankara et al., 2009; Rockström et al., 2009).

The realization of the impacts of nutrient release to waters and have led to stricter regulations in Europe imposed by the Urban Wastewater Treatment (UWT) Directive (91/271/EEC Council Directive, 1991). For Denmark, the Baltic Sea catchment requires more stringent nitrogen removal. Despite substantially reduced emission of reactive nitrogen through tertiary treatment since the 1990s, wastewater treatment plants (WWTP) still contribute as the third highest emitter of urban nitrogen release in Europe (Svirejeva-Hopkins and Reis, 2011). The environmental burden of discharging nitrogen-rich effluents is thus significant, yet many industrialized countries as well as developing countries still lack tertiary treatment. The main reason is economic: even though it is possible to remove up to 95% of the reactive nitrogen, the cost of removal increases faster than the degree of treatment achieved. Treating streams with concentrated ammonium means larger energy demand, high-

er level of know-how transfer and, ultimately, higher costs. Furthermore, with climate change considerations, the overall ecological footprint of the processes becomes important. This includes greenhouse gas emissions, either directly as through nitrous oxide, or indirectly as carbon dioxide equivalents of energy production. Under the IPCC guidelines of 2006 and based on limited number of studies, emissions of nitrous oxide from WWTP are considered to be minor. Since then, however, many emission measurement campaigns revealed that if they are indeed small compared to the total anthropogenic emissions (3%), they were largely underestimated and in fact do contribute significantly to the greenhouse gas footprint of a WWTP (Desloover et al., 2011; Kampschreur et al., 2009b; Law et al., 2012). In a WWTP, aeration is the highest energy consuming process, amounting from 30 and up to 75% of the whole plant consumption (Reardon, 1995; Zessner et al., 2010). Therefore energy- and cost-effective solutions are still needed. So far, chemical N recovery techniques require more energy and are more costly than biological removal techniques as they cannot yet compete in the fertilizer market due to their poor quantitative and qualitative yield (Siegrist, 1996).

Microorganisms, on the other hand, have been driving the global N cycle until the Haber-Bosch process was applied massively. Therefore engineers can benefit from their millions of years of evolution to catalyse the treatment of nitrogen-rich wastewaters. Biological nitrogen removal is achieved through the sequential functioning of a number of microbial groups. In a WWTP, this requires appropriate unit/process configurations to meet their specific ecophysiological requirements, especially in terms of regulation of aeration and organic carbon supply, which bring the above-mentioned setbacks of tertiary treatment. One long-hidden set of actors in the global nitrogen cycle that has been predicted as early 1977 (Broda, 1977), has been relatively recently discovered. The anaerobic ammonium oxidizing or “anammox” bacteria, which have, ironically, been first discovered in a wastewater treatment setting (Mulder et al., 1995) can support the struggle against energy footprint and costs. Since 1995, use of the anammox process in combination with the required preceding partial nitrification process - which enables fully autotrophic nitrogen removal - has been intensively explored. Currently, there are over 30 full-scale applications utilising autotrophic nitrogen removal with promising efficiency and capacity. However, all these systems also present issues to be resolved, mostly regarding process stability (Joss et al., 2011; Lackner et al., 2014) and evaluation of the so far little accounted for nitrous oxide emissions.

In order to clarify the distinction and advantages of autotrophic nitrogen removal over conventional biological nitrogen removal processes; the bottlenecks in start-up, operation, stability, maintenance of reduced footprint, and the opportunities for engineering solutions, we need to recognize and comprehend the key microbial actors of nitrogen conversion in this system.

1.1.1 Aerobic ammonia oxidizing prokaryotes

Metabolism and Stoichiometry

Aerobic ammonia oxidizers are primarily chemolithoautotrophs that utilize inorganic carbon for biomass synthesis (CO_2 fixation) and convert ammonium to nitrite for energy in presence of oxygen as presented with the overall (catabolic + anabolic) reaction (1.1) (Barnes and Bliss, 1983):

$$\begin{aligned} &NH_4^+ + 1.382\ O_2 + 1.982\ HCO_3^- \rightarrow \\ &0.018 C_5H_7NO_2 + 0.982\ NO_2^- + 1.036\ H_2O + 1.891\ H_2CO_3 \end{aligned} \quad (1.1)$$

The overall conversion namely "nitritation" is carried out by two sets of enzymes in sequence (Bock and Wagner, 2006). First ammonia, the true substrate (Suzuki et al., 1974), is oxidized to hydroxylamine (NH_2OH) catalysed by the enzyme ammonia monooxygenase (AMO) (1.2). This is followed by hydroxylamine oxidation to nitrite by the hydroxylamine oxidoreductase (HAO) (1.3). The electrons released from the final step are used in the respiratory chain and back in ammonia oxidation step.

$$NH_3 + O_2 + 2H^+ + 2e^- \xrightarrow{AMO} NH_2OH + H_2O \quad (1.2)$$

$$NH_2OH + H_2O \xrightarrow{HAO} HNO_2 + 4H^+ + 4e^- \quad (1.3)$$

Being autotrophs, their growth rates are quite low, ca. $2d^{-1}$. The majority grow at optimal temperatures of 25-35^oC (except *N. cryotolerans*) and pH 7.9-8.2. Their activity releases protons and consumes alkalinity, hence pH control may be crucial; lower pH leads to free nitrous acid (FNA) while higher pH leads to free ammonia (FA) both of which can be inhibitory (Anthonisen et al., 1976).

Phylogenetic diversity and ecophysiology

Ammonia oxidizing prokaryotes are mainly bacterial (AOB) but recently ammonia oxidizing archaea (AOA) have been discovered. These archaea are members of the *Chrenarchaeota* and *Thaumarchaeota* phyla (Pester et al., 2012) and are indeed found in WWTP (with sequential aeration, low DO, high solids retention time (SRT) and hydraulic residence time (HRT) (Park et

al., 2006)) but their contribution to ammonium oxidation in typical wastewater treatment is considered insignificant. AOA are expected to be more adapted to relatively low concentration of ammonia. AOB found in wastewaters are mainly affiliated to the *Nitrosomonas* and the *Nitrosospira* genera, both belonging to the subphylum of the beta-proteobacteria (Purkhold et al., 2000).

The distribution of AOB in the environment is mostly driven by their salt requirement, salt tolerance and substrate affinities (Koops and Pommerening-Roser, 2001). Within nitrosomonads, members of four clusters are significantly found in WWTP. *Nitrosomonas europaea* and *Nitrosomonas (Nitrosococcus) mobilis* lineage (Cluster 7) mainly consist of salt tolerant bacteria with low affinity to ammonia (K_{NH3}=0.42-0.85 mgN/L). *N. europaea* and *N. eutropha* are strong halotolerants without obligate requirements, while *N. halophila* and *N. mobilis* are obligate halophiles. *N. halophila* are also alkali tolerant up to pH=10. *N. europaea* and *N. eutropha* are the most commonly found in WWTP (Wagner et al., 2002). An interesting feature of *N. europaea* is that it cannot synthesize siderophores (Fe binding chelator) although they are very dependent on Fe for electron transport via the cytochromes (Chain et al., 2011). It is suggested that, with many Fe-siderophore receptors, they instead harvest siderophores produced by other bacteria, point to be considered for association with other bacteria, such as *N. eutropha* that can indeed produce their own (Stein et al., 2007). *N. mobilis* is motile and more abundant than *N. europaea* in industrial WWTP (Juretschko et al., 1998) as well as in nitrifying biofilms (H Daims et al., 2001).

The *Nitrosomonas communis* lineage (Cluster 8) also has a relatively low ammonia affinity (K_{NH3}=0.20-0.64 mgN/L) comparable to *N. europaea/mobilis* (Koops and Pommerening-Roser, 2001). The *Nitrosomonas oligotropha* lineage (Cluster 6a) has extremely high ammonia affinity compared to the other lineages (K_{NH3}=0.03-0.06 mgN/L) but also appears to be inhibited by higher ammonium concentrations (Bollmann and Laanbroek, 2001). Both *N. oligotropha* and *N. nitrosa* are regularly found in industrial WWTP. *N. oligotropha* found in aggregates heavily excrete extracellular polymers which is considered to help them tolerate heavy metals (Stehr et al., 1995). *Nitrosomonas marina* lineage (Cluster 6b), despite being identified as marine obligate halophiles, are also found in WWTP (Purkhold et al., 2000) with comparable ammonia affinity as the *N. europaea*/*mobilis* lineage.

Finally, the *Nitrosospira* (Cluster 0-4) constitute a relatively newly discovered group of AOB that has so far 3 genera, *Nitrosospina*, *Nitrosolobus*, *Nitrosovibrio*. Their affinity constants and salt tolerance/requirements are not yet well determined. *Nitrosospir*a were found abundant and outcompeting *Nitrosomonas* in nitrifying fluidized bed aggregates (Schramm et al., 1998), probably because they are better adapted to low ammonium concentrations with in-situ K_{NH3}=0.56mgN/L (Schramm, 1999). *Nitrosospira briensis* has especially high ammonia affinity (K_{NH3}=0.02-0.04 mgN/L) close to that of *N. oligotropha* and even higher affinity in biofilm mode which possibly allows fast recovery after long term ammonium starvation (Bollmann et al., 2005).

In an intermittently aerated WWTP (Kraftisried) operating with low DO and nitrite peaks, for simultaneous aerobic and anoxic processes to remove a high load of 5000 mgNH_4-N/L, *N. mobilis* was singularly dominant in the AOB guild, forming aggregates of 5-20µm that were significantly brighter than municipal activated sludge when identified with fluorescence in-situ hybridization (Purkhold et al., 2000; Wagner et al., 1995). While in another study surveying 12 WWTP, *N. communis* was found in all intermittently anoxic activated sludge plants (Limpiyakorn et al., 2005).

Studies on oxygen affinities are rather inconclusive. Considering the environmental habitats and gradients in the biofilms they inhabit, *Nitrosospira* are believed to have higher affinity to oxygen than *Nitrosomonas* (Laanbroek and Gerards, 1993; Schramm et al., 1999, 1998), as for ammonia. Within *Nitrosomonas*, Gieseke and colleagues suggest that *N. oligotropha* has higher affinity than *N. europaea* (Gieseke et al., 2001), dominating the deeper biofilms while other researchers enriched *N. europaea* over *N. oligotropha* under oxygen limitation (<0.24 mg O_2/L) (Park and Noguera, 2004). *N. europaea* may be able to cope with low oxygen through alternative pathways of denitrification (Yu and Chandran, 2010). *N. oligotropha* dominated both high (1.5-3.5 mg/L) and low DO (0.5 mg/L) enrichments (Bellucci et al., 2011). Recently, it has been suggested that acclimation to low DO enhances expression of an unidentified heme-protein in *N.europaea* that would enable higher oxygen uptake (Arnaldos et al., 2013). AOB adaptation to low DO has been investigated by many, yet differences even at strain level and metabolic versatility of these bacteria make it hard to generalize.

Alternative metabolisms and nitrous oxide

N. europaea has a versatile metabolism and can use organic substrates such as pyruvate, amino acids, fructose as source of carbon (Clark and Schmidt,

1966). Both *N. europaea* and *N. eutropha* can be mixotrophic: in presence of organic carbon they are able to nitrify and denitrify simultaneously, giving higher yields provided that ammonia is present (Bock et al., 1995). Under anoxia *N. eutropha* can denitrify, using hydrogen as electron donor and nitrite as electron acceptors (Bock et al., 1995), but *N. europaea* lacks the necessary genes to do so (Chain et al., 2011).

One component of AOB metabolism is currently of much interest due to potential alternative pathways catalysed that lead to nitrous oxide (N_2O) production. Depending on the availability of electron donors (ammonium or hydroxylamine) and electron acceptors (oxygen or nitrite), especially with transiency in oxygen levels, *N. europaea* may i) oxidize NH_2OH to nitric oxide (NO) instead of HNO_2 catalysed by HAO ii) reduce HNO_2 to NO catalysed by nitrite reductase (NIR) and ultimately reduce either NO to N_2O by nitric oxide reductase (NOR). The first mechanism is called the hydroxylamine oxidation pathway and the second is called the nitrifier denitrification pathway of N_2O production; the second pathway is similar to the anaerobic denitrifier pathway (see Section 1.1.3).

Although an important intracellular signal molecule, NO is also a cytotoxic compound (Richardson et al., 2009); hence, its detoxification to N_2O is crucial for most microorganisms. For AOB, the major driving condition for N_2O production is the presence of ammonia, and the reimposition of oxygen after transient anoxic conditions (Yu and Chandran, 2010). Recently, the cause of this diversion was proposed to be the imbalance in electron equivalents (electron overproduction with no acceptor under anoxia) while excess electron donor (ammonium) activates nitrifier denitrification its co-depletion with the electron acceptor (oxygen) causes a shift to the hydroxylamine oxidation pathway (Perez-Garcia et al., 2014). The exact mechanisms, enzymes involved and environmental conditions for nitrous oxide production are still under exploration (Kozlowski et al., 2014). Among the AOB, *Nitrosomonas* and *Nitrosopira* produce nitrous oxide, but the rates may differ even between strains. For instance ATCC19718 strain of *Nitrosomonas europaea* was found to produce N_2O at rates four times higher than other *Nitrosomonas* spp. and *Nitrosospira* (Shaw et al., 2006).

In *Nitrosomonas*, HAO can also catalyse the reduction of NO back to ammonia through NH_2OH, a switch from aerobic to anaerobic metabolism (Kostera et al., 2008). HAO may therefore limit accumulation of NO and NH_2OH if oxygen is limiting, as an energetically wasteful detoxification measure. If low

oxygen conditions are prolonged, the organism switches to anaerobic metabolism which may be induced by NO concentrations of 30 ppm (Schmidt et al., 2004).

Detection and Quantification

AOB can be detected and quantified with molecular methods from DNA extract of biomass samples through quantitative PCR amplification with primers for key AMO coding functional genes like *amoA*; with primers targeting 16S rRNA-encoding genes. Alternatively, they can detected and quantified or on whole cell samples with fluorescence in-situ hybridization with cluster specific rRNA-targeted oligonucleotide probes (Adamczyk et al., 2003; Almstrand et al., 2013; Gieseke et al., 2001; Mobarry et al., 1996).

1.1.2 Nitrite oxidizing bacteria

Metabolism and Stoichiometry

Nitrite oxidizing bacteria (NOB) are also primarily chemolithoautotrophs, fixing CO_2 and oxidizing nitrite to nitrate in aerobic conditions as presented with the overall (catabolic + anabolic) reaction (1.4) (Barnes and Bliss, 1983):

$$\begin{aligned} &NO_2^- + 0.0025\ NH_4^+ + 0.4875\ O_2 + 0.01\ H_2CO_3 + 0.0025\ HCO_3^- \rightarrow \\ &0.0025\ C_5H_7NO_2 + NO_3^- + 0.0075\ H_2O \end{aligned} \quad (1.4)$$

The nitrite conversion to nitrate namely “nitratation” is catalysed by the nitrite oxidoreductase (NXR) (1.5) which can also catalyse the reverse reaction, paving the way for heterotrophic growth. The produced electrons pass to respiratory chain yielding energy for biosynthesis (1.6).

$$NO_2^- + H_2O \xrightarrow{NXR} NO_3^- + 2H^+ + 2e^- \quad (1.5)$$

$$0.5\ O_2 + 2H^+ + 2e^- \longrightarrow H_2O \quad (1.6)$$

Also autotrophs, their growth rates are even lower than those AOB, ca. 1.4 d^{-1}. The majority grow at optimal temperatures of 25-35^{o}C (except *Nitrotoga*) and pH 7.2-7.6, and they are inhibited by FNA and FA (Anthonisen et al., 1976).

Phylogenetic diversity and ecophysiology

Nitrite oxidizing bacteria can be found in the *Alphaproteobacteria, Deltaproteopbacteria*, *Nitrospirae* and *Chloroflexi* phyla. These organisms are notoriously hard to culture (Spieck and Lipski, 2011), so that the knowledge on their diversity and physiology has only been relatively recently obtained. The

most recently discovered species are *Nitrospina gracilis,* affiliated to *Deltaproteobacteria* and isolated from an oxygen minimum marine zone (Lücker et al., 2013) and *Nitrolancetus hollandicus*, affiliated to the *Chloroflexi* and isolated from a nitrifying bioreactor (Sorokin et al., 2012). The latter is characterized by its low nitrite affinity, its temperature optimum (25 to 63^{o}C), and its ability to use formate as energy and carbon source.

The majority of the metabolic, kinetic and ecophysiological studies have focused on the *Nitrobacter* genus of the *Alphaproteobacteria*. WWTPs, however, *Nitrospira* genera (of the *Nitrospira* phylum) form the majority of the NOB, along with coexisting *Nitrobacter spp*.

Nitrobacter contains the known species *Nitrobacter winogradskyi*, *Nitrobacter hamburgensis, Nitrobacter vulgaris* of which complete genome information is available for the first two (Starkenburg et al., 2006). They are considered as fast growers with low affinity for nitrite compared to *Nitrospira* (Schramm et al., 1998). Most *Nitrobacter* spp. have the ability to grow mixotrophically that brings them higher fitness with faster growth rates when multiple substrates are available. They are also capable of heterotrophic growth under oxic and anoxic conditions but the growth rates are much lower than under lithoautotrophic growth (Bock and Wagner, 2006). *Nitrobacter vulgaris* can synthesize NirK (Ahlers, 1990) though the exact functionality has not ascertained, and no known nitric oxide reductase has been found in completed genomes, hence their contribution to nitrous oxide emissions next to AOB in WWTP are insignificant (Kampschreur et al., 2009b).

Within the *Nitrospira* genus *Nitrospira moscoviensis, Nitrospira defluvii* are known and considered under sublineage I and sublineage II, respectively. Members of sublineage I that have lower nitrite affinity seem to be more dominant in WWTP. In-situ observations of nitrifying biofilms revealed members of sublineage I residing in close proximity to AOB while members of sublineage II formed another niche at a distance (Maixner et al., 2006). Correlating with *in situ* nitrite measurements, sublineage II is considered to have higher affinity for nitrite (Schramm et al., 1999) and considering evolutionary relations to microaerophilics, possibly they also prefer lower oxygen concentrations (Okabe et al., 1999; Schramm et al., 2000). The *Nitrospira defluvii* genome suggests that it has the potential to grow mixotrophically like *Nitrobacter*.

Detection and Quantification

NOB can be detected with qPCR amplifying with primers for key NXR coding functional genes, with both *nxrA* proposed for *Nitrobacter* and *nxrB* proposed for *Nitrospira* coverage (Pester et al., 2014); with primers targeting 16S rRNA-encoding genes or with fluorescence in-situ hybridization with phylum (*Nitrobacter* or *Nitrospira*) (Holger Daims et al., 2001; Wagner et al., 1996) and *Nitrospira* sublineage (I or II) specific rRNA-targeted oligonucleotide probes (Maixner et al., 2006).

1.1.3 Denitrifying prokaryotes

Metabolism and Stoichiometry

Denitrifiers are conventionally defined as heterotrophic bacteria (HB) oxidizing biodegradable organic carbon while converting nitrate (1.7) or nitrite (1.8) (nitrogenous oxides) to dinitrogen gas under anaerobic conditions. However, a broad variety of bacteria as well as archaea with different physiologies, including autotrophs, as discussed for aerobic ammonia and nitrite oxidizing bacteria, are capable of denitrifying (Shapleigh, 2006).

$$NO_3^- + 1.08CH_3OH + 0.24\ H_2CO_3 \rightarrow 0.056C_5H_7NO_2 + 0.47N_2 + 1.68H_2O + HCO_3^- \quad (1.7)$$

$$NO_2^- + 0.67CH_3OH + 0.53\ H_2CO_3 \rightarrow 0.04C_5H_7NO_2 + 0.48N_2 + 1.23H_2O + HCO_3^- \quad (1.8)$$

Complete denitrification is the combination of four partly independent respiratory modules, catalysed by different enzymes (Figure 1). If nitrate is the primary electron donor, it is first converted to nitrite by the nitrate reductase (NAR). Then, in sequence, the nitrite reductase (NIR), the nitric oxide reductase (NOR) and the nitrous oxide reductase (NOS) can reduce nitrite to nitric oxide, nitrous oxide and dinitrogen gas (1.9). Denitrification is an alkalinity producing process therefore the pH should be controlled.

$$NO_3^- \xrightarrow{NAR} NO_2^- \xrightarrow{NIR} NO \xrightarrow{NOR} N_2O \xrightarrow{NOS} N_2 \quad (1.9)$$

Many organisms are able to reduce nitrate all the way to dinitrogen gas, while others contain only parts of the pathway. The processes catalysed by NAR and NOS can be carried out independently of the rest, under the control of different regulators, while the activities of NIR and NOR are interdependent possibly to prevent accumulation of NO (Zumft, 1997).

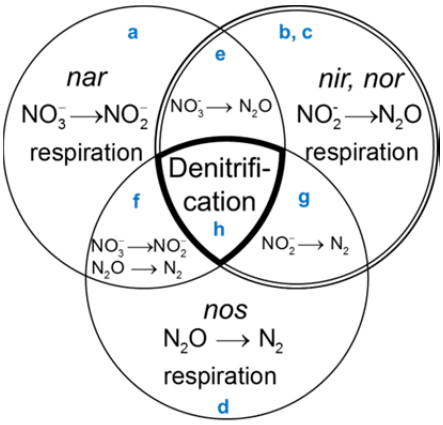

Figure 1. The four respiratory modules (a, b, c, d) for complete denitrification (h), adapted from (Zumft, 1997)

Most denitrifiers are facultative aerobes, meaning that they would use oxygen if present since it is energetically more favourable than nitrogen oxides (N_xO). Correspondingly, oxygen is also found to inhibit the activity of NAR, NIR and NOR to a great extent for denitrifiers (Cavigelli and Robertson, 2000), but denitrification, although incomplete, can still be active under microaerophilic conditions (Körner and Zumft, 1989). In these cases, emission of nitrous oxide is an issue. Another cause of incomplete denitrification can be lack of organic carbon (Hanaki et al., 1992; Park et al., 2000).

Phylogenetic diversity and ecophysiology

Denitrifier diversity spans over many phyla but most frequently gather under alpha and beta *Proteobacteria*. They differentiate according to their carbon substrate preference and are grouped according to their growth mode or dominant physiological feature. Recently, carbon isotope labelled incubations combined with fluorescence in-situ hybridization (MAR-FISH) revealed more about the preferred carbon substrates (eg. methanol vs ethanol vs acetate) and their ecophysiology (Morgan-Sagastume et al., 2008; Osaka et al., 2006). In wastewater, the genera *Alcaligenes*, *Pseudomonas*, *Methylobacterium*, *Bacillus*, *Paracoccus*, *Hyphomicrobium*, *Azoarcus* have been identified as denitrifiers (Wagner et al., 2002).

Detection and Quantification

The functional genes *nirS* and *nirK* have been used as molecular identifiers of denitrifying heterotrophs (Hallin and Lindgren, 1999; Philippot et al., 2002; Throbäck et al., 2004). The 16S rRNA gene is typically not a good target because denitrifiers are a paraphyletic group, as *nirK* has been suggested to have been horizontally transferred (Heylen et al., 2006).

1.1.4 Anaerobic ammonium oxidizing bacteria

Metabolism and Stoichiometry

Anaerobic ammonium oxidizing, namely "anammox" bacteria (AnAOB), are primarily chemolithoautotrophs that fix inorganic carbon and get their energy by oxidizing ammonium to dinitrogen gas with nitrite as electron acceptor under anaerobic conditions (1.10) (Strous et al., 1998).

$$\begin{aligned} &NH_4^+ + 1.32\ NO_2^- + 0.066HCO_3^- + 0.13\ H^+ \rightarrow \\ &0.26\ NO_3^- + 1.02\ N_2 + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O \end{aligned} \quad (1.10)$$

The overall conversion is carried out in three steps. First, nitrite is reduced to NO, a step catalysed by NIR (1.11). Then NO and ammonium are condensed to hydrazine (N_2H_4), by the hydrazine synthase (HZS) (1.12). Finally, hydrazine is oxidized to dinitrogen gas by the hydrazine dehydrogenase (HZO) (1.13). The electrons produced in the last step are used to sustain the nitrite and nitric oxide reduction and carbon fixation. In addition, some nitrite is oxidized to nitrate by the nitrate reductase (NAR) in order to supply electrons that are directed to carbon fixation. Hence nitrate formation always accompanies cell synthesis.

$$NO_2^- + 2H^+ + e^- \xrightarrow{NIR} NO + H_2O \quad (1.11)$$

$$NO + NH_4^+ + 2H^+ + 3e^- \xrightarrow{HZS} N_2H_4 + H_2O \quad (1.12)$$

$$N_2H_4 \xrightarrow{HZO} N_2 + 4H^+ + 4e^- \quad (1.13)$$

One interesting aspect of AnAOB metabolism is the hydrazine intermediate which is a highly toxic and strong reductant. Hence AnAOB have an intracellular organelle called the anammoxosome inside which reactions involving hydrazine take place (Neumann et al., 2014) (1.12-13).

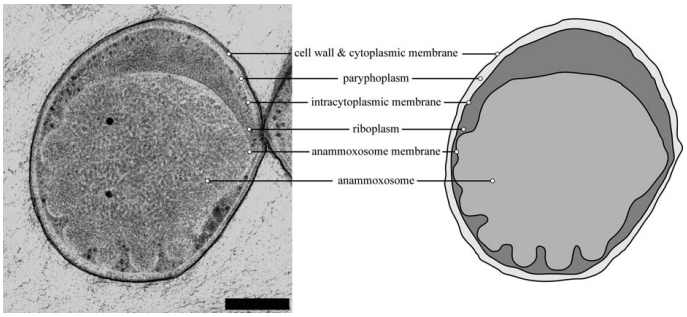

Figure 2. Anaerobic ammonium oxidizing bacteria with the intracellular organelle "anammoxosome" surrounded by the rest of the cellular material (including ribosomes), from (van Niftrik, 2013).

Anammox bacteria are hard to cultivate and so far only enrichments could be obtained. Generation times of 11-12 days or even longer have been reported (Strous et al., 1998; van de Graaf et al., 1996). However it has recently been proposed that generation times are intrinsically much shorter (3-4 days), attributing the previous observations to mass transfer limitations (Lotti et al., 2014b). They are strictly anaerobic and inhibited by very low concentrations of oxygen, but this inhibition is reversible (Strous et al., 1997). At the same time high nitrite concentrations also inhibit AnAOB in some cases reversibly, with a wide range of threshold values ranging from 10 to 182 mgN/L have been reported, possibly depends on the specific species (Dapena-Mora et al., 2007; Egli et al., 2001; Lotti et al., 2014a; Wett, 2007). Optimal growth temperatures of most anammox bacteria range between 37-40^{o}C and optimal pH is around 8 (Egli et al., 2001; Strous et al., 1999). As they fix carbon dioxide the alkalinity should be controlled and pH buffered.

Phylogenetic diversity and ecophysiology

The existence of AnAOB was predicted by Broda in 1977, based on the thermodynamically favourable reaction of nitrite reduction coupled to ammonium oxidation and N_2 generation (Broda, 1977), but AnAOB have only been recently discovered (Mulder et al., 1995). They form a monophyletic group in *Planctomycetes* and are divided into five genera *Ca. Brocadia*, *Ca. Kuenenia*, *Ca. Scalindua*, *Ca. Anammoxoglobus* and *Ca. Jettenia*. Apart from *Scalindua* which is found in saline marine environment, all the other genera are found in enrichments from wastewater treatment, most commonly *Brocadia* and *Kuenenia*.

Kuenenia stuttgartiensis was the first AnAOB species for which the full genome was sequenced (Strous et al., 2006) followed by *Jettenia* affiliated KSU-1 strain (Hira et al., 2012), *Scalindua profunda* (van de Vossenberg et al., 2013) and finally *Brocadia fulgida* (Ferousi et al., 2013). Information derived from the genome allowed the development of a conceptual model of their metabolism. *K. stuttgartiensis* can also perform dissimilatory nitrate reduction to ammonium (DNRA) using formate as electron donor and nitrate as electron acceptor (Kartal et al., 2007a). Many AnAOB species are shown to oxidize organic acids such as formate, acetate and propionate when supplemented to ammonium, nitrite, nitrate (Kartal et al., 2008, 2007b). *Anammoxoglobus propionicus* can utilize propionate at rates double of *Ca. Brocadia fulgida* and ca. 6 times of *Brocadia anammoxidans* and *Kuenenia*

stuttgartiensis. While *Brocadia fulgida* can utilize acetate at higher rates than the others.

The reported apparent half-saturation constants of AnAOB for nitrite range from K_{NO2}=0.028-0.042 mgN/L for Ca. *Kuenenia stuttgartiensis* (van der Star et al., 2008a), <0.070 mgN/L for Ca. *Brocadia anammoxidans* (Strous et al., 1999), 1.2 mgN/L for Ca. *Brocadia sinica* (Oshiki et al., 2011) while intrinsic values can be even as low as 0.035mgN/L for *Brocadia* (Lotti et al., 2014b). With higher affinity, *Kuenenia* is expected to thrive in low nitrite concentrations while *Brocadia* would dominate in high concentrations. Nitrite affinity as well as the organic acid utilization capacity are considered to be the causes of strong niche differentiation of AnAOB, and they appear to be present as single main species in the different environments they have been detected.

Nitric oxide is found to be an intermediate for the anammox pathway, which can be directly utilized by *Ca. Brocadia fulgida* without inhibition and without nitrous oxide production (Kartal et al., 2010). *Ca. Brocadia fulgida* can also disproportionate hydroxylamine directly without nitrite in to dinitrogen gas and ammonium followed by hydrazine accumulation upon depletion (van der Star et al., 2008b). Therefore, from a nitrous oxide perspective AnAOB act as sinks rather than sources.

Detection and Quantification

AnAOB can be detected via qPCR with 16S rRNA targeting primers or functional gene primers *hzoA* and *hzs* coding for the HZO and HZS (Harhangi et al., 2012) enzymes as well as a *nirS* primer, which differs from the typical denitrifier NIR coding gene (Li et al., 2011). Oligonucleotide probes for FISH target most of the known AnAOB species whereas specific species targeting probes also exist. With FISH, AnAOB cells have a characteristic "dough-nut ring" shape due to the localization of ribosomes around the central anammoxosome. It should also be noted that *Brocadia fulgida* is reported to secrete autofluorescent extracellular polymers with double excitation and emission maxima at 390-630nm and 352-442nm, respectively that might impair FISH studies with FLUOS and Cy3 labelled probes (Kartal et al., 2008). The distinct cell ultrastructure AnAOB also provides other biomarkers; such as unique ladderane lipids which make up the membrane of anammoxosome, but are quite elaborate to extract (Rattray et al., 2008). The presence of cytochrome c, a hemeprotein which contains a ferrous ion, gives AnAOB have a characteristic bright red colour (Jetten et al., 1998).

1.1.5 Autotrophic nitrogen removal in one reactor – the choice

Multiple configurations exist to remove ammonium from wastewaters using aerobic and anaerobic ammonium oxidizers, nitrite oxidizers and denitrifiers. Nitrification is the combined process of nitritation by AOB with nitratation by NOB. The combined process carried out under aerobic conditions converts ammonium to nitrate autotrophically. Then denitrification, under anoxic conditions, to convert nitrate to unreactive dinitrogen gas by heterotrophs requires organic carbon. Organic carbon removal by aerobic heterotrophs has been the earliest wastewater treatment strategy; however, in the case of nitrogen removal, the organic carbon to nitrogen ratio (C/N) does not always match the stoichiometric requirement of these bacteria, resulting in incomplete nutrient removal.

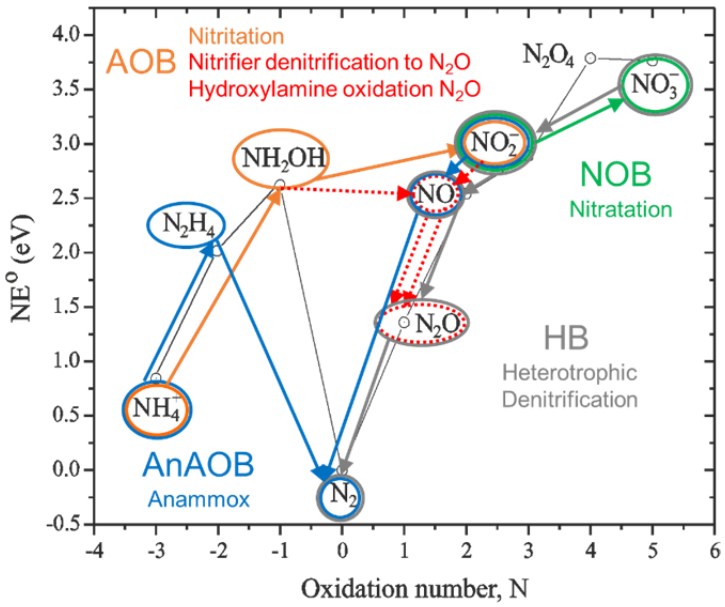

Figure 3. Frost diagram for nitrogen species at pH=7.0 showing relative energies and conversion paths by AOB, AnAOB, NOB and HB, adapted from (Kostera et al., 2008).

In the 1960s, the first activated sludge system utilizing autotrophs for nitrogen removal was developed using a nitrification/denitrification configuration (Ludzack and Ettinger, 1962), but this process required dosing of additional organic carbon such as methanol to achieve complete denitrification. Then, the configuration was modified to pre-denitrification (MLE), recycling the nitrate from nitrification to the beginning in order to utilize the organic carbon coming with the influent (Barnard, 1975). Lastly, to further diminish the nitrate in MLE effluent, 4-stage Bardenpho with secondary anoxic and reaeration configuration was developed and conventional nitrification/denitrification became widespread. Nevertheless, the space requirement,

pumping, aeration and fitting the C/N remain as costly disadvantages. Also more treatment means more biomass; ultimately more sludge to handle and additional costs.

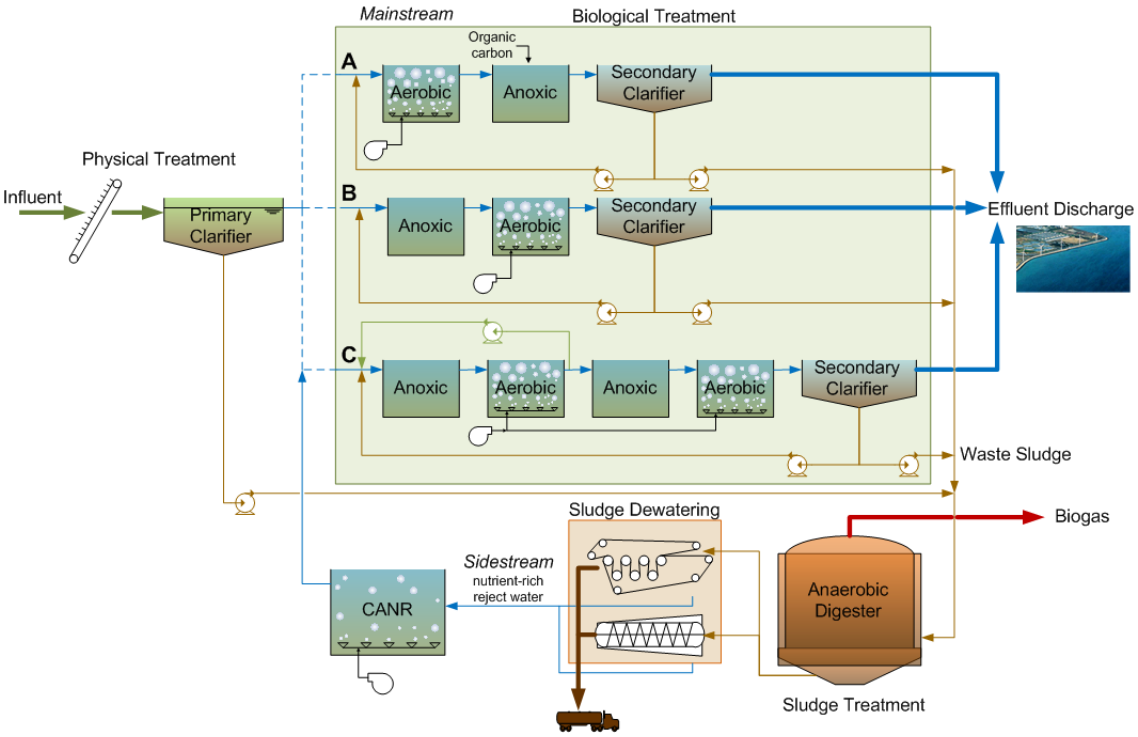

Figure 4. Wastewater treatment plant scheme for conventional mainstream biological nitrogen removal A) Ludzack-Ettinger, B) Modified Ludzack-Ettinger (MLE), C) 4-stage Bardenpho, and sidestream CANR

Space requirement and pumping can be reduced by sequentially operating batch systems and with biofilm-based technologies, where processes can take place simultaneously within the microniches of the biomass. Aeration can be reduced by short-cut denitrification ("nitrite-shunt") which proceeds from nitritation to denitritation, bypassing nitrate production step by NOB, driving the nitrite produced by AOB directly to denitrifiers (Abeling and Seyfried, 1992). In this case, the produced sludge (biomass) that would need further handling is also reduced.

The organic carbon requirement however cannot be removed completely as long as heterotrophs are part of the treatment train. For the wastewater streams with high ammonium and little organic carbon this is a problem. A major nitrogen rich stream in WWTP is the reject water from the dewatering of digested sludge that has little to no organic carbon left but is richer in phosphate and ammonium (300-1500 mgN/L) than the WWTP influent. This stream is commonly recycled back to the beginning of the plant and entails

10-30% up to 50% of the total nitrogen load to the WWTP. Especially for existing WWTP to upgrade the facility for strict nitrogen regulations this means additional unit requirements. Also industrial streams such as food and agriculture which utilize anaerobic digestion for their wastes, semiconductor effluents (Tokutomi et al., 2011) or landfill leachate (Ruscalleda et al., 2008) generate these low C/N wastewater streams.

Completely autotrophic nitrogen removal (CANR) combines nitritation by AOB with anammox by AnAOB. This treatment train not only cuts the organic carbon requirement but also decreases the aeration demand and sludge production significantly (Table 1). The autotrophic bacteria require only inorganic carbon which is already present as digestate alkalinity and they have a much smaller yield than heterotrophs; therefore, the amount of biomass synthesized per substrate removed is much lower. Since only half of the ammonium needs to be oxidized to nitrite, the oxygen input is much smaller than for conventional or short-cut nitrification/denitrification trains. Furthermore, unlike AOB and heterotrophs, nitrous oxide is not part of AnAOB metabolism hence reduces the likelihood of its emission. Overall, autotrophic nitrogen removal is a cost-effective, low footprint treatment alternative for nitrogen rich wastewater streams. Another aspect related to carbon and energy in WWTP scheme is that, by diverging more nitrogen to digestate liquor stream, more of the incoming carbon (primary sludge) can be diverted to anaerobic digestion in the upstream that can be recovered as energy (methane) rather than supporting denitrification and producing CO_2 (Siegrist et al., 2008).

Table 1. Comparison of oxygen, organic carbon requirements and sludge production per ammonium nitrogen removed for conventional nitrification/denitrification, shortcut nitritation/denitritation and nitritation/anammox.

	Conventional nitrification/denitrification	Short-cut nitritation/denitritation	Completely autotrophic nitritation/anammox
Aeration demand (kgO_2/kgN) [1]	4.24	3.16	1.81
Organic carbon demand C/N (kgCOD/kgN) [1]	3.63	2.26	0
Sludge production biomass yield (kgVSS/kgN) [1]	0.61	0.46	0.12

[1]Stoichiometry from equations

Many CANR configurations have been independently developed, with many claiming their own process acronym. Major categorization has been as two-

stage or single-stage operation. Since nitrate-shunt or nitritation was already of interest, SHARON (single reactor high activity ammonia removal over nitrite) was developed (Hellinga et al., 1998). Originally designed as a CSTR with no biomass retention to oxidize reject water only to nitrite, the effluent was directed to mainstream denitritation. Later, operating the process to nitritate only half of the ammonium, it was proceeded to a single ANAMMOX reactor and this became the first full scale application. On the other side, following (Mulder et al., 1995), in the absence of proper identification of AnAOB apart from ammonium:nitrite stoichiometry (Strous et al., 1998; van de Graaf et al., 1996), many single-stage systems have been developed. Aerobic deammonification (Hippen et al., 1997), OLAND (oxygen limited autotrophic nitrification-denitrification) (Kuai and Verstraete, 1998), nitrogen loss in a nitrifying biofilm (Siegrist et al., 1998) and aerobic/anoxic deammonification (Seyfried and Hippen, 2001) were among the first systems that reported autotrophic nitrogen removal taking place in a single reactor. Then with proper identification of anammox followed CANON (completely autotrophic nitrogen removal over nitrite) (Sliekers et al., 2002; Third et al., 2001), SNAP (single-stage nitrogen removal using anammox and partial nitritation) (Lieu et al., 2005) and DEMON (pH controlled deammonification) (Wett, 2007).

Another aspect differentiating the different single-stage systems is the biomass immobilization method. Since the process depends on two autotrophic groups with low yield, the growth and retention of the biomass is crucial and systems often benefit from biofilm technologies. For this purpose, different suspended and attached growth systems emerged. OLAND and CANON processes use suspended biomass. Suspended aggregates/granules are the most common in sequencing batch reactor (SBR) schemes like CANON and DEMON (Strous et al., 1997). Continuous systems include plastic carriers in moving bed biofilm reactor (MBBR) (Helmer et al., 2001; Hippen et al., 1997), also the full scale ANITAMox (Christensson et al., 2013) including hybrid IFAS configuration (integrated fixed-film activated sludge) (Veuillet et al., 2014); rotating biological disc (RBC) biofilm (Pynaert et al., 2004; Siegrist et al., 1998); fixed-bed biofilm acryl-resin fibre SNAP (Furukawa et al., 2006; Lieu et al., 2005); membrane aerated biofilm reactor (MABR) (Pellicer-Nàcher et al., 2010; Terada et al., 2007).

Currently almost 100 full-scale plants are in operation and the majority employ single reactor option (Lackner et al., 2014; Vlaeminck et al., 2012). The obvious advantage of a single reactor is the reduced space and instrumenta-

tion requirement. While in two-stage systems, conditions can be optimized at steady-state for either nitritation or anammox, a single reactor provides more flexibility to control internal dynamics. As will be discussed in the section on management of the microbial community, intermediate handling can be more advantageous in single reactor system. Majority of the full-scale systems utilize sequencing batch reactors. SBR enables high SRT, ease in handling variable loads and reduced space. For rehabilitation or upgrading of existing WWTP, it is a more adaptable configuration than air-lift reactors. However, so far air-lift granular systems handle higher specific nitrogen loads. Treatment ranges of single-stage systems range from 40 to 650 $g/m^3.d$ in suspended SBR systems, up to 1200 $g/m^3.d$ in MBBR and strictly granular systems (Lackner et al., 2014).

1.2 Microbial community management in single-stage CANR

1.2.1 Interactions between key functional guilds

The microbial communities in wastewater treatment systems are mixed culture with complex interactions among their constituting guilds. For single-stage CANR process, the major community members of interest are AOB and AnAOB, yet the success of the process relies on their correct management as well as the other community members involved in the nitrogen cycle; NOB and HB.

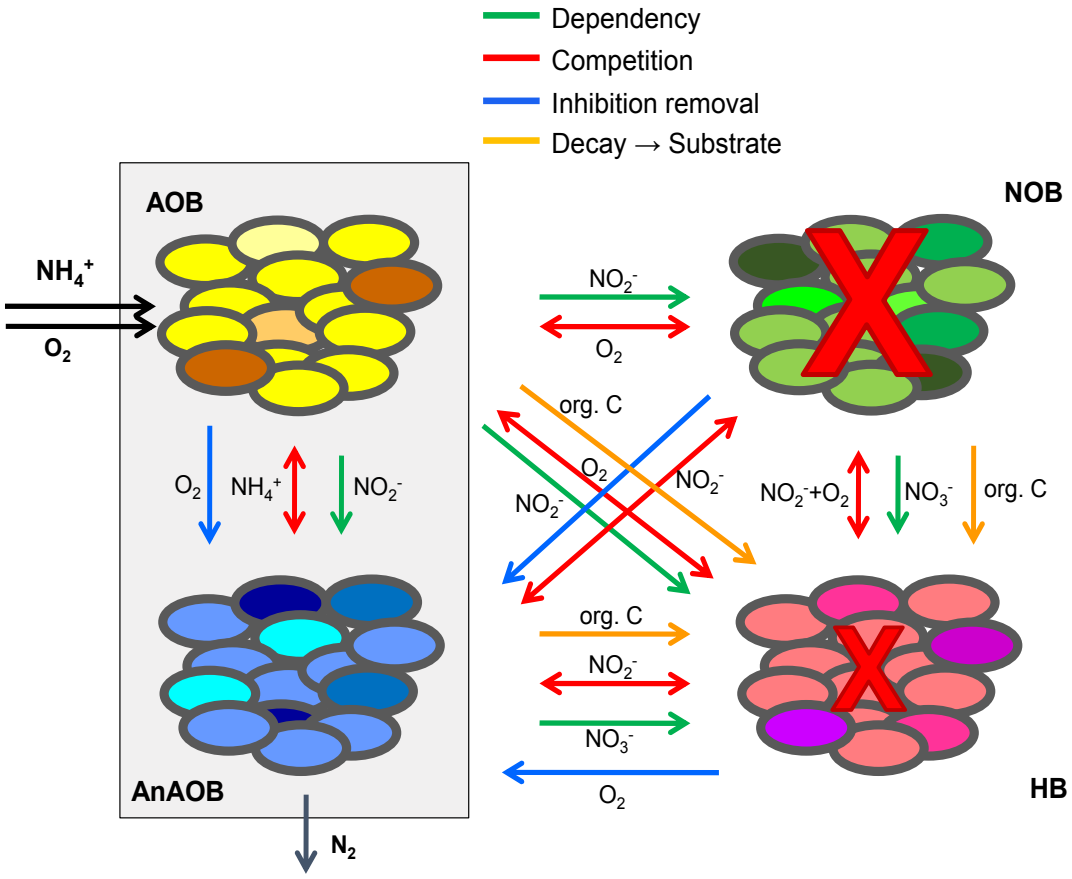

Figure 5. Interactions between key functional guilds in CANR system, adapted from (Vangsgaard, 2013).

In the ideal case, AOB would be the limiting members; their performance depending only on the ammonium and oxygen loading to the system. AOB produce nitrite, the electron acceptor for AnAOB, yet they compete for the ammonium substrate. Nitrite production should be commensurate with consumption by AnAOB, otherwise AnAOB can be inhibited irreversibly. At the

same time, AOB are required to completely consume the oxygen which would otherwise inhibit AnAOB reversibly.

A major complexity in CANR management is the suppression of NOB. NOB depend on AOB for nitrite substrate and compete for oxygen. Most disconcertingly, NOB compete with AnAOB for nitrite, although this can be beneficial when nitrite is at inhibitory levels for AnAOB.

Presence of HB is in most cases inevitable. Even if no or little organic carbon is present in the feed, decay products of other microbial groups, which fix inorganic carbon, are always released; providing organic carbon substrate for HB. Denitrifying HB compete with both AnAOB and NOB for nitrite produced by AOB, while aerobic HB compete with both AOB and NOB for oxygen. At the same time, as denitrifiers they can consume the nitrate produced by AnAOB and NOB. In this sense, HB working under limitation could be beneficial to the overall system performance, helping AnAOB by removing oxygen and diminishing the effluent nitrate. However, with their yield higher than autotrophs, it is likely that they take over if not well kept under control.

The substrate requirements and growth stoichiometry are not the only determinant of competition in microbial systems. The kinetics of the different functional groups as well as the diversity within each group are also key to the management of these community. Microbial growth kinetics in relation to substrate availability is often described by Monod equation (Equation 1.14), where μ is the specific growth rate, S the substrate concentration and K_s the substrate half-saturation constant. In terms of kinetics, two types of ecological strategies are commonly contrasted. The r-strategists have high maximum growth rate but low substrate affinity, while K-strategists have the opposite characteristics and typically higher yield than the r-strategists (Figure 6). As a consequence, K-strategists would thrive under oligotrophic conditions while the r-strategists dominate nutrient-rich habitats.

$$\mu = \mu_{max} \frac{S}{K_s + S} \tag{1.14}$$

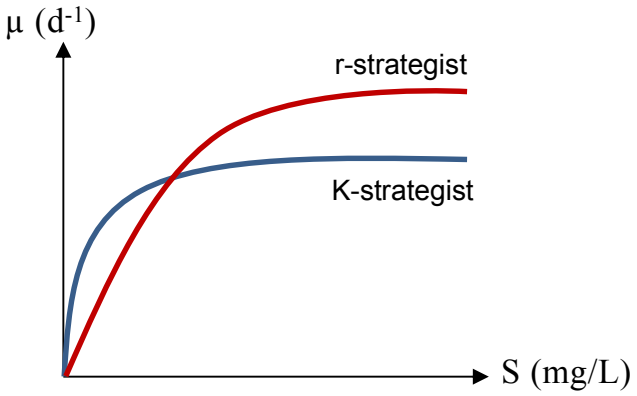

Figure 6. Growth strategies r- and K- for bacteria with respect to available substrate concentration

AOB versus NOB

Nitrite oxidation is thermodynamically less favourable than ammonia oxidation ($\Delta G^{0\prime}$=-75 kJ/mol versus -275 kJ/mol), resulting in lower cell yields than ammonia oxidation (Y=0.08gVSS/gNO_2^--N versus 0.33gVSS/gNH_4^+-N). Although the maximum growth rate (μ_{max}) of AOB is higher, mixotrophic growth of NOB can result in rates comparable with that of AOB. The affinity of AOB to oxygen is most of the time reported to be higher than that of NOB. The half–saturation constant for oxygen (K_{O2}) has been a key selective parameter for shunting nitrite oxidation. However, like for other kinetic parameters, the diversity within the functional groups is considerable. For instance, the K_{O2} values reported for *Nitrobacter* (0.17-8.2mgO_2/L) (Blackburne et al., 2007; Laanbroek and Gerards, 1993; Laanbroek et al., 1994; Prosser, 1989) seem to be high compared to *Nitrosomonas* (0.033-1.21mgO_2/L) (Blackburne et al., 2008; Laanbroek and Gerards, 1993; Prosser, 1989). *Nitrospira* may have higher oxygen affinity than *Nitrosomonas* but lower than *Nitrosospira*. Even within the same lineage, these constants may differ at strain level. The higher versatility of *N. hamburgensis* towards mixotrophy compared to *N. winogradsky* brings it additional fitness, enabling higher growth rates, as opposed to AOB (Laanbroek et al., 1994). Another issue is the bias from mass transfer limitations when determining these constants. The large variance in reported K_{O2} is possibly caused by the inadequate consideration of biomass structure (whether in single cell or aggregate form). As a consequence, many affinity values in the literature are to be considered as "apparent" rather than "intrinsic". Hence, there is not a consensus on nitrification kinetics (Lackner and Smets, 2012).

Free nitrous acid (FNA) and free ammonia (FA) are also known to inhibit both AOB and NOB. NOB have a lower inhibition threshold level than AOB; 0.011-0.22 versus 0.42-1.72 mgHNO_2-N/L (Vadivelu et al., 2007, 2006; Zhou et al., 2011) and 6.0 mgNH_3-N/L versus 16 mgNH_3-N/L (Vadivelu et al., 2007, 2006) where *Nitrospira* can be more sensitive 0.3mg HNO_2-N/L and 0.04-0.08mgNH_3-N/L (Blackburne et al., 2007).

On the other hand, both AOB and NOB have been shown to acclimate to limiting environmental conditions of low DO (Arnaldos et al., 2013; Park and Noguera, 2004) or high FA and FNA (Turk and Mavinic, 1989, 1986) especially when SRT is high; hence, the community can manifest different affinity and rates after being exposed for long periods to a certain limiting condition. In addition, hydroxylamine, the intermediate of AOB during ammonia oxidation, can reportedly inhibit NOB (*Nitrobacter* spp.) (Stüven et al., 1992).

AOB and NOB metabolic activity is also differentially affected by temporal variation in oxygen supply. One consistent observation is the accumulation of nitrite when a mixed nitrifying culture is exposed to transient anoxia. In processes with intermittent aeration there appears to be a lag in nitrite oxidation activity after a transient anoxia. The extent of NOB lag has been related to the long duration of anoxia as well as the high acclimated DO concentrations (Gilbert et al., 2014; Kornaros et al., 2010; Mota et al., 2005). Correspondingly, shorter duration of aeration has led to incomplete recovery of NOB, while AOB were not affected (Abeling and Seyfried, 1992; Turk and Mavinic, 1989).

AnAOB versus NOB

The anammox reaction is energetically very favourable ($\Delta G^{0\prime}$=-357 kJ/mol) but affords extremely slow growth rates compared to NOB. Therefore any condition favourable for NOB has the potential to select against AnAOB. As nitrite, the common substrate is produced under aerobic conditions, NOB have a considerable advantage over AnAOB unless outcompeted by AOB or suppressed by a discriminant inhibitor. AnAOB activity can support NOB suppression as long as nitrite is available under anoxic conditions. Correspondingly, the lag phase of NOB after anoxia provides an advantage to AnAOB as they can utilize more of the produced nitrite during the next anoxic period or at deeper anoxic parts of a biofilm where nitrite may diffuse. The reported apparent half-saturation constants of AnAOB for nitrite range from K_{NO2}=0.028-0.07 mgN/L while intrinsic values can be even lower. In either

case, AnAOB seem to have higher affinity to nitrite than NOB whose K_{NO2} range from 0.9-17.4 mgN/L with higher affinity of *Nitrospira* compared to *Nitrobacter* (Blackburne et al., 2007; Laanbroek and Gerards, 1993; Laanbroek et al., 1994). Again considering K- and r- strategists, *Nitrospira* possibly have an advantage over *Nitrobacter* in competition against AnAOB. At the same time, like NOB, AnAOB also risk inhibition by nitrite and by FNA at even lower concentrations of 0.006mgHNO_2-N/L for *Brocadia* (Strous et al., 1999) and 0.04mgHNO_2-N/L for *Kuenenia* (Egli et al., 2001).

AOB versus AnAOB

Anaerobic ammonia oxidation is energetically more favorable than aerobic ammonia oxidation and they afford similar cell yields; however, AnAOB are again the slow growers compared to AOB. AOB are rather self-sufficient in this system, while AnAOB have a commensal association to AOB for providing nitrite and keeping oxygen concentration low. While AnAOB have higher affinity for ammonium (K_{NH3}=0.0012 (Strous et al., 1999) versus lowest 0.03 mgN/L (Koops and Pommerening-Roser, 2001)); they are dependent on AOB to obtain nitrite. It is however important that the nitrite production rates of AOB are consistent with AnAOB utilization rates, otherwise AnAOB can be irreversibly inhibited by elevated nitrite concentrations. One benefit of AnAOB for AOB could arise in the presence of toxic nitric oxide, which can be produced by AOB under oxygen limitation, but can be utilized by AnAOB without any toxic effects (Kartal et al., 2010).

HB versus autotrophs

Denitrification with organic carbon by HB is much more energetically favourable than autotrophic catabolic reactions and therefore HB grow 2-3 times faster and have up to 4 times higher yield than autotrophs. The high yield of heterotrophs brings great advantage over autotrophs. On the other hand, their higher anoxic decay rates are in disadvantage to autotrophs (Geets et al., 2006). Although they are dependent on autotrophs they are equipped to compete for multiple substrates. Aerobic heterotrophic metabolism can also be a major disturbance as competition for oxygen will affect the precursor of the whole process carried out by AOB.

Different AnAOB species can outcompete HB oxidizing organic acids in presence of ammonium, nitrite and nitrate (Kartal et al., 2008). Higher nitrite affinity of AnAOB (0.06-0.35mgN/L for HB (Almeida et al., 1995)) can in fact overthrow heterotrophic denitrifiers with organoheterotrophic AnAOB activity in the system (Winkler et al., 2012).

Heterotrophs can coexist with autotrophs and contribute to the nitrogen removal. Though they would mask the extent of NOB activity the process may achieve higher overall nitrogen removals.

1.2.2 Biomass architecture: aggregation and spatial heterogeneity

Biofilms enable process intensification, higher removal capacity with enhanced loading rates due to spatial specialization in dense structures. In CANR systems this could be achieved by providing an inert substratum (plastic carriers, fibers, oxygen diffusive membranes, etc.) or by inducing aggregation of bacteria to form granules. Granulation has been a widespread biomass retention technique and is preferable over substratum attached biofilms as it does not require additional carrier material. Although analogous to surface biofilms in terms of spatial heterogeneity, but of spherical form, the concept of granulation is more complex. What exactly triggers aggregation and what is needed to maintain the aggregate architecture, are neither well-understood nor described concretely. Hence, in this section the subject will be discussed in the broad context of microbial granulation in wastewater treatment systems.

Extracellular polymeric substances: The gluing matrix

Essentially, granules are dense aggregates of cells. An integral part of these microbial architectures is the interconnecting matrix composed of a conglomerate of extracellular polymeric substances (EPS). This matrix, which protects cells from the bulk environment forms over 90% of the dry mass (Flemming and Wingender, 2010) and is mainly composed of mainly carbohydrates, proteins, extracellular DNA, lipids and humic acids. The wide range of polymers with many charged sites harbor adhesive, sorptive as well as cohesive and repulsive properties to form three-dimensional structures at molecular level. Such can be as electrostatic or ionic attractive forces, van der Waals forces, hydrogen bonding. For example, alginate, a well-studied exopolysaccharide, is thought to stimulate strongly cell-to-cell aggregation into microcolonies when substituted with acetyl groups. Also, carboxylic acid groups of anionic EPS interacting with multivalent cations such as Ca^{2+} can be mechanically determinant in architecture. Another important function of EPS is the capacity of this matrix to retain water, nutrients, enzymes as well as genetic material. The retention capacity serves as source of energy under starvation, enables metabolic turnover also provided by degradation of EPS with exoenzymes. EPS can be actively secreted by bacteria but can also be considered to constitute remains of decayed cells. Structurally, EPS has been

classified according to ease of extraction as tightly-bound capsular EPS immediate to cell surface forming strong clusters, as detachable loosely-bound EPS interconnecting microcolonies and as soluble EPS that is lightly adsorbed and easy to extract (Nielsen and Jahn, 1999). From the perspective of production and turnover, a further classification regards soluble EPS as soluble microbial products (SMP) (Laspidou and Rittman, 2002). SMP is further divided as utilization-associated products (UAP) which is a side-product of substrate utilization for growth and as biomass-associated products (BAP) which is a product of cell decay or hydrolysis of bound EPS. Bound EPS is also regarded to be actively synthesized by the cells in proportion to substrate utilization like UAP but is considered to be part of active cell. While SMP are biodegradable hence can be used as electron donors, there is also an inert fraction that is residue of true dead cells.

For aerobic granulation, gel-forming alginate-like polysaccharides and Granulan extracted from carbon, nitrogen and phosphorous removing granules have been suggested as major inducers (Lin et al., 2010; Seviour et al., 2009). Calcium can structurally stabilize alginate or bridge between negatively charged moieties (Sobeck and Higgins, 2002) and has been suggested to be essential for granulation of anammox biomass (de Graaff et al., 2011). On the other hand, polysaccharides are not the only gel forming components; proteins namely gelatine and mucins also have gel-forming properties, though with higher critical gelling concentrations than polysaccharides (Seviour et al., 2012). Indeed in wastewater treatment biofilms, proteins were found to be more abundant than polysaccharides (Frølund et al., 1996) and can have structural importance such as cell-surface associated lectins that bind carbohydrates, or amyloid adhesins that are insoluble and highly resistant to denaturation (Larsen et al., 2008a). Extracellular DNA (eDNA) also seems crucial in wastewater biofilms (Frølund et al., 1996) and can indeed be actively secreted as adhesin as is found to show differences from genomic DNA (Molin, 2003). Nitrifying bacteria are known to form especially strong and dense microcolonies with a large amount of EPS. It is tempting to relate this property with the extreme resistance of *Nitrosomonas oligotropha* and *Nitrospira* spp. to extreme stresses caused by shear, alkaline, acidic or chelator (to remove divalent cation) treatments (Larsen et al., 2008b). eDNA has been most abundantly identified around denitrifiers as well as *Nitrosomonas* and *Nitrospira* microcolonies (Dominiak et al., 2011), while amyloids were found to be produced mainly by denitrifiers of *Alpha-* and *Betaproteobaacteria* and many filamentous bacteria but not by nitrifiers (Larsen et al., 2008a). Compared to

AOB, AnAOB enriched biomass has been found to contain higher fraction of proteins than polysaccharides and specifically no β-polysaccharides (Vlaeminck et al., 2010; Yin et al., 2015).

Mechanisms and triggers for aggregation and granulation

The initial step of aggregate formation is through cell-to-cell interactions similar to bacterial adhesion to surface. The transport of cells to each other could be due to Brownian motion, gravitation, diffusion, convection or intrinsic motility (Bos et al., 1999). Then initial adhesion is overall governed by long-range interactions that depend on the physico-chemical surface properties and the liquid medium properties such as ionic strength (Loosdrecht and Zehnder, 1990), and can be explained by colloidal mechanisms such as DLVO (Derjaguin, Landau, Verwey, Overbeek) where Lifshitz-van der Waals and electrostatic forces are accounted for. These forces are rather weak hence initial adhesion is reversible (Bos et al., 1999), yet they allow time for stronger interactions to evolve. The irreversible attachment involves stronger short-range interactions like hydrophobic attractive and hydrophilic repulsive forces as included in extended DLVO with Lewis acid-base interactions (Van Oss et al., 1986). Nevertheless, the bacterial adhesion is different than adhesion of inert substances as the surface and physico-chemical properties of a biological entity is more dynamic and responsive to environmental conditions (Hermansson, 1999). Bacteria are overall negatively charged at their surface and at the diffuse layer, while certain surface appendages may have different moieties. Correspondingly, multivalent cations can also support reduction of the electrostatic barrier or bridge between negative moieties (Hoygaard Bruus et al., 1992). Cell surface hydrophobicity (CSH), which may change with growth rate (Loosdrecht et al., 1987), typically differs between species. CSH can also vary with the growth substrate; proteins and carbohydrates enriched granules and aerobic cells tend to be more hydrophilic while fatty acid enriched granules and anaerobic cells tend to be more hydrophobic. Also, within granules, a layering of hydrophobicity has been hypothesized (Daffonchio et al., 1995). As previously explained, EPS can have a major impact on interactions and is greatly a time-dependent factor. EPS synthesis is found to be upregulated upon initial attachment leading to irreversible attachment and to remain so (Davies and Geesey, 1995). With irreversible attachment and sustained production of EPS, aggregates can then grow by adhered binary division or recruit other planktonic cells (Stoodley et al., 2002). The strength of these interactions still follow colloidal chemistry depending on the composition of the exopolymers.

As outlined, the intermolecular interactions can be complex. For certain species these properties may be innate under certain environmental condition; however, the triggers (genetic or environmental) for actively switching from planktonic to biofilm mode in bacteria is still a black box. In fact, it has been argued that biofilm mode of life could be the default one (Jefferson, 2004; McDougald et al., 2012). Aggregative behavior does provide advantages over planktonic mode. Bacteria could respond by aggregative behaviors to environmental adversities such as physical or chemical stress, in defense from predators, to sequester nutrients against starving, to maximize substrate availability in syntrophic or symbiotic interactions with other. In this sense, bacteria can bring about phenotypic changes, down regulating expression of motility related genes and upregulating the expression of genes that lead to surface properties favorable to adhesion, as mentioned above (Bossier and Verstraete, 1996; Jefferson, 2004). Shear stress can cause conformational changes in membrane bound lectins and induce adhesion (Thomas et al., 2002). Nitric oxide is also an important intracellular signalling molecule regulator for biofilm dispersal (Barnes et al., 2013) but has also been shown to induce biofilm growth in planktonic *N. europaea* (Schmidt et al., 2004). Lastly, the role of quorum sensing and signaling has been debated to ascertain whether aggregative behaviors are based on individually experienced responses or whether there exist a population scale coordination. In many species quorum sensing signaling with *N*-acyl-homoserine-lactones (AHLs) is involved in regulation of EPS secretion through c-di-GMP cycle (McDougald et al., 2012; Pamp et al., 2007). Increased AHLs in medium have been correlated to granulation (Tan et al., 2014) but whether if it is a cause or effect has not been clarified.

The stability of aggregates largely depends not only on the composition and strength of EPS but also on other factors, like biological activity. The morphological development of aggregates are mostly determined by the balance between growth rate and detachment forces (Loosdrecht and Eikelboom, 1995). There is a general consensus, validated by mathematical models and experiments, that fast growth and low shear stress will lead to loose, porous, open aggregates while slow growth or high shear on fast growing aggregates would lead to more compact structures (Picioreanu et al., 2000).

In many wastewater systems, such as in activated sludge, floc is the most common bacterial aggregate structures. Although possibly evolving from similar initial cell-to-cell interactions, they differ greatly from granules. Granules have much more compact structure with biofilms-like high density

and fast settleability, while flocs are more loosely bound fractal structures with lower quality of settling. These properties partly derive from the microbial composition of the biomass such as higher abundance of filamentous organisms in floccular biomass leading to irregular structures. The compact structure of granules is accompanied by high spatial heterogeneity, forming well organized microniches driven majorly by diffusion. Most distinctively, unlike flocs, granules do not coagulate under reduced hydrodynamic shear (de Kreuk et al., 2007), which relates to the irreversibility of adhesion characteristics. The differentiating mechanisms behind these two types of aggregative structures remain to this date largely debatable.

Granules for wastewater treatment

Granules in wastewater treatment have been first realized in upflow anaerobic sludge blanket (UASB) reactors utilized for anaerobic treatment which requires high biomass retention capacity (Lettinga et al., 1980). Initially thought to be specific to certain methanogenic bacteria and upflow mixing pattern, granules have later been formed from different groups of anaerobic and aerobic microorganisms (Beun et al., 1999; Hulshoff Pol et al., 2004), in different reactor configurations and with different mixing patterns (de Kreuk and van Loosdrecht, 2004; Morgenroth et al., 1997; Weissbrodt et al., 2013). Specific to aerobic granules is the existence, in addition to substrate gradients, of redox gradients that enable anaerobic and aerobic metabolisms to take place within the same granule. Many theories, prerequisite conditions and mechanisms of aggregation and evolution of architecture that depend on physical, chemical and biological aspects have been proposed (Adav et al., 2008; Beun et al., 1999; de Kreuk et al., 2007; Hulshoff Pol et al., 2004; Vlaeminck et al., 2010). Nevertheless, there is some consensus on supporting operational conditions and influencing parameters. Substrate composition and loading regimes, hydrodynamic conditions, physical selection (settling/washout) and composition of seed sludge count among the most influential parameters.

Substrate composition and loading regime can differentiate the microbial community composition and diversity through selection of bacteria with different metabolism and growths rates. As in aerobic C, N, P removing granules, feast-famine cycles which alternate aeration regime can select for slow growing bacteria that can store the substrate under anaerobic conditions over fast growing bacteria that lack this ability hence cannot access the substrate (de Kreuk and van Loosdrecht, 2004). At the same time the composition of the medium is important in terms of ionic strength and availability of polyva-

lent cations. Low settling times allow selection of well settling aggregates over less compact floccular aggregates which can accommodate fast growing organisms that would compete with slower growing compact aggregates. Hydrodynamic conditions can be considered as mixing and shear. Mixing is crucial for increasing chances of aggregate coalescence as well as for control of mass transport and substrate distribution in the reactor. Shear forces enable compaction and shaping of loose aggregates by increasing detachment of protuding structures. Although shear stress has been suggested to induce granulation through enhanced EPS production (Liu and Tay, 2001) yet there is no mechanistic evidence at cellular response level. Here it should be noted that, aeration regime can be classified as influential both at the substrate loading regime level, determining the oxygen concentration, and at the hydrodynamics level, where it affects the mixing and shear regime. In many studies, this duality of aeration is not considered. In conclusion, operational conditions and parameters for granulation are possibly not universal to all types of granules due to the microbial composition and interactions that are different for anaerobic to aerobic and to nitritation-anammox systems.

Microbial composition and spatial dynamics

In terms of community composition, specific interactions have been reported between certain bacterial groups. At strain level, bacterial autoaggregation, defined as aggregation of genetically identical cells, and coaggregation, for genetically different cells, have been most scrutinized in dental biofilms and mostly attributed to lectin-carbohydrate interactions on their surfaces (Kinder and Holt, 1994; Kolenbrander, 1988). Coaggregation could be more stably driven by syntrophic relations that are unlikely in autoaggregates (Gieseke et al., 2003; Rickard et al., 2004). In anaerobic granules, the tightly coupled mutualistic interaction between fermentative bacteria and methanogenic archaea through hydrogen or formate exchange supports conserving energy (Sieber et al., 2012). In nitrifying biofilms, coaggregation of NOB with AOB as in the case for *Nitrospira* with *Nitrosomonas oligotropha* can be related to the affinity of NOB to nitrite produced by AOB (Juretschko et al., 1998; Schramm et al., 1999). Despite being at the same trophic level, *Nitrosomonas europaea* and *Nitrosomonas mobili*s have also been reported to coaggregate (Gieseke et al., 2003) which might be explained by siderophore parasitism of *N. europaea* from *N. mobilis* (Almstrand et al., 2013), a kind of symbiosis.

As compact aggregates grow, spatial heterogeneities form due to diffusion limitations. Driven by chemical heterogeneities, the community composition and architecture also differentiates and may mature into a substantially dif-

ferent community composition. Major selection criteria are result from the gradients of substrate and redox conditions inside the aggregate (de Beer et al., 1994; Schramm et al., 2000). The biological stratification in aggregate can thus exist between different functional guilds but also at physiological level between the members of the same species since they can be of different age or with different genetic expression (Stewart and Franklin, 2008).

In CANR biofilms, the co- or counter- diffusion of oxygen and ammonium determine the spatial distribution of different functional guilds (Figure 6). In a co-diffusion biofilm like in suspended aggregates or MBBR carriers, the precursor AOB reside on the rim in contact with the bulk liquid where the substrate ammonium and oxygen are provided; while AnAOB reside deeper into the biofilm where inhibitory oxygen is depleted yet ammonium and nitrite produced by AOB can diffuse inside. The size of the aggregate or the thickness of the biofilm are also then determined by the extent of substrate diffusion. On the other hand, in a counter-diffusion biofilm as in MABR, AOB grow on the membrane substratum through which oxygen is supplied with ammonium diffusing from the bulk liquid on the other side. AnAOB proliferate on the biofilm-liquid interface where ammonium supply is ample, while nitrite is produced and oxygen is depleted by AOB on the other side (Pellicer-Nàcher et al., 2014) .

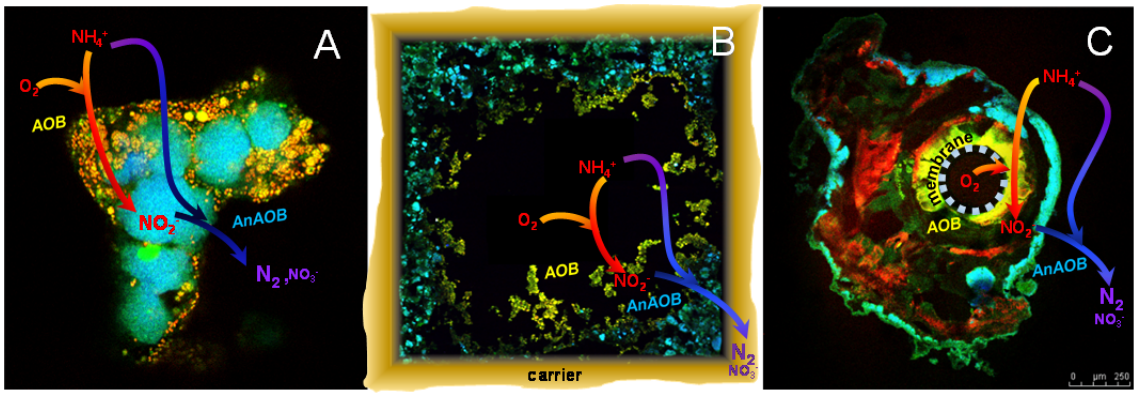

Figure 7. Different biomass immobilization types forming co-diffusion biofilm as in (A) granular aggregates (this study) (B) on MBBR carrier (Almstrand et al., 2014) or counter-diffusion biofilm (C) on MABR membrane (Pellicer-Nàcher, 2013).

Despite this ideal kinetic/mass-transfer concept, in reality architectures in CANR systems do not always have this stratification. The evolution of architecture may be quite different when the starting inoculum is anammox or aerobic/nitrifying granule/biofilm rather than floccular sludge. (Cho et al., 2011, 2010), showed that start-up by aerating anammox granules and biofilm led to stratified AOB while by decreasing DO for nitritating biofilm led to irregular

AOB architecture. Similar to (Veuillet et al., 2014) the newly grown AOB may wash out if not coaggregating with AnAOB layering on the oxygenated surface in anammox based; whereas, AnAOB can grow in any oxygen depleted parts of the nitritating biofilm that is defined more by diffusion limitation and homogeneity of mass transfer. On the other hand, (Vázquez-Padín et al., 2009)and (Winkler et al., 2011) achieved stratified architecture by incorporating AnAOB into aerobic granules with additional anammox seeding under pulse-aeration and with additional nitrite feeding under anoxia, respectively. In these cases, AnAOB dependence on AOB in terms of substrate is slightly leveraged while the oxygen inhibition and size selection are the main drivers for AnAOB niche within the granules architecture, as long as the small AOB are washed out.

1.2.3 Monitoring, operation and control

Monitoring

Autotrophic nitrogen removal in one reactor is a complex system due to the interactions of different functional groups that are sensitive to intermediates and redox conditions. Simultaneous activity of these microorganisms also requires close monitoring of environmental variables. Many systems employ online monitoring as well as offline measurements to ensure successful operation.

Dissolved oxygen (DO) is a key variable as it governs the extent of the overall process. Considering the effect of oxygen on each functional group as a driver or inhibitor and as a potential cause of nitrous oxide emissions, its monitoring is essential. The DO relative to half-saturation concentrations of different species can also have a selective role. In an oxygen-limited system like single-stage CANR, the operational DO ranges from 0.05-1.5 mg/L to below detection limit of sensors. Therefore, it may not provide sensitive feedback for the system operation unless for extreme aeration cases. This is especially true considering that the sensitivity of many commercial DO probes based on Clark-type electrode decreases with decreasing concentrations. In this sense, optodes with higher sensitivity at lower range or Clark-type microsensors with lower range could be beneficial, but their sensitivity to stirring, life-time and calibration requirements should be considered for long-term and full-scale use.

The pH is also a key variable affecting microbial metabolism. For most of the essential functional guilds in autotrophic nitrogen removal, the optimal range lies between ca 7.0 and 8.0. The alkalinity provided by bicarbonate as inor-

ganic carbon and the buffering capacity of the solution are regulators of pH in the system. Additionally, stripping caused by stirring and aeration background charge should also be accounted for in pH monitoring (Vangsgaard et al., 2013). Monitoring of pH can be used as an indicator of substrate consumption due to the concomitant alkalinity consumption or production (Wett, 2007). The equilibrium between ammonium and FA, nitrite and FNA, rely on pH. Hence pH, together with nitrogen species, can be crucial for selective inhibition of bacterial groups. High and low pH inhibit AnAOB and AOB, respectively.

Temperature is important as it affects all the biochemical reactions as well as physico-chemical transport rates. A majority of side-stream application utilize relatively high temperatures of 25-32^{o}C associated with anaerobic digestion. Low temperature is an important factor for consideration in colder climates and for mainstream applications, which may require changing other operational parameters and range of set-points.

The concentrations of nitrogen species, mainly ammonium, nitrate and nitrite provide direct information about process performance. Ammonium and nitrate sensors are relatively accessible while nitrite sensors have been recently developed. Unlike for the more conventional DO, pH and ORP (oxidation-reduction potential, see below) sensors, there is still room for improving the robustness and stability of theses sensors. However, when available, they directly provide process efficiency values. The relative consumption and production rates provide valuable feedback on the balance between the activities of the functional groups.

A differentiating variable that incorporates many aspects is the oxidation-reduction potential (ORP). ORP can differentiate between anoxic and oxic conditions but there are many chemical species in water that contribute to ORP, so ORP sensors require system specific calibration (Lackner and Horn, 2012; Lackner et al., 2012). It can be correlated to air flow rate and ammonium depletion. Although employed in conventional nitrification/denitrification systems, its use in nitritation/anammox systems is more complicated since the process is operated at intermediate conditions such as shunting nitrate which cannot be differentiated with ORP. However a swinging strategy provides indirect yet rather cheaper feedback.

Nitrous oxide is a relatively new parameter in WWTP monitoring (Kampschreur et al., 2009b); however, it is increasingly considered quite critical for nitritation-anammox systems. The measurement methods are still un-

der development, it is most often measured in liquid phase with Clark-type sensors or in gas phase emission with gas chromatography with floating flux chambers at full-scale. In order to determine gaseous emissions from liquid phase the stripping rate (correlated to stirring and aeration rates) need also to be known. Apart from increased footprint measures, N_2O can be used as an indicator for imbalanced or limited performance of different functional guilds, especially of AOB (Wunderlin et al., 2013). Considering different pathways it can also indicate the presence and contribution of heterotrophs in the system.

In suspended systems the importance of particle size distribution (PSD) is becoming more and more recognized. Unlike fixed biofilm systems, granular aggregate systems show differentiating activities with size. Due to extent of substrate and redox gradients (Nielsen et al., 2005; Vázquez-Padín et al., 2010), different aggregate sizes can provide different microniches and house different ratios of functional groups and species. Together with extant potential activity assays and molecular techniques such as quantitative polymerase chain reaction (qPCR) and fluorescence in-situ hybridization (FISH) targeting different functional groups revealed different distribution and activity trends in different aggregate sizes (Gilbert et al., 2013; Vázquez-Padín et al., 2010; Vlaeminck et al., 2010; Winkler et al., 2011). Hence the overall size distribution which can also be an indicator of transitions between planktonic, floccular or granular biomass can have major impacts on the performance as also predicted with modeling studies (Hubaux et al., 2014; Vangsgaard et al., 2012; Volcke et al., 2012). Measuring PSD can be fast and straight-forward with a laser diffractometer, yet is rather expensive and impractical for a WWTP. Instead, automatized image analysis with microscopy could be useful. Sludge volume index (SVI) is used for settleability characterization but to determine degree of granulation over flocs, consolidation index as SVI_5/SVI_{30} is a better indicator (Schwarzenbeck et al., 2004). Quantitative PCR has been deemed useful especially in start-up phases when enriching AnAOB (van der Star et al., 2007) but also for quantification of the NOB population (Mota et al., 2005; Nogueira and Melo, 2006) despite their suppression as reflected on overall performance. The relative low activities of NOB manifested during default operation may withhold potential to shift the overall performance in their favour when acute disturbances, such as oxygen overload, take place in the system.

Operation and Control

In order to optimize the effluent quality and removal efficiency of single-stage CANR, balanced high activity of the key functional groups AOB and AnAOB as well as suppression of NOB and, secondarily, of heterotrophic denitrifying bacteria are necessary. At the same time, the process should be able to handle pulse or pressed disturbances such as variable loading without compromising removal efficiency.

For the control of community, the primary task is the suppression of NOB and successful nitritation. To this end, many strategies have been implemented to favour AOB over NOB, starting with the short-cut denitritation process. Apart from oxygen, the concentrations of ammonium and nitrite substrates are crucial, considering the affinity of each group to their own energy source. Together with substrates, pH is important because it determines FA and FNA concentrations, it should be high enough for FA inhibition and low enough for FNA inhibition of NOB. In addition, temperature can discriminate the two guilds in terms of kinetics. AOB can outgrow NOB at temperatures above 25^{o}C (Hellinga et al., 1998), hence keeping a minimum SRT for AOB that is too just short for NOB enables AOB dominance. The SHARON process successfully exploits all these: continuous operation at high temperature, high FNA and short SRT. NOB suppression has also been demonstrated for a short-cut denitritation system in an intermittently aerated SBR. The operation, under high FNA and keeping high nitrite concentrations by controlling DO and pH achieved shunting nitratation (Lemaire et al., 2008). On the other hand, in many cases such as for CANR in one reactor, the FNA route does not work, due to the working pH range and the risk of nitrite inhibition of AnAOB. Also it has been reported that NOB can adapt to increasing FNA concentrations (Turk and Mavinic, 1989). Operation under low DO concentrations is the other most relied upon nitritation strategy which is based on exploiting the higher oxygen affinity of AOB over NOB. This has been supported by many observations (Blackburne et al., 2008; Dongen et al., 2001) but equally often it has been deemed unreliable and inconsistent (Jardin and Hennerkes, 2012; Joss et al., 2011). A few observed that nitritation can be achieved at high DO as long as intermittent aeration is applied (De Clippeleir et al., 2013; Pellicer-Nàcher et al., 2010; Regmi et al., 2014; Wett et al., 2013).

In systems where NOB have been suppressed, nitrite accumulation can cause upsets, due to insufficient AnAOB activity compared to the production by

AOB. In many cases, simply reducing the DO or introducing longer anoxic periods for AnAOB to gain activity is a successful strategy.

Consequently, aeration can have a high impact on the system. Aeration encompasses many factors. DO set-points can be unreliable for control, but air flow rate, which sets the oxygen loading to the system and the relative oxygen to ammonium loading, is essential in determining the extent of nitritation. At the same time, depending on the oxygen consumption rate, the DO of bulk liquid is set by the aeration rate even if DO may be undetectable by the sensors. Inducing intermittent aeration results in cyclic switching of redox conditions, which can induce the suppression of obligate aerobic organisms, production of N_2O, accumulation or depletion of intermediates like nitrite and nitrous oxide. On the physical side, aeration provides mixing, sets mass transfer rates and the shear applied on particles/biomass. Stripping of CO_2 by aeration can set the pH of the system, and stripping also affect emission rates of N_2O.

The majority of control strategies rely on manipulation of aeration. The main goals are not to inhibit AnAOB and to exploit the lag phase of NOB after transient anoxia to minimize nitratation. Major control parameters are air flow rate and in case of sequential or intermittent operation the duration of aerated and non-aerated periods. In different systems, these aeration parameters are set depending on the feedback from different online monitored variables. In the DEMON system, aeration duration is set based on discrete set-points for pH variance as an indicator of aerobic and anaerobic ammonium oxidation, incorporating stripping rate (Wett, 2007). Similarly pH variance has been used but overruled by time set-points (Jardin and Hennerkes, 2012). Other feedback parameters have been proposed to set aeration duration, mostly in suspended sludge systems. They include more direct activity indicators such as the ratio of nitrite produced to ammonium consumed (Jeanningros et al., 2010), effluent nitrite concentrations and conductivity as indicator for ammonium (Joss et al., 2009), and as more practical indicators for ammonium depletion and nitrate levels, swinging ORP amplitudes (Lackner et al., 2012). The DO set-point in the majority of these intermittently aerated systems was used as a secondary measure, and set to remain at values ranging from 0.3 to 0.8 mg/L. For the air flow rate, Joss and colleagues also used effluent ammonium, nitrite and nitrate to ammonium consumed ratio as feedback in a suspended system (Joss et al., 2011). Effluent ammonium (Abma et al., 2010) and extant potential activities for AOB, AnAOB and NOB (Vázquez-Padín et al., 2009) proved useful as feedback for control in granu-

lar systems. The amount of nitrate produced to ammonium consumed was used to set the DO through air flow rate in carrier based ANITAMox MBBR (Christensson et al., 2013). Lastly, in a continuous system, oxygenated recycling flow rate served as a control for air supply rate, successfully using the theoretical oxygen to ammonium loading rate as a set-point (Kwak et al., 2012).

SRT is another control parameter to selectively wash out certain community members. As mentioned above, this is done in SHARON process to selectively wash out NOB but this can also be exploited by applying different SRT for different particle sizes (Veuillet et al., 2014; Wett, 2007; Winkler et al., 2011). Some researchers washed out the less dense biomass particles containing more NOB. Others washed out small particles containing AOB in suspended sludge (Wett, 2007), while yet some other retained the AOB rich small suspended biomass in MABRs (Veuillet et al., 2014). Beyond selection of community member, SRT is also important for granulation and selection of well-settling dense biomass (Beun et al., 1999).

In terms of feeding regime for granulation it has been evaluated that high volumetric exchange ratios drive aerobic granulation (Liu et al., 2005) while for CANR low volumetric exchange ratios have been identified as more efficient (De Clippeleir et al., 2009; Lackner and Horn, 2012; Schaubroeck et al., 2012). Also short settling times practiced for most granulation systems does not seem to promote granular aggregates in CANR (De Clippeleir et al., 2009). Feast-famine in SBR may physiologically enhance aggregation.

N_2O emissions being a relatively new concern, control strategy to mitigate them are in their infancy. N_2O is mostly induced by transient conditions with nitrite peaks, anoxic-oxic cycling (Kampschreur et al., 2009b). Emission can either be caused by oxygen limitation for AOB and presence of oxygen or lack of organic carbon for heterotrophs. Apart from producers, consumers of intermediates must also be considered: AnAOB and NOB for nitrite, AnAOB for NO. Continuous operation with sufficient DO has been proposed as potential route to limit N_2O emissions (Vlaeminck et al., 2012). However, contradicting results exist on the comparative effects of continuous versus intermittent aeration (Joss et al., 2009; Yang et al., 2013). It is important to resolve these contradictions in order to optimize aeration and correctly evaluate the footprint of the system as compared to conventional alternatives.

1.3 Motivations and objectives of the PhD study

Many different measures are taken by operators in order to remediate imbalances or disturbances that impair maximal nitrogen removal efficiency. Although aeration is recognized as an important parameter that has effective consequences and is relatively easy to manipulate, the majority of the actions rely on the conventional - yet harder to monitor - DO set-points rather than on the air supply regime. In this sense, diagnosis of disturbances most of the time lacks an integrated process understanding. A systematic strategy needs to be devised to guide the manipulation of aeration regimes. Especially, a rationale for choosing among the different means of modifying oxygen loading (e.g. by modifying the instantaneous air supply rate or by introducing intermittent versus continuous aeration) is currently lacking.

Such rationale ideally needs to integrate the interplay between oxygen delivery strategy and the composition and architecture of the community. Due to their ability to enrich slow-growing microorganisms, SBRs are ubiquitously used for single-stage CANR. By nature, SBRs enable time-wise sequential operation and, with intermittent aeration, temporal gradients of oxygen as well as nitrogen species can be achieved. At the same time, the biomass in SBR can form granular aggregates that harbour spatially segregated metabolic activities. This provides opportunity to create oxic-anoxic redox gradients necessary for the process both temporally and spatially. We lack an understanding of how granules form in nitritation/anammox reactors and how their dynamic is affected by aeration regime, considering that aeration not only affects oxygen loading but also intermediate dynamics, mass transfer rates and shear stress on the granules.

The magnitude of nitrous oxide emissions is of critical importance for the advancement of CANR systems in biological nitrogen removal (BNR) market. The benefit of reduced cost and energy consumption by reduced aeration must indeed offset any potential increased N_2O emission footprint. Conditions of low dissolved oxygen, high nitrite and ammonium concentrations and dynamic aeration, which are typical present in single-stage CANR, require thorough evaluation for overall optimization of the process for low cost and footprint.

Dissolved oxygen and aeration appear to be crucial variables for management of nitritation/anammox SBRs, by their influence on attained kinetics, on mass transfer, and on biofilm/bioaggregate dynamics. This study investigates oper-

ational manipulation of community architecture, community performance and footprint through aeration strategies, addressing the following questions:

- Can nitrogen stoichiometry be used to derive reliable monitoring parameters to recognize disturbances and sources of imbalance in nitritation/anammox reactors?
- What are the impacts of discrete aeration parameters on the activity of different functional groups involved in single-stage nitritation/anammox?
- Can discrete aeration parameters be used to steer the system away from disturbances and imbalanced community activities to high rates of complete autotrophic nitrogen removal?
    - Can nitrite accumulation and NOB suppression be achieved through manipulation of aeration parameters?
- What does overall reactor performance tell about the process stability?
- How do aggregate size distribution and architecture contribute to optimal oxygen to ammonium loading rate?
- Does aeration regime affect the aggregate size distribution, community composition and architecture? Can aeration regime be used to drive towards a desired community architecture?
- Does aeration regime affect nitrous oxide emissions? Can aeration regime be used to drive reduced nitrous oxide emissions?

In order to answer these questions

- An operational protocol with stoichiometry based diagnosis and aeration regime based actions was developed for a SBR, implemented and improved. (**Paper I-II**)
- The evolution of aggregate size distribution, community composition and architecture under varying operational conditions was investigated. (**Paper II-III**)
- A conceptual model of aggregation and architecture evolution controlled by aeration regime was developed. (**Paper III**)
- A strategy to reduce nitrous oxide emissions based on aeration regime was devised. (**Paper IV**)

2 Diagnostic-action tool for start-up and process enhancement

System start-up can take several months (van der Star et al. 2007), and system stability may be compromised by many factors. Operating the system for selection of the desired microbial community and maintaining stable process performance can be difficult. Controls only based on set point values for DO, ORP, nitrogen species and pH may not be sufficient to deduce whether microbial community activities are adequate or balanced (Bürgmann et al., 2011; Joss et al., 2011; Lackner and Horn, 2012). Stoichiometric ratios of produced or consumed nitrogen species have been used to interpret process performance, but such have not been implemented as monitoring tools in decision-making.

In **Paper I** the aim was to develop a decision making protocol for single-stage nitritation/anammox reactor operation in order to i) manipulate the key functional groups towards balanced nitritation/anammox during start-up or during recovery from upsets, ii) increase the treatment capacity during loading increases while maintaining balanced nitritation/anammox.

2.1 Reaction stoichiometries – diagnosis

The protocol was based on the process performance stoichiometry derived from ammonium, nitrite and nitrate concentrations measured in the influent and the effluent. From stoichiometric analyses, we could infer the nitrogen conversion dynamics and the relative contribution of three different core microbial groups (AOB, AnAOB, NOB), which allows rapid and appropriate identification of required changes in operational conditions that can be recursively implemented.

Considering that all nitrogen removal is mediated by partial nitritation-anammox, the overall reaction stoichiometry for "balanced CANR" (1.15) was deduced and used to define theoretical boundary and target stoichiometric ratios.

$$\begin{aligned} &1.15\ NH_4^+ + 0.91\ O_2 + 1.34\ HCO_3^- \rightarrow \\ &0.13\ NO_3^- + 0.5\ N_2 + 0.02\ \text{cellular-N} + 1.68\ H_2O + 1.25\ H_2CO_3 + 0.06\ H^+ \end{aligned} \quad (1.15)$$

$$R_{AmmTot} = \left| \frac{\Delta NH_4^+\text{-N}}{\Delta TN} \right| = 1.15 \text{ target; } \geq 1.15 \text{ boundary} \quad (1.16)$$

$$R_{NitAmm} = \left| \frac{\Delta NO_2^- \text{-N}}{\Delta NH_4^+ \text{-N}} \right| = 0 \text{ target; } \leq 1 \text{ boundary} \quad (1.17)$$

$$R_{NatTot} = \left| \frac{\Delta NO_3^- \text{-N}}{\Delta TN} \right| \leq 0.16 \text{ target; } \geq 0.13 \text{ boundary} \quad (1.18)$$

The ratios are calculated with absolute values of consumption and production (1.16-18). R_{AmmTot} is ammonium consumed per total nitrogen removed i.e. it measures AnAOB activity versus AOB and NOB. R_{NitAmm} is nitrite produced to ammonium consumed. Unless zero, as in balanced cases, it measures prevalence of AOB activity over AnAOB and NOB. R_{NatTot} is the nitrate produced per total N removed i.e. it differentiates between AnAOB and NOB activity and is given a 20% extra as allowable limit. If the value of ΔTN is low or approaching zero, R_{NitAmm} would be the appropriate indicator of the system to diagnose whether only nitritation is achieved or if full nitrification takes place.

2.2 Aeration parameters – actions

The system was defined as an intermittently aerated sequencing batch reactor (Figure 8). Discrete aeration parameters for oxygen loading with respect to ammonium loading that can have unique effects on reactor performance were defined for this system. Total duration of one cycle (t_{cycle}), together with the influent ammonium concentration (NH_{4inf}) and exchange ratio (ER), determine the overall ammonium loading rate per cycle (L_{NH4}). Intermittent aeration was characterized by the duration of a single aerated period (t_{on}), the duration of a single non-aerated/anoxic period (t_{off}), the frequency of redox cycling/aeration (f_{redox}), the total aerated fraction of the whole cycle (R_{on}) and the air flow rate (Q_{air}).

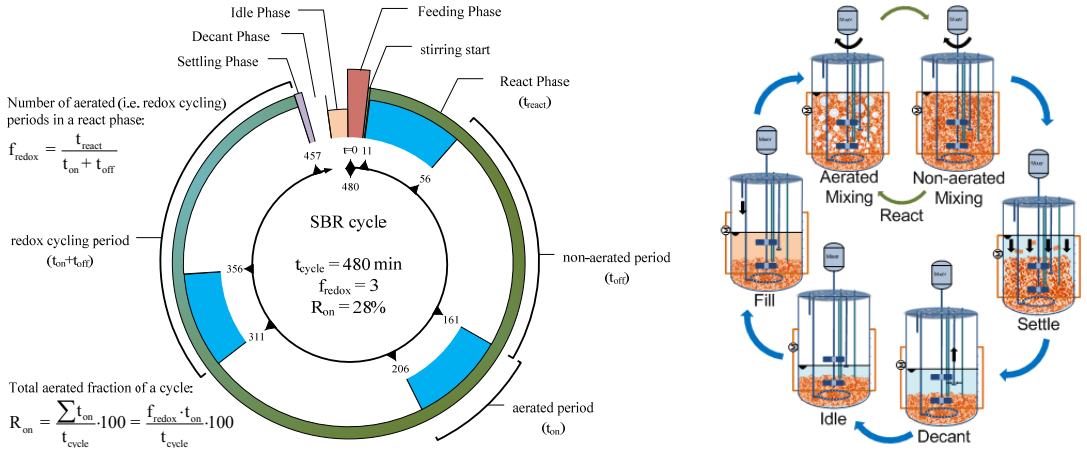

Figure 8. Exemplary operational schedule of an intermittently aerated SBR (**Paper II**).

The f_{redox} and R_{on} were derived as:

$$f_{redox} = \frac{t_{react}}{t_{on} + t_{off}} \tag{1.19}$$

$$R_{on} = \frac{f_{redox}\, t_{on}}{t_{cycle}} \cdot 100 \tag{1.20}$$

The overall oxygen to ammonium loading to the system ($L_{O_2}/L_{NH_4^+}$) was derived from these parameters.

The t_{on} is important to exploit the relatively longer lag phase of NOB over AOB when returning to aerobic conditions, as observed before. By keeping t_{on} short enough, nitrite accumulation for subsequent use by AnAOB can be achieved, reducing the ability of NOB to utilize it within the aerated period. The t_{off} must be sufficient for AnAOB to deplete the nitrite produced by AOB, and it can also differentially inactivate AOB and NOB, characterized by higher anoxic decay rate than AnAOB. Increasing the frequency of change in redox conditions could enable exploiting differences in lag phase faster. On the other hand, a largely increased frequency would eventually mimic continuous operation and the advantages of intermittence would be lost. R_{on} is determined by t_{on} and its frequency over a complete operational cycle. This parameter together with Q_{air} determines the overall oxygen loading rate and must be concurrent with the overall ammonium loading rate.

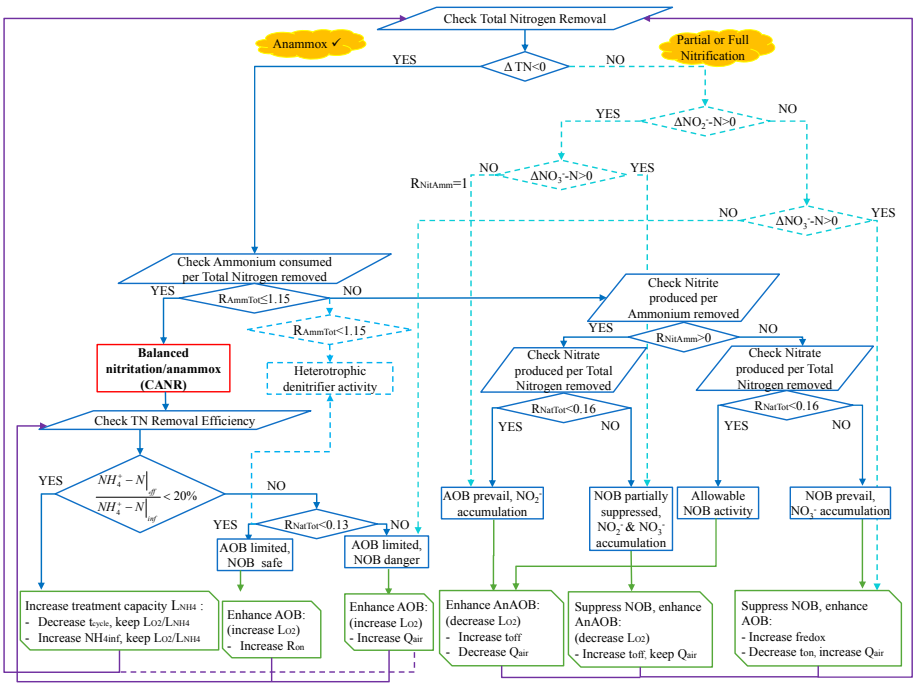

Figure 9. Operational protocol (diagnosis-action tree) (**Paper I-II**)

Once the dominant community activity is inferred from the stoichiometric analysis, a number of actions are defined (Figure 9). Depending on the response of the system, the same action can be repeated incrementally. If the response is not sufficient, the next action in line is chosen. Briefly, in order to attain balanced activities, anoxic conditions should be favoured by increasing f_{redox} and t_{off} to enhance AnAOB activity; the overall oxygen loading should be decreased by decreasing Q_{air}, t_{on} and R_{on} to control aerobic activity (AOB and NOB); the intensity of oxygen loading should be increased by shortening t_{on} and increasing Q_{air} to select AOB over NOB. A target upper limit for $L_{O_2}/L_{NH_4^+}$ was set as the theoretical demand of $1.81 gO_2/gN$, to keep operating the system in oxygen-limited manner.

2.3 Protocol implementation on lab-scale sequencing batch reactors

Short and long term evaluations of the protocol (**Paper I and II,** respectively**)** were carried out during the start-up and process enhancement of two sequencing batch reactors, ultimately leading to improvements of the protocol (Figure 9).

The two SBRs namely, SBR_f (or 1) and SBR_t (or 2) were inoculated with biomass from a nitritation-anammox biofilm reactor and operated over 1 to 1.5 years utilizing the operational protocol. The reactors were automated (Figure 10); process parameters as well as microbial community compositions were monitored throughout the whole operation phase (Figure 11).

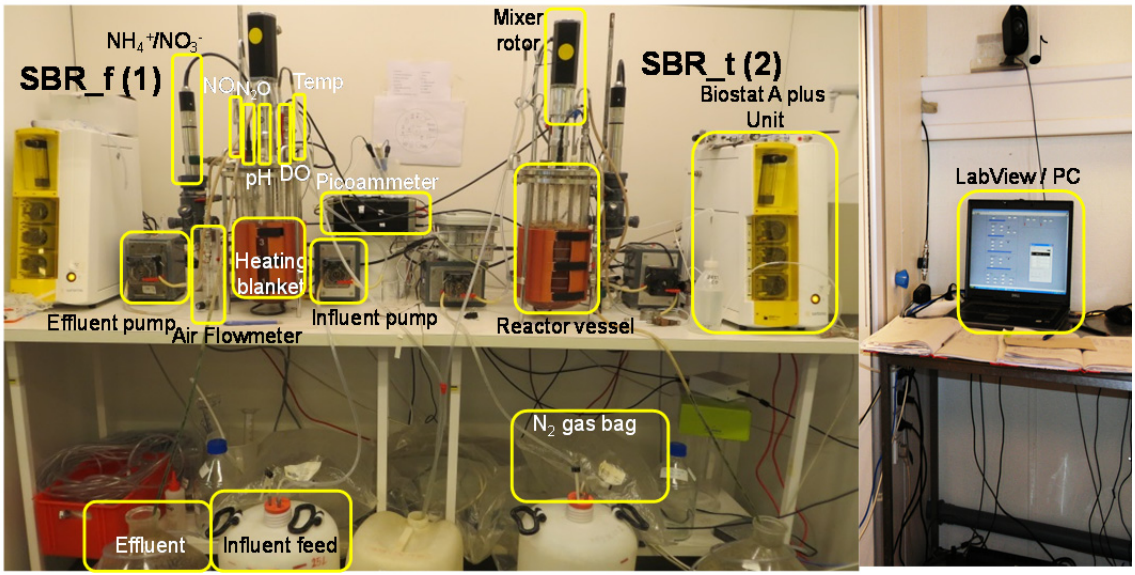

Figure 10. Setup of the two lab-scale sequencing batch reactors

The oxygen loading to the system was calculated from the oxygen transfer rate (OTR) through experimentally determined volumetric oxygen mass transfer coefficient, k_La (min^{-1}) as a function of the air flow rate. Then, the $L_{O_2}/L_{NH_4^+}$ per cycle was determined as in (1.21):

$$\frac{L_{O_2}}{L_{NH_4^+}} = \frac{k_L a(S_{O_{2,sat}} - S_{O_2}) t_{on} f_{redox}}{NH^+_{4,inf} ER} \quad (1.21)$$

Both reactors recovered from upset conditions of nitrite accumulation, nitrate accumulation, ammonium starvation and oxygen overloading. Ultimately, process performances were safely enhanced up to ca. 635 $gN/m^3.d$ with 85% TN removal efficiencies.

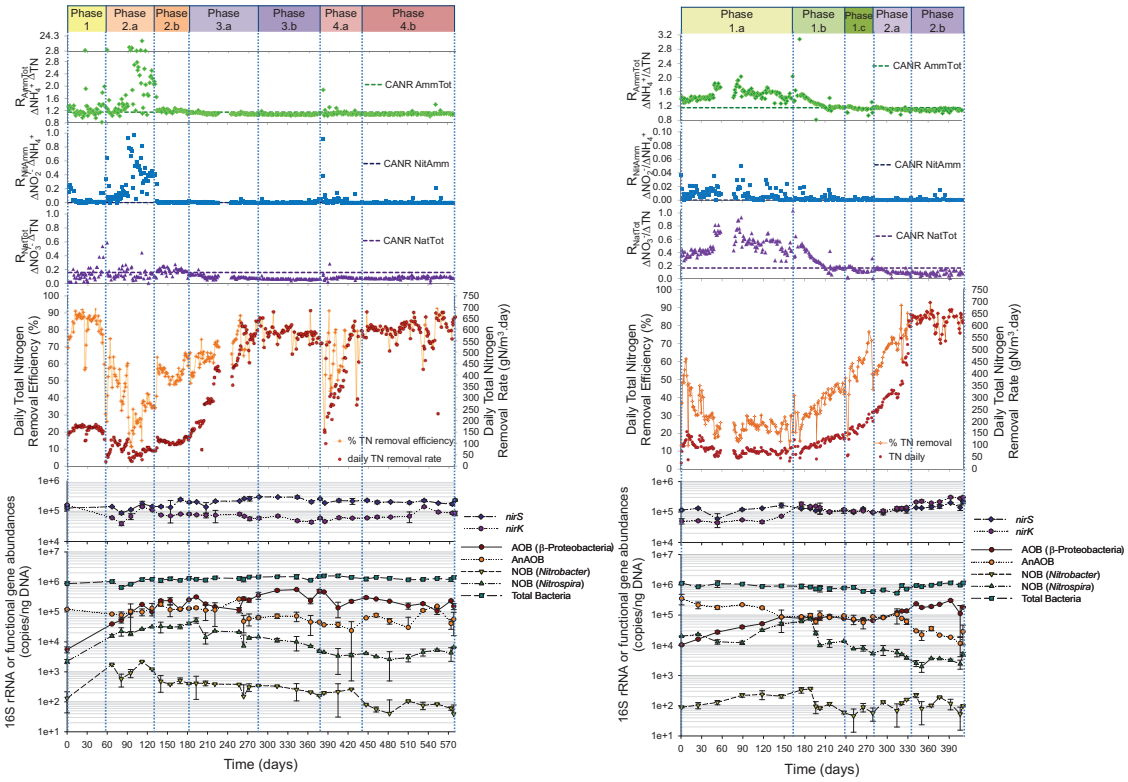

Figure 11. Overlay of performance and community composition throughout operation of SBR-1 for 578 days (left) and SBR-2 for 410 days (right) (**Paper II**).

2.4 NOB suppression and mitigation of nitrite accumulation

NOB suppression was successful only by intermittent aeration and increasing non-aerated periods, where wash-out was also confirmed by qPCR. Decreasing oxygen loading by air flow rate alone did not always suppress NOB and even enhanced nitratation unless the aerated fraction was sufficiently low.

During process performance enhancement, NOB suppression was maintained by increasing the oxygen loading through air flow rate rather than the duration of aeration.

Nitrite accumulation due to excessive AOB activity compared to AnAOB or NOB activity was prevented by decreasing the overall oxygen loading either by decreasing the air flow rate or by switching to intermittent aeration and decreasing the aerated fraction of a cycle. Decreasing air flow rate may not be effective when there is concurrent NOB activity, while introducing anoxic periods to create conditions exclusively for AnAOB activity was successful. This applies when there is no significant spatial separation of the processes. Loss of treatment capacity with excessive nitritation due to extreme oxygen loading (high DO) was redeemed by systematically adjusting substrate loading rates. The process impairment was due to reversible oxic inhibition of AnAOB activity rather than to a loss of AnAOB biomass. Hence, AnAOB recovery determined the restoration strategy and the degree of each step for improving system performance.

In both reactors, DO was not reliable as sole control parameter to track process stability and diagnose risks because of the prevalence of micro-oxic conditions. Air flow supply rate could be used as a substitute for DO while regulating the oxygen loading rate, as proposed before for oxygen limited operational conditions.

3 Community composition and architecture response to aeration strategies

Microbial community architecture of single-stage CANR aggregates can be challenging to control. The process intensification benefits largely from the architecture where spatial stratification of redox conditions are required if not mediated by temporal variation. The aggregate size and architecture of biomass determines the balance in microbial activity; hence, to a certain extent, the performance stability (stress resistance). Distinct redox-stratified architectures develop in systems that incorporate AnAOB into aerobic/nitrifying granules (Vázquez-Padín et al., 2010; Winkler et al., 2011) or incorporate AOB onto AnAOB granules (Cho et al., 2010; Sliekers et al., 2002). However there are also aggregate-based systems with a distribution of sizes (Vlaeminck et al., 2010) where functional groups are not ordered that show heterogeneous architectures with no clear redox-stratification.

3.1 Architecture evolution during long term performance enhancement

During the long term process enhancement of our two SBRs, aggregates reaching 2 mm in diameter evolved in both reactors, despite a weak size-selection imposed by settling velocity control. In one of the SBRs (SBR-1), the transition from floccular to granular biomass was more obvious (Figure 12) while in the other reactor larger compact aggregates were present from start-up.

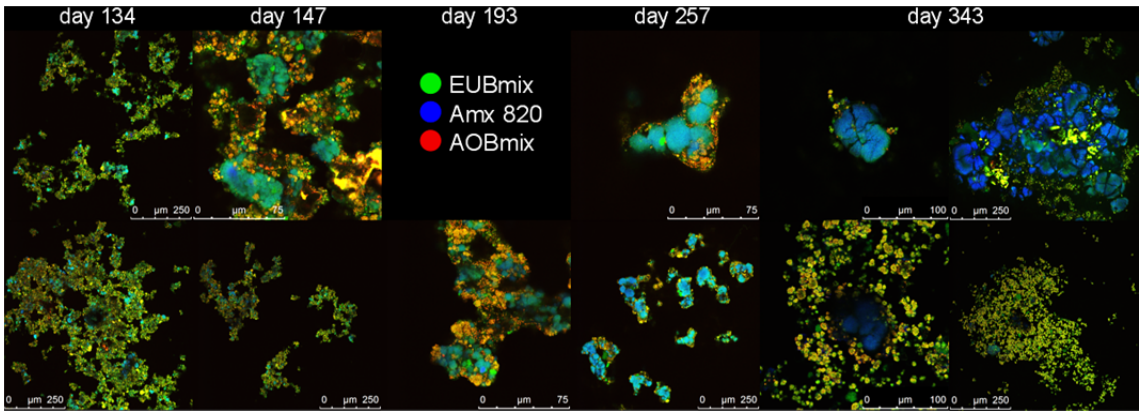

Figure 12. Floc to aggregate evolution in SBR-1 through day 134 to 343 (**Paper III**).

As presented in **Paper II,** the two SBRs, while showing overall similar performances had different biomass architectures and instantaneous (in-cycle) activities, which led to different optimal oxygen to ammonium loading rates despite similar aeration regime. The difference in average aggregate sizes (85.8µm vs. 194.9µm) could explain the difference in the final oxygen loadings (1.09gO_2/gN vs 1.20gO_2/gN). AOB residing on larger aggregates experienced more mass transfer limitations for oxygen, compared to the small AOB aggregates. Furthermore, the in-cycle nitrogen conversion dynamics and community compositions of biomass size-fractions (Figure 13) determined by qPCR strongly supported a *stratified* architecture of AOB and AnAOB in the large aggregate reactor with no in-cycle nitrite accumulation. In contrast, in the small aggregate reactor with in-cycle nitrite accumulation, the evidence points at a *size-segregated* distribution of AOB and AnAOB. Subsequent in-situ hybridizations (FISH) of functional groups also confirmed these architectural differences (Figure 15).

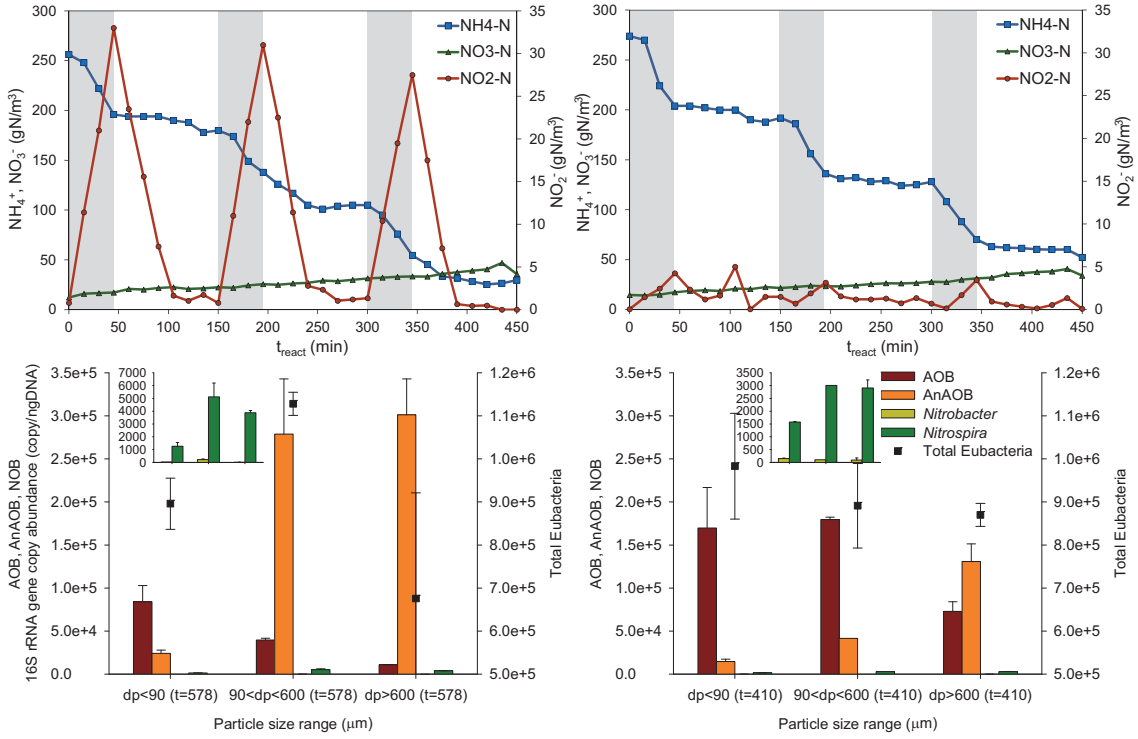

Figure 13. In-cycle nitrogen conversion dynamics and functional guild abundances (via qPCR) in different aggregate size fractions in SBR-1 (left) and SBR-2 (right) (**Paper II**).

Hence it was concluded that both the aggregate size distribution and the stratification within these aggregate, via their impact on mass transfer, set the optimal oxygen loading demand for a given ammonium loading. To further investigate this interplay, we imposed gradually changing aeration regimes on both reactors while keeping equivalent overall oxygen to ammonium loading

rates (**Paper III**). We wanted to explore if we can drive redox-stratification by changing the aeration regime. AOB-AnAOB functional guild co-aggregation or stratification was a key interest, since it enables process intensification by nitrite neutral autonomous biomass and possibly provides stress-resistance to AnAOB.

3.2 Functional guild stratification and NOB community with aeration regime

We speculated that increasing frequency of redox-cycling with short aerated periods would select for stratified aggregates because non-stratified AOB would consistently expose themselves to cytotoxic NO while the ones in proximity to AnAOB would survive thanks to AnAOB acting as NO and NH_2OH sinks. We also hypothesized that increasing the duration of aeration towards continuous aeration would further promote stratified aggregates as the relatively constant redox conditions in the bulk environment would need to be compensated spatially and would enable continuous simultaneous TN removal. Lastly, we speculated that frequent redox-cycling would be more successful than extending aeration duration in suppressing NOB proliferation, due to consistent exploitation of NOB lag behind AOB upon anoxia.

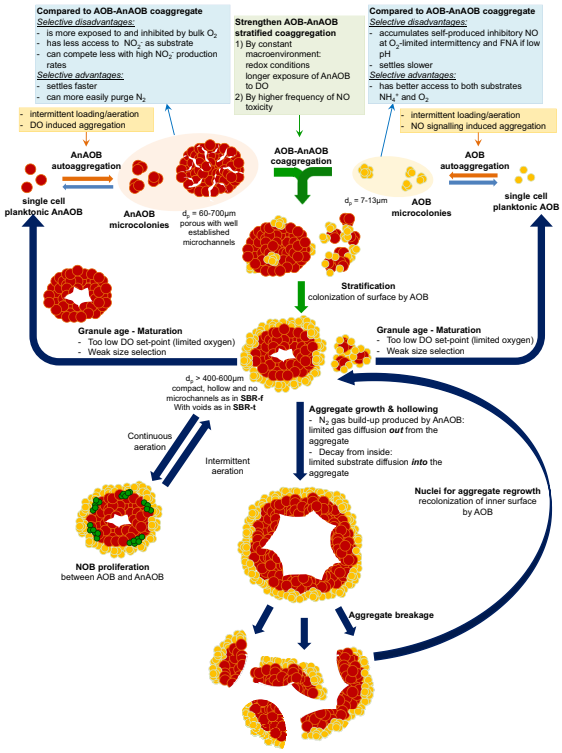

Figure 14. Conceptual control of major functional group distributions over different aggregate size ranges (**Paper III**).

Correspondingly, we gradually increased the redox-cycling frequency (f_{redox}) from 3 to 25 in the reactor with size-segregated biomass (SBR_f) and the aerated fraction of a cycle (R_{on}) from 28% to 93% towards continuous aeration in the reactor with already stratified aggregates (SBR_t).

With increasing f_{redox}, the average aggregate size increased, stratified aggregates emerged and TN removal efficiency gradually decreased to ca. 75%. However the decline in performance was recovered by increasing the oxygen loading through higher air flow rate. The transitory decline in TN removal rates was deemed to be caused by the limitation of the surface available for oxygen diffusion from bulk liquid to stratified AOB populations.

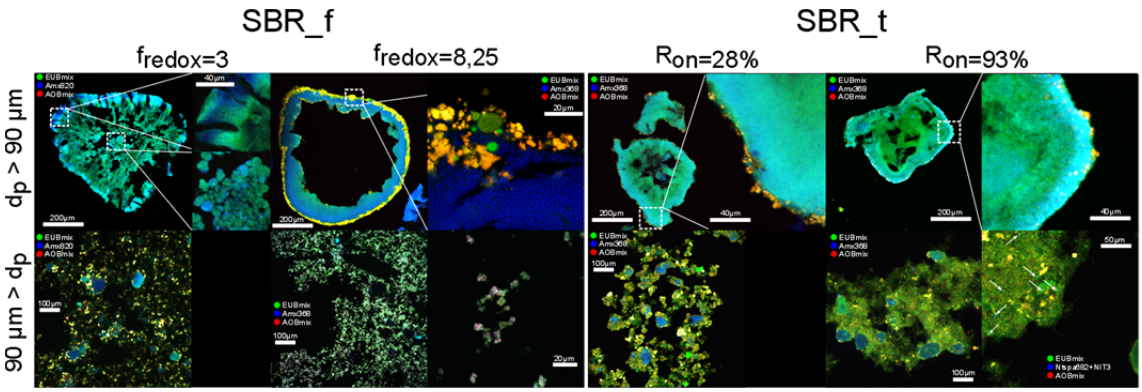

Figure 15. Size-segregation of labour by AOB (yellow) and AnAOB (blue), stratification in co-aggregates, red arrow NOB, white arrow putative heterotrophic filaments (**Paper III**)

With increasing R_{on}, AOB stratified AnAOB coaggregates remained unchanged, although smaller size was promoted. On the other hand, the NOB suppression seemed to weaken and members of this guild proliferated in the coaggregates at kinetically anticipated depths between AOB and AnAOB. NOB relative abundance raised with increasing aerated fraction and ultimately aerating continuously at lowered air flow rate (or DO). A slight increase in R_{NatTot} provided an early warning of this situation, but the fact that the stoichiometry of the system was below that expected from a system with AnAOB only metabolism implied that the NOB were not yet a threat to the TN removal. However, their increasing abundances, especially in size-segregated biomass, support that insufficient anoxic time hampered their counter selection over AOB, leading to their slow accumulation in the system.

3.3 Effect of aeration regime on community diversity

With increasing aeration frequency community richness and diversity increased and the community became more even which could be related to the increased operational dynamics through frequent redox-cycling. In contrast, with increasing aeration duration no significant change in diversity indices was observed. The abundant taxa within each autotrophic functional group did not change significantly with aeration regimes and were possibly determined by the sequential ammonium feeding and aeration under oxygen limitation (i.e., overall low oxygen and high ammonium concentrations).

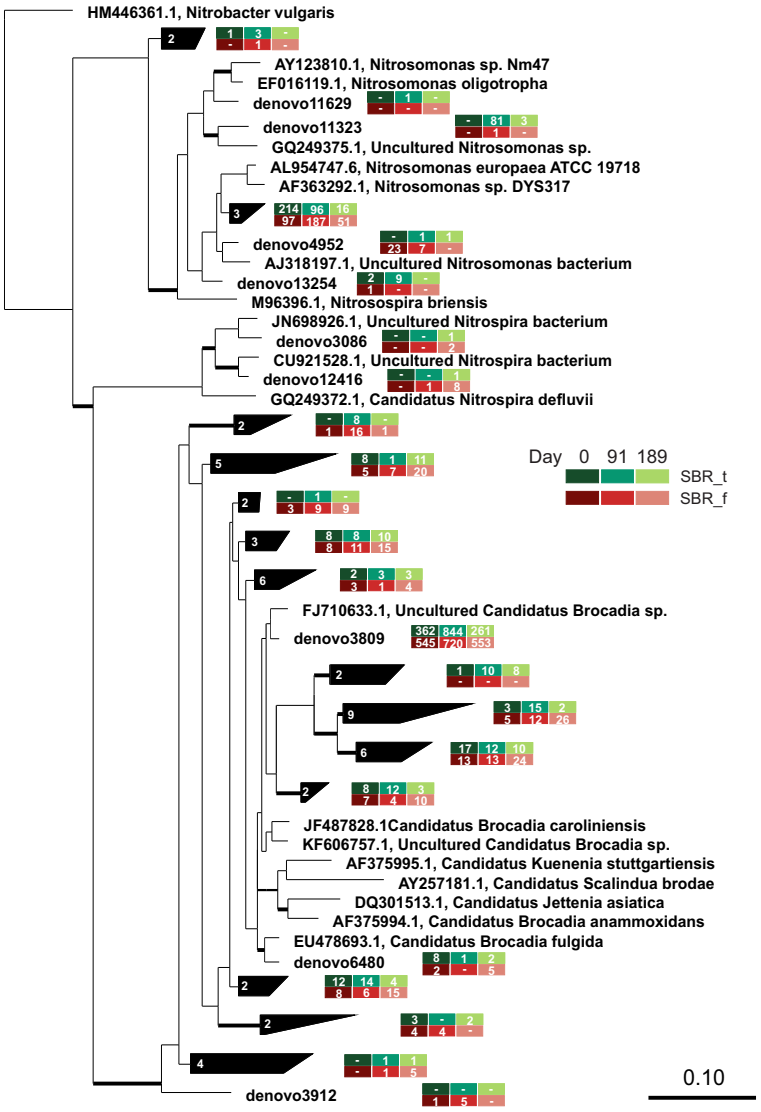

Figure 16. Phylogenetic trees with abunadances for AOB, NOB and AnAOB obtained from pyrosequencing of SBR_f and SBR_t biomass on days 0, 91, 189 f before (f_{redox}=3; R_{on}=28%), mid-way (f_{redox}=8, R_{on}=62%) and after (f_{redox}=25, R_{on}=93%) (**Paper III**).

Nitrosomonas europaea were selected over *Nitrosomonas oligotropha* and *Nitrosospira briensis*, possibly since they grow faster under non-limiting ammonia concentrations. The capability of *N. europaea* to carry out nitrifier denitrification at much higher rates possibly endowed this lineage with higher fitness under oxygen-limited conditions and with the ability to recover faster after ammonium starvation, despite its lower ammonia affinity. *Nitrospira* were selected over *Nitrobacter* as they have higher affinity for nitrite and oxygen. *Nitrospira defluvii* is considered well adapted to substrate-limited conditions because it has evolved from microaerophilic or anaerobic bacteria. Hence it would have strong survival chances in our system. *Brocadia* was selected over *Kuenenia*. The relatively persistent high ammonium concentrations as well as the nitrite peaks should have favoured the r-strategist *Brocadia* since the K-strategist *Kuenenia* are inhibited at even low nitrite concentrations. Despite the absence of organic carbon in the feed, the reactors contained diverse heterotrophic clades. Ecophysiological cross-feeding patterns, a metabolic network including hydrolysis and fermentation of decay products and EPS from autotrophs for subsequent use by heterotrophic denitrifiers, probably exist.

Our study showed that manipulating the aeration regime while keeping all other conditions (settling time, volumetric exchange ratio, SRT, influent characteristics) constant can induce changes in the architecture and aggregate size distribution. These changes, in turn, are reflected in different demands for overall oxygen to ammonium loading rates. Continuous aeration seems more effective for high rate nitrogen removal once aggregates are redox-stratified, since stratification persists and non-aerated periods do not contribute to the nitrogen removal. However, we conclude that intermittent aeration is required in order to keep NOB suppressed at a certain level.

4 Nitrous oxide emission response to aeration strategies

The cases of limited aeration by low oxygen loading rates, imposing transient anoxia (Schmidt et al., 2004; Yu and Chandran, 2010) by intermittent aeration and nitrite accumulation (Kampschreur et al., 2009b) as commonly confronted in single-stage CANRs may promote production of nitrous oxide by AOB via the previously mentioned mechanisms introduced in Section 1.1.1. Correspondingly, uniform operational conditions rather than sequential ammonium and intermittent oxygen loading are deemed to produce less N_2O (Vlaeminck, 2012). Documented N_2O emissions from lab and full-scale single-stage nitritation/anammox systems have been higher than those measured from conventional BNR processes (Desloover et al., 2011; Kampschreur et al., 2009b). While simulation studies have suggested that optimum conditions for autotrophic ammonium removal with granular biomass favour higher N_2O production (Van Hulle et al., 2012), measurements with continuous versus intermittent aeration regimes reached contradictory results (Joss et al., 2009; Yang et al., 2013). The N_2O emission is also physically governed by stripping rate, hence the air flow rate is an important contributor to the emission rates (Kampschreur et al., 2008). Consequently, is no consensus on the impact of alternative aeration strategies and how the process can be optimized gearing towards emission mitigation.

Accordingly in **Paper IV** we investigated whether the aeration intermittency and intensity could be optimized to minimize N_2O production, while retaining maximum liquid phase nitrogen removal efficiencies in our two SBRs. Online measurements of NO and N_2O concentrations in liquid phase were used to calculate the instantaneous net N_2O production rates including emission via stripping (1.22). Stripping was quantified through experimental determination of volumetric liquid-gas mass transfer coefficient for N_2O (k_La, min^{-1}) under varying air flow rates. The net rates were integrated over the whole react phase and normalized to cyclic TN removal rate and N-loads. For comparison, values were also integrated per total aerated periods, total non-aerated periods and per % interval of each aerated periods.

$$r_{N_2O,i} = \frac{\Delta N_2O_i}{\Delta t} + k_L a_{N_2O} \cdot N_2O_i \qquad (1.22)$$

With higher aeration frequencies, the N_2O production rates as well as the average emissions decreased more than three-folds, ranging from 0.17 to 0.71

mgN_2O- N/gVSS·h and from 1.7 to 7.0% $\Delta N_2O/\Delta TN$, respectively. Whereas the increased duration of aeration did not affect the cycle averaged N_2O production of each Ron condition, ranging from 0.80 to 1.56 mgN_2O-N/gVSS·h with no significant difference between the lowest and highest emissions ranging from 8.6 to13.9% $\Delta N_2O/\Delta TN$ either. The N_2O production rates and emissions in our reactor operated with high aeration frequency was in the same range as pilot and full-scale single-stage CANR systems (Castro-Barros et al., 2013; Desloover et al., 2011; Kampschreur et al., 2009a; Yang et al., 2013), while the reactor with high aeration duration had higher emissions.

N_2O formation and the majority of emissions (>95%) were primarily observed during aerated periods in both systems. The presence of N_xO peaks at the beginning of each aerated period indicates that the imposition of aeration, and not the nonreactive part of the cycle, was the main contributor of N_xO peaks. Higher aeration rates increased N_2O production but only transiently. No obvious effects in N_2O emissions were seen upon transition from intermittent to continuous aeration. Correspondingly, gas stripping during aerated periods was responsible for the majority of emissions regardless of the aeration conditions.

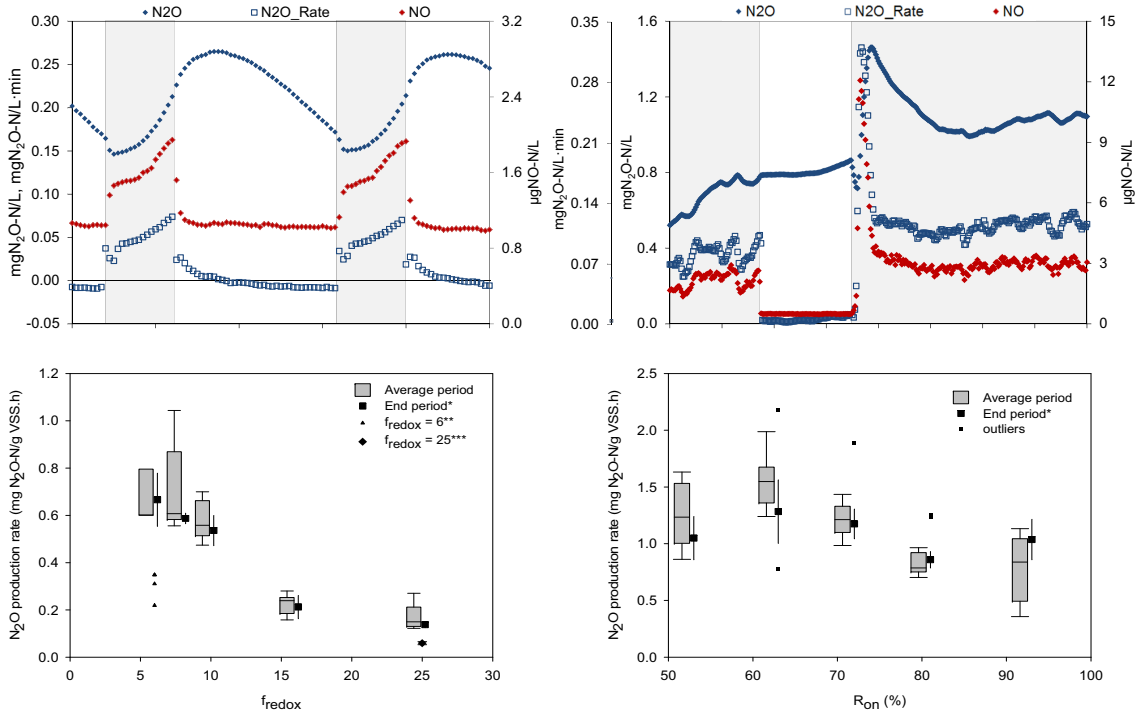

Figure 17. Detailed and cycle-average N_2O production profiles Top: Close-up of a redox-cycling period for N_xO concentration and production rate profiles, Bottom: Cycle-integrated N_2O production rates for SBR_f (left) and SBR_t (right) (**Paper IV**).

Since the N_2O production rate increased almost linearly with time from the onset until the end of aeration, longer aerated periods resulted in progressively higher N_2O production (Figure 17). Moreover, initial (first 5 min) N_2O production rates decreased with increasing aeration frequencies. Clearly, the frequency of aeration affected the initial N_2O production under aerated conditions. The decline in TN removal at higher frequencies was quickly remediated by increasing the air flow rate and N_2O productions declined even further. This indicates that the lower N_2O production at higher aeration frequencies was not due to a lower nitrogen removal, but to the imposed aeration regime.

During aerated periods, the calculated N_2O production profiles mimicked the NO concentration profiles, in agreement with the notion that NO serves as precursor in N_2O production. Indeed, N_2O production rates, but not N_2O concentrations, correlated strongly with measured NO concentrations. On the other hand, neither nitrite nor FNA correlated strongly with the N_2O production rates and were not good indicators of N_2O formation.

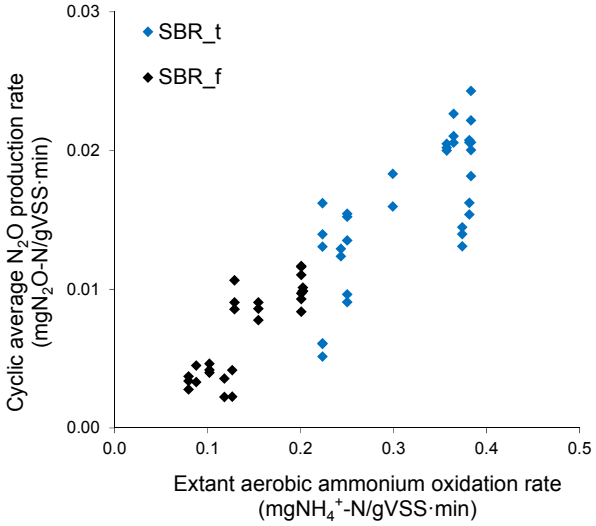

Figure 18. Cyclic average N_2O production rates correlated to extant aerobic ammonia oxidation rates (**Paper IV**).

The reaction conditions in our study, such as aerated/non-aerated periods, carbon-limited feeding, non-limiting FA and nitrite, support the possibility of N_2O production by all known pathways: incomplete denitrification, nitrifier denitrification, and hydroxylamine oxidation. While the N_2O peaks observed after recovery from anoxic conditions can be attributed to the oxidative pathway as a short-term response to changing conditions, the low NO concentrations before detecting initial NO peaks indicated that NO was produced at the beginning of aerated periods as a metabolic response and not generated during anoxic phases and subsequently stripped upon aeration. Before the restart

of aeration, phases of net N_2O consumption were detected, likely due to heterotrophic denitrifying activity. Additionally, a clear correlation between N_2O production and extant ammonium oxidation rates was observed (Figure 18). Hence, AOB activity may provide a simple estimator of N_2O emission rates in single-stage nitritation/anammox systems.

Our findings highlight that high frequency of aeration switching is a potential strategy to minimize N_2O emissions. Frequently switching aeration shortens aeration times, limiting N_2O production rates while maintaining the system-wide ammonium removal capacity. The lowest N_2O emissions were achieved when the system was operated at low potential AOB activities and with indirect assistance from other functional guilds and especially AnAOB utilization of the precursor NO and of nitrite. Performance would then be AOB-limited with AnAOB in excess.

5 Conclusions

The single-stage nitritation/anammox process is excellent for treating nitrogen rich wastewater streams. With lower aeration needs compared to conventional systems, they can have a significantly reduced carbon footprint. It is increasingly adopted for energy-efficient nitrogen removal in sidestream as well as mainstream wastewaters. However, reliable process monitoring and control strategies against instabilities are still challenging and systematic remedies are lacking. Furthermore, the optimal working conditions, especially in regard to the mode of aeration, are those known to favour nitrous oxide emissions. As nitrous oxide is a potent ozone depleter, this leads to a trade-off between energy - and hence, cost-savings- and environmental impact. On account of process stability, the driver of the process from micro- to macroscale, the microbial community architecture of nitritation/anammox aggregates are of critical importance, yet little is known about their formation and evolution mechanisms.

In this PhD project, aeration strategies were rigorously assessed in order to mitigate disturbances caused by community imbalances and safely enhance capacity in two SBRs operated over long term. At steady performance states, controlled gradual changes in aeration regimes were implemented in the two systems, either towards high intermittence or towards continuous aeration, without changing the overall oxygen loading. The goals were to evaluate hypotheses on aeration regime driven mechanisms that control microbial community architecture and strategies to alleviate nitrous oxide emissions. Consequently, a diagnosis-action protocol, a conceptual model for aggregation mechanism and architecture control, and a nitrous oxide mitigation strategy were developed. The following findings were made:

- Regulation of aeration with nitrogen mass balance stoichiometry feedback is a reliable approach for recovery of process efficiency and long term operation of oxygen-limited nitritation/anammox systems.
- DO concentration monitoring is a weak control variable for systems operated under microaerobic conditions. A better control variable is the oxygen loading relative to the nitrogen loading at a stoichiometric ratio regulated by air flow rate.
- Nitrite accumulation can be mitigated by adjusting the overall oxygen loading by decreasing the air flow rate or the aerated fraction of a cycle.

- Short and intense aeration periods followed by sufficiently long anoxic periods successfully suppress NOB. Continuous aeration under equivalent oxygen-limited loadings alleviates the suppression, leading to NOB proliferation. This approach can be used effectively when selectively limiting NOB by SRT control is not possible
- Under the same aeration regime, differences in reactor scale efficiencies and optimal oxygen to ammonium loadings can be explained by temporally resolved process dynamics within a cycle.
- The aggregate size distribution and architecture of the biomass directly impact the overall aeration demand. This explains why seemingly parallel systems demand different optimal oxygen to ammonium loadings. Correspondingly, an intermittently aerated system with time-segregated activity and size-segregated distribution of aerobic and anaerobic ammonia oxidizing communities can be as efficient as a system composed of continuously aerated redox-stratified aggregates with simultaneous activity, under lower oxygen loading.
- With settling time, volumetric exchange ratio, sludge retention time and influent characteristics kept constant, the aeration regime itself causes changes in aggregate architecture and aggregate size distribution.
- Increasing aeration frequency is effective in steering a size-segregated nitritation/anammox community to a more redox-stratified architecture with larger aggregates.
- Increasing the duration of aeration does not significantly alter the original redox-stratified architecture, but allows proliferation of unwanted nitrite oxidizing bacteria.
- Increased frequencies also alter aggregate morphology and settleability leading to compact but hollow aggregates that transiently accumulate nitrogen gas.
- Despite the absence of organic carbon in the feed an abundant heterotrophic fraction can proliferate alongside the autotrophic community.
- Frequent short shifts in aeration alleviate the overall nitrous oxide emissions in oxygen-limited autotrophic nitrogen removing reactors.
- The correlation between extant aerobic ammonium oxidation rates and nitrous oxide production rates is likely the key for mitigation of nitrous oxide in these systems.

- Minimizing nitrous oxide emissions is possible by simply controlling the air flow regime to a nitritation/anammox reactor while maintaining the nitrogen removal capacity of the system.

6 Future Research and Perspectives

The findings of this research contribute to the establishment of strategies that can be adopted in practice to operate single-stage nitritation/anammox reactors. The knowledge gained here can be extended in future research and applications as follows:

The iterative diagnosis-action protocol and N_2O mitigation

The protocol presented here (Paper I & II) is a framework that can be applied to a single-stage SBR system regardless of the biomass structure. The iterative nature provides flexibility to apply action ranges specific to the systems. In practice, a quantitative starting range for aeration parameters could be determined for a certain biomass structure and aggregate size distribution model predictions. Models including aggregate size distribution and structure (floc *versus* granule) showed these parameters may indeed account for the differences in optimal oxygen to ammonium loading (Hubaux et al., 2014; Volcke et al., 2012) and therefore can be used to deduce limiting values. Furthermore, size distribution could be introduced as a dynamic variable and measured values could be implemented into the diagnosis stage. On the other hand, consensus on biokinetic parameter as well as consideration of different species within a functional guild would enable a more precise directionality while taking actions. Such quantifications can be, for instance, determination of a minimum anoxic period duration for successful lag of *Nitrospira* (Gilbert et al., 2014) versus *Nitrobacter*. Also, a statistical analysis (eg. redundancy analysis, principal component analysis) of discretized aeration parameters, stoichiometric indicators and community abundances would strengthen the protocol matrix.

For the application of the diagnosis part, both a fuzzy-logic framework was adapted from the protocol presented here (Boiocchi et al., 2014; Vangsgaard, 2013) and a feed-forward-feedback strategy using the stoichiometric indicators was successfully validated to control optimal cycle-averaged oxygen to nitrogen loadings targeting high TN removal efficiencies (Mauricio-Iglesias et al., 2015; Vangsgaard, 2013).

The protocol could be further enhanced by inclusion of N_2O as an indicator N-species, as a measure of imbalances in aerobic ammonia oxidation, as was also previously proposed (Wunderlin et al., 2013). Actions such as reducing aerated period or decreasing air flow rate can be suggested to remediate the imbalance. However, for this, a better mechanistic understanding of N_2O pro-

duction and the interactions with other microbial guilds in terms of utilization or toxicity of NO and N_2O is required. The development of robust online N-species sensors will enable the successful implementation of the protocol in full-scale systems.

Aggregation and architectural implications

The granulation mechanisms, and especially the triggers of aggregative behavior, are a subject of discussion in many research fields that involve biofilms. In this scope, the chemical/physical stressors, signaling (eg. quorum sensing, NO), response regulation, and dynamics as a part of the biofilm "life cycle" are key unresolved questions. For nitritation/anammox aggregates, the formation mechanisms, ecophysiological characteristics as well as the mechanistic basis of EPS production, EPS composition, characteristics and role in architecture are not studied as thoroughly as for heterotrophic aerobic or anaerobic granules. Considering the diverse architectures and morphologies observed in this study (Paper III), it will enhance our understanding to identify if there is any phylogenetic or physiological differentiation that results in proximity patterns between and within functional groups. Furthermore, these different morphologies can provide a framework of quantitative descriptors for individual-based models studying cell-to-cell interactions and further to multi-scale models (Ofiţeru et al., 2014; Xavier et al., 2007). Another point to resolve is how and to what extent heterotrophic bacteria affect the autotrophic guilds and process. Their presence despite the unfavorable conditions due to the absence of organic carbon supply and their potential to compete with autotrophs needs to be assessed.

The architectures of different size aggregates observed in this study (Paper III) also necessitate a more precise definition of "granules" and indeed many putatively granular systems constitute of floccular/suspended biomass effectively changing the overall performance (Hubaux et al., 2014) and they should be considered as hybrid systems. The definition of granule per (de Kreuk et al., 2007) as "cells that do not coagulate under reduced hydrodynamic shear" underlines their most definitive difference from flocs, while size and settling velocity are not as useful as distinctive parameters. The definition of granules as biomass "settling significantly faster than flocs" is ambiguous while faster than biomass "of the same size" would be more appropriate, as then it refers to the density and compactness. Flocs can range from 10-150 μm, yet compact aggregates with sharp gradients as biofilm or nucleation of granules can be at 50μm scale (Paper III). Practically, the settling definition is more useful as the aim is to have well settling sludge, but in con-

cept, small dense aggregates settling as slow as large flocs yet with different regular architectures may indeed grow into larger granules unlike flocs. What separates a microcolony from an aggregate and a granule should be defined in terms of macro-environment as well as density, not only by size. Low DO conditions allow redox-stratification for small dense cellular aggregates.

Overall characterization of the conditions and microbial ecology under "low DO/ microaerophilic" seems to be critical for single-stage nitritation/anammox systems. However, precise quantification of key environmental parameters (DO, NO, N_2O) is difficult with conventional equipment. These conditions could cause the microbial community to be in a more dynamic situation resulting in adaptation to stress conditions (or functional redundancy). Especially, the physiological changes associated with the biofilm mode of life, and a better understanding of adaptation to environmental stress conditions is warranted.

Finally, validation experiments would be beneficial to confirm the observed effect of aeration regimes on conceptual aggregation/stratification models and N_2O mitigation strategies. Changing aeration regimes in systems with identical biomass composition, architecture, size distribution and activity with no history would strengthen confidence in these proposed strategies. Nonetheless, switching the operations between the two studied reactors or reversing the aeration regimes back to the beginning frequency and duration could also provide valuable results. Such efforts would show whether effects of aeration regimes on architecture and N_2O emissions are truly reversible or depend on operational history.

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8 Papers

I **Mutlu, A. G.**, Vangsgaard, A. K., Sin, G., Smets, B. F. (2013). An operational protocol for facilitating start-up of single-stage autotrophic nitrogen-removing reactors based on process stoichiometry. *Water Science and Technology*. **(68)** 3: 514-521.

II **Mutlu, A. G.**, Vangsgaard, A. K., Sin, G., Smets, B. F. (2015). Spatial and temporal differentiation of microbial composition and activity during long term performance enhancement in two single-stage nitritation/anammox sequencing batch reactors. *Manuscript in preparation.*

III **Mutlu, A. G.**, Lv, C., Domingo-Félez, C., Gülay, A., Smets, B. F. (2015). Impact of aeration regimes on activity, community composition and biomass architecture in sequencing batch reactors performing single-stage nitritation/anammox. *Manuscript in preparation.*

IV Domingo-Félez, C., **Mutlu, A. G.**, Jensen, M. M., Smets, B. F. (2014). Aeration strategies to mitigate nitrous oxide emissions from single-stage nitritation/anammox reactors. *Environmental Science and Technology*. **(48)** 15: 8679-8687.

In this online version of the thesis, the papers are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from.

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