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„Innovative strategies for enhanced deammonification performance and reduced nitrous oxide emissions“

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Für meine Familie und Freunde,
und all jene, die unsere Natur lieben und in ihrer Schönheit erhalten möchten.

„Die meisten Menschen wissen gar nicht, wie schön die Welt ist
und wie viel Pracht in den kleinsten Dingen, in irgendeiner Blume, einem Stein,
einer Baumrinde oder einem Birkenblatt sich offenbart.

Die erwachsenen Menschen, die Geschäfte und Sorgen haben,
sich mit lauter Kleinigkeiten quälen,
verlieren allmählich ganz den Blick für diese Reichtümer.

Es geht eine große und ewige Schönheit durch die ganze Welt,
und diese ist gerecht über die kleinen und großen Dinge verstreut.“

– Rainer Maria Rilke –

Abstract

Autotrophic biological nitrogen removal by deammonification systems is a promising wastewater treatment process for the purpose of reduced operational costs in comparison to conventional nitrification/denitrification. Furthermore, it offers the added benefit of an increased nitrogen removal capacity of the entire wastewater treatment plant (WWTP) if it is implemented as a side-stream treatment. However, favorable conditions for the deammonification process are also likely to favor the formation of nitrous oxide (N_2O) as an undesired byproduct which in turn imposes the risk of increased N_2O emissions. N_2O is not only a potent greenhouse gas with a global warming potential 298 higher than that of carbon dioxide, but also an ozone-depleting substance. Therefore, effective N_2O mitigation strategies are crucial to minimize negative long-term effects on the environment. As N_2O emissions from WWTPs are so far not legislatively restricted, a voluntarily implementation of these N_2O mitigation strategies by WWTP operators could be incited by a simultaneous enhancement of the deammonification performance.

For these reasons, the key research objectives of this dissertation were twofold, namely developing strategies for both an enhanced performance for nitrogen removal and reduced N_2O emissions of deammonification processes. To cover as many system configurations as possible, experiments with one- and two-stage deammonification systems employing both suspended sludge operated as sequencing batch reactor (SBR) and biofilm carriers employed in a moving bed biofilm reactor (MBBR) at laboratory and pilot scale have been conducted.

Results regarding an enhanced deammonification performance demonstrated that even small quantities of residual suspended biomass in a MBBR severely influenced the nitrogen removal rate. A complete withdrawal of suspension from the biofilm carriers resulted in a decreased nitrogen removal rate by a factor of 3.5. Chemical and fluorescence *in-situ* hybridization (FISH) analyses revealed that the suspension was almost completely composed of ammonium oxidizing bacteria (AOB). Due to their high specific growth rates, the system was able to recover after a partial wash-out within hours. In contrast, the biofilm comprised both AOBs and anoxic ammonium oxidizing bacteria (AnAOBs). A positive correlation between the volatile suspended solids (VSS) concentration and the nitrogen removal rate was revealed, which highlighted the possibility to further improve the deammonification performance by accumulating suspended biomass in such systems.

For two-stage deammonification systems, a sufficient partial nitritation as first step of the progress as close as possible to the theoretical ammonium-nitrite-ratio of 1:1.32 was demonstrated to be crucial for a well performing overall process. The produced nitrite serves as substrate for the subsequent anoxic ammonium oxidation as a second step and therefore limits the minimal possible ammonium effluent concentrations. Due to a moderate partial nitritation, the twofold implemented second stages operated as SBR and as MBBR were additionally aerated by the WWTP operators to initiate an additional one-stage deammonification side-process for performance enhancement. This approach was successful with nitrogen removal rates and degradation rates exceeding 0.50 $kg_N/(m^3 \cdot d)$ and 80 %, respectively. However, an aeration in the second stage is highly uncommon and not recommended for two-stage deammonification systems as it sacrifices the advantage of two distinctly developing

microbial communities in the first and second stage. Nevertheless, this modification enabled a comparison of the SBR and MBBR hypothetically being operated as one-stage deammonification systems. Results revealed that the nitrogen removal rates of the MBBR slightly exceeded those of the SBR (0.39±0.15 $kg_N/(m^3 \cdot d)$ vs. 0.33±0.11 $kg_N/(m^3 \cdot d)$). This could be explained by an operation of the MBBR in a more favorable pH range (pH 7.60±0.26 vs. pH 7.84±0.15) as revealed during an additional study.

Here, the influence of the aeration and feeding strategy as well as the cycle's initial pH value on the nitrogen removal rate and the N_2O emissions of an one-stage deammonification system was systematically investigated by applying a design of experiment (DoE) method. Two models were developed which could be used to derive effective process control strategies for an efficient and environmentally friendly deammonification operation. The aeration strategy was identified to have the highest effect on the nitrogen removal rate. Settings of an intermittent feeding and aeration strategy operated at an initial pH value of pH 7.46 was predicted to maximize the nitrogen removal rate to 0.49±0.03 $kg_N/(m^3 \cdot d)$.

With respect to reduced N_2O emissions, minimal N_2O emissions were predicated at operational settings of single feeding, continuous aeration, and an initial pH value of pH 7.80 with the pH value having the highest effect. These deviating settings for an enhanced deammonification performance and reduced N_2O emissions already indicated that a simultaneous improvement might be hardly achievable under the tested conditions. Combining the two models confirmed this assumption and demonstrated that the deammonification performance and the N_2O emissions exhibited a weak positive relation. Thus, a single operational set-point for an overall optimization did not exist. Nevertheless, several settings meeting a desired compromise between an economic and ecologic deammonification process could be defined using the two models.

In the two-stage deammonification system, the buffer tank interconnecting the first and second stage exhibited the highest dissolved N_2O concentrations of all reactors. Elevated nitrite and ammonium concentrations, carbon limiting and anoxic conditions, as well as a constant supply of microorganisms from the first stage were identified as ideal conditions for N_2O formation. A decreased hydraulic retention time (HRT) and frequent removal of biomass were proposed as N_2O mitigation strategies for the buffer tank. Anoxic phases in the second (uncommonly aerated) stage proved to enable N_2O reduction. Thus, an aeration re-arrangement with unaerated phases after the influent high in accumulated dissolved N_2O or a transition of the second stage to a complete anoxic ammonium oxidation process without aeration would reduce stripping effects.

Despite all efforts to mitigate N_2O emissions, a complete avoidance of N_2O formation will most likely not be feasible, since N_2O is not only produced biologically, but also abiotically. A potential solution to render N_2O into non-hazardous N_2 could be a complete catchment and incineration of the deammonification off-gas together with biogas which would additionally make use of the 37 % higher energy yield of N_2O in comparison to oxygen.

Key words: Anammox; Deammonification; Design of experiment (DoE); Greenhouse gas emissions; Nitrogen removal efficiency; Nitrous oxide (N_2O); N_2O mitigation strategies; Moving bed biofilm reactor (MBBR); Partial nitritation; Sequencing batch reactor (SBR)

Zusammenfassung

Die autotrophe Deammonifikation zur biologischen Stickstoffelimination aus Abwässern ist im Vergleich zur konventionellen Nitrifikation/Denitrifikation ein vielversprechendes Verfahren, um Betriebskosten zu senken. Darüber hinaus kann die Stickstoffeliminationskapazität der gesamten Kläranlage durch deren Anwendung im Seitenstrom erweitert werden. Allerdings können für die Deammonifikation vorteilhafte Betriebsbedingungen die Bildung von unerwünschtem Lachgas (N_2O) begünstigen, was wiederum ein Risiko an erhöhten N_2O-Emissionen birgt. N_2O ist nicht nur ein 298-fach stärkeres Treibhausgas als Kohlendioxid, sondern trägt auch zum Ozonabbau bei. Daher sind wirksame N_2O-Minderungsstrategien essentiell, um negative Umweltauswirkungen zu minimieren. Da N_2O-Emissionen von Kläranlagen gesetzlich bislang nicht begrenzt sind, könnte eine mit den N_2O-Minderungsstrategien gleichzeitig einhergehende Verbesserung der Deammonifikationsleistung einen Anreiz schaffen, diese freiwillig umzusetzen.

Aus diesen Gründen verfolgte diese Dissertation zwei Hauptziele: die Entwicklung von Strategien a) zur Verbesserung der Stickstoffeliminationsleistung und b) zur Reduktion der N_2O-Emissionen bei der Deammonifikation. Um möglichst viele Systemkonfigurationen abzudecken, wurden Experimente im Labor- und Pilotmaßstab mit ein- und zweistufigen Deammonifikationsanlagen durchgeführt, die sowohl als Sequencing-Batch-Reaktoren (SBR) mit suspendierter Biomasse, als auch als Moving-Bed-Biofilm-Reaktoren (MBBR) mit Biofilm betrieben wurden.

Die Untersuchungen bzgl. einer verbesserten Deammonifikationsleistung zeigten, dass bereits geringe Mengen an suspendierter Biomasse die Stickstoffeliminationsrate des MBBRs positiv beeinflussten. Ein vollständiger Abzug der Suspension aus dem MBBR führte zu einer 3,5-fach geringeren Stickstoffeliminationsrate. Chemische Analysen und eine Fluoreszenz in situ Hybridisierung (FISH) der Biomasse zeigten, dass die Suspension fast ausschließlich aus ammoniumoxidierenden Bakterien (AOB) bestand. Wurde diese teilweise ausgewaschen, konnte sich das System dennoch binnen weniger Stunden erholen, was auf die hohen spezifischen Wachstumsraten der AOBs zurückzuführen war. Im Gegensatz dazu war der Biofilm sowohl aus AOBs, als auch anoxischen ammoniumoxidierenden Bakterien (AnAOBs) zusammengesetzt. Die organische Trockensubstanz und Stickstoffeliminationsrate korrelierten positiv, weshalb die Leistungsfähigkeit von MBBRs durch eine Akkumulation der suspendierten Biomasse weiter verbessert werden könnte.

In einer Pilotanlage zur zweistufigen Deammonifikation auf der Kläranlage Kempten wurde aufgezeigt, dass eine partielle Nitritation als erster Teilschritt des Prozesses möglichst nahe am theoretischen Ammonium-Nitrit-Verhältnis von 1:1,32 entscheidend für einen gut funktionierenden Gesamtprozess ist. Das produzierte Nitrit dient als Substrat für die anschließende anoxische Ammoniumoxidation im zweiten Schritt und begrenzt somit die minimal mögliche Ammoniumablaufkonzentration. Aufgrund einer moderaten partiellen Nitritation wurden die zweifach implementierten zweiten Stufen (ausgeführt als SBR und MBBR) von den Kläranlagenbetreibern intermittierend belüftet, um eine zusätzliche partielle Nitritation zur Leistungssteigerung zu ermöglichen. Dieser Ansatz war mit Stickstoffeliminations- und Abbauraten von mehr als 0,50 $kg_N/(m^3 \cdot d)$ bzw. 80 % erfolgreich. Allerdings ist eine Belüftung in der zweiten Stufe äußerst ungewöhnlich und für zweistufige Deammonifikationsanlagen nicht empfehlenswert, da dadurch der Vorteil zweier sich verschieden entwickelnder Biozönosen in der ersten und zweiten Stufe verloren geht. Dennoch ermöglichte dies, die Leistungsfähigkeit

des SBRs und MBBRs (hypothetisch als einstufig betriebene Deammonifikation) zu vergleichen. Die Ergebnisse zeigten, dass die Stickstoffeliminationsrate des MBBRs die des SBRs leicht überstieg (0,39±0,15 $kg_N/(m^3 \cdot d)$ vs. 0,33±0,11 $kg_N/(m^3 \cdot d)$). Dies könnte durch den Betrieb des MBBRs in einem günstigeren pH-Bereich erklärt werden (pH 7,60±0,26 vs. pH 7,84±0,15), wie in einer zusätzlichen Studie aufgezeigt werden konnte.

Hierbei wurde systematisch der Einfluss der Belüftungs- und Fütterungsstrategie sowie des pH-Wertes bei Zyklusbeginn auf die Stickstoffeliminationsrate und die N_2O-Emissionen der einstufigen Deammonifikation mit Hilfe einer experimentellen Versuchsplanung untersucht. Zwei Modelle wurden entwickelt, um effektive Prozesskontrollstrategien für einen effizienten und umweltfreundlichen Betrieb abzuleiten. Dabei konnte gezeigt werden, dass die Belüftungsstrategie den höchsten Einfluss auf die Stickstoffeliminationsrate ausübte. Eine maximale Stickstoffeliminationsrate von 0,49±0,03 $kg_N/(m^3 \cdot d)$ wurde für einen Betrieb mit intermittierender Fütterungs- und Belüftungsstrategie sowie einem pH-Wert von 7,46 bei Zyklusbeginn vorhergesagt.

Die Versuche bzgl. der N_2O-Emissionen zeigten, dass minimale N_2O-Emissionen hingegen mit Hilfe einer einmaligen Fütterungsstrategie, kontinuierlicher Belüftung und einem anfänglichen pH-Wert von 7,80 erwartet werden können, wobei der pH-Wert bei Zyklusbeginn den höchsten Effekt auf die N_2O-Emissionen hatte. Diese voneinander abweichenden Einstellungen für eine verbesserte Deammonifikationsleistung und reduzierte N_2O-Emissionen wiesen bereits darauf hin, dass eine gleichzeitige Verbesserung unter den getesteten Bedingungen kaum erreichbar sein wird. Die Kombination der beiden Modelle bestätigte diese Annahme und zeigte eine schwache positive Korrelation zwischen der Deammonifikationsleistung und den N_2O-Emissionen auf. Somit konnte für die Gesamtoptimierung nicht ein einzelner Betriebspunkt festgelegt, jedoch mehrere Einstellungen definiert werden, die einen gewünschten Kompromiss zwischen einer ökonomischen und ökologischen Betriebsweise der Deammonifikation erfüllten.

In der zweistufigen Deammonifikation hatte der Pufferspeicher zwischen der ersten und zweiten Stufe die höchsten Konzentrationen an gelöstem N_2O aller Reaktoren. Erhöhte Nitrit- und Ammoniumkonzentrationen, kohlenstofflimitierte und anoxische Bedingungen sowie eine konstante Zufuhr von Mikroorganismen aus der ersten Stufe stellten offenbar ideale Voraussetzungen für die N_2O-Bildung dar. Eine reduzierte hydraulische Verweilzeit und ein regelmäßiger Abzug der Biomasse aus dem Pufferspeicher sollten zur N_2O-Minimierung beitragen. Auch die Einführung einer anoxischen Phase in der zweiten (unkonventionell belüfteten) Stufe ermöglichte eine N_2O-Reduktion. Somit würde die Umstellung auf eine Belüftungsstrategie mit unbelüfteten Phasen direkt nach der Zugabe des mit gelöstem N_2O angereicherten Zulaufs oder auf einen vollständig anoxischen Prozess der zweiten Stufe komplett ohne Belüftung die Stripping-Effekte verringern.

Trotz aller Bemühungen die N_2O-Emissionen weitestgehend zu reduzieren, wird eine vollständige Vermeidung der N_2O-Bildung höchstwahrscheinlich nicht möglich sein, da N_2O nicht nur biologisch, sondern auch abiotisch produziert wird. Das umweltschädliche N_2O könnte dennoch sicher in inertes N_2 umgewandelt werden, indem die Abluft aus der Deammonifikation vollständig erfasst und zusammen mit Biogas verbrannt werden würden, wodurch zusätzlich die 37 % höhere Energieausbeute von N_2O im Vergleich zu Sauerstoff genutzt werden könnte.

Research Papers and Author Contributions

This cumulative doctoral thesis is based on the following peer-reviewed research papers, which are presented in Chapter 4-6. Their respective Roman numerals (**Paper I – Paper III**) are used to refer to them throughout the text.

Paper I:

Leix, C., Drewes, J. E. & Koch, K. 2016a The role of residual quantities of suspended sludge on nitrogen removal efficiency in a deammonifying moving bed biofilm reactor. Bioresource Technology, 219, 212–218.

- Chapter 4
- Author contributions: Carmen Leix designed and conducted all experiments including chemical and microbial analyses and imaging. She was also responsible for the preparation of this manuscript. Jörg E. Drewes and Konrad Koch supervised this study and reviewed the manuscript.

Paper II:

Leix, C., Hartl, R., Zeh, C., Beer, F., Drewes, J. & Koch, K. 2016b Performance and N_2O formation of the deammonification process by suspended sludge and biofilm systems—A pilot-scale study. Water, 8(12), 578.

- Chapter 5
- Author contributions: Carmen Leix and the operators (Christian Zeh and Franz Beer) of the wastewater treatment plant (WWTP) in Kempten (Allgäu) designed and conceived the experiments. The team of the WWTP operated the pilot plant including data acquisition and chemical analyses. Carmen Leix and Rebecca Hartl were responsible for the dissolved N_2O measurements and analyzed all data. Carmen Leix wrote the manuscript. Jörg E. Drewes and Konrad Koch supervised this study and reviewed the manuscript.

Paper III:

Leix, C., Drewes, J. E., Ye, L. & Koch, K. 2017 Strategies for enhanced deammonification performance and reduced nitrous oxide emissions. Bioresource Technology, 236, 174-185.

- Chapter 6
- Author contributions: Carmen Leix designed and conducted all experiments including chemical analyses, gaseous measurements, and model development. Jörg E. Drewes and Konrad Koch supervised this study. Carmen Leix was responsible for the preparation of this manuscript which was reviewed by Jörg E. Drewes, Liu Ye, and Konrad Koch.

Topic Related Research Papers and Other Scientific Contributions

Topic related publications:

The following topic related peer-reviewed papers have also been prepared and published during this PhD study. However, they are not presented as entire manuscripts in this dissertation, but serve as a supportive element in the respective sub-chapters:

- Zhu, B., Bradford, L., Huang, S., Szalay, A., **Leix, C.**, Weissbach, M., Tancsics, A., Drewes, J.E. & Lüders, T. 2016 Unexpected diversity and high abundance of putative nitric oxide dismutase (Nod) genes in contaminated aquifers and wastewater treatment systems. Applied and Environmental Microbiology, 83(4).
  ↳ supportive element in Chapter 2.1, Chapter 2.3.2, and Chapter 7.4
- Thaler, K., Berger, C., **Leix, C.**, Drewes, J., Niessner, R. & Haisch, C. 2017 Photoacoustic spectroscopy for the quantification of N_2O in the off-gas of wastewater treatment plants. Analytical Chemistry, 89(6), 3795-3801.
  ↳ supportive element in Chapter 2.3.4 and Chapter 7.4

In the following, the most important topic related scientific contributions including German publications, presentations, and posters are listed.

Topic related German publications:

- Koch, K., Weißbach, M., **Leix, C.**, Horstmeyer, N. & Drewes, J. 2015 Gezielte Erzeugung von Lachgas als alternative Behandlung stickstoffreicher Abwasserteilströme einschließlich einer Energierückgewinnung. In: Umwelttechnologie und Energie in Bayern. München, S. 50–53.
- **Leix, C.**, Koch, K., Ebertseder, F., Lindenblatt, C. & Drewes, J. 2017 Alternative Verwertungsmöglichkeiten flüssiger Gärrückstände aus der anaeroben Abfall- und Schlammbehandlung. In: 11. Biogastagung Dresden, Institut für Abfall- und Kreislaufwirtschaft (ed.).

Presentations:

- Koch, K., **Leix, C.**, Weißbach, M., Drewes, J., Haisch, C., Berger, C., Thaler, K. & Nießner, R. 2013 Enabling Energy Savings and Recovery in Contemporary Wastewater Treatment Facilities through Photoacoustic-Based N_2O Monitoring and Control Strategies. International Graduate School of Science and Engineering (IGSSE) Kick-off Meeting, 2-3 December 2013, Munich.
- Koch, K., **Leix, C.**, Weißbach, M., Berger, C., Thaler, K., Haisch, C., Nießner, R. & Drewes, J. 2014 Optimization and reduction of N_2O emission in wastewater treatment. World's Leading Trade Fair for Water, Wastewater and Solid Waste Management (IFAT), 5 May 2014, Munich.
- Weißbach, M., Wolfram, D., **Leix, C.**, Koch, K. & Drewes, J. 2015 Untersuchung der Umgebungsbedingungen bei der biogenen Lachgasproduktion. Jahrestagung der

Wasserchemischen Gesellschaft - Fachgruppe in der Gesellschaft Deutscher Chemiker; 11-13 May 2015, Schwerin.

- **Leix, C.**, Drewes, J. E. & Koch, K. 2016 Nitrous oxide formation of a two-stage deammonification pilot-plant – What lessons can we learn? Nitrous oxide emissions from biological wastewater treatment. Expert Meeting and Workshop, 22-23 September 2016, Bochum.
- **Leix, C.**, Drewes, J. E. & Koch, K. 2016 Strategies for the enhancement of the deammonification‘s performance and the reduction of N_2O emissions. The University of Queensland (UQ), 1 November 2016, Brisbane.
- **Leix, C.**, Drewes, J. E. & Koch, K. 2016 Innovative strategies for both the enhancement of the deammonification‘s performance and the reduction of N_2O emissions. The University of New South Wales (UNSW), 17 November 2016, Sydney.

Posters:

- **Leix, C.**, Weißbach, M., Koch, K., Drewes, J., Berger, C., Thaler, K., Haisch, C. & Nießner, R. 2014 Innovative monitoring and minimization/maximization strategies for N_2O emissions from wastewater treatment processes. Annual International Graduate School of Science and Engineering (IGSSE) Forum, 16-18 July 2014, Burghausen.
- **Leix, C.**, Koch, K. & Drewes, J. 2015 N_2O emissions of deammonification: Development of minimization strategies. Doctoral Candidates Day, 21 January 2015, Munich.
- **Leix, C.**, Weißbach, M., Koch, K., Drewes, J., Berger, C., Thaler, K., Haisch, C. & Nießner, R. 2015 N_2O emissions from wastewater treatment processes – Innovative monitoring and minimization/maximization strategies. 11th IWA Leading Edge Conference on Water and Wastewater Technologies, 26-29 May 2015, Abu Dhabi.
- Weißbach, M., **Leix, C.**, Koch, K., Drewes, J., Berger, C., Thaler, K., Haisch, C. & Nießner, R. 2015 Innovative monitoring and utilization of N_2O emissions in wastewater treatment processes. The University of Queensland - Technische Univerisät München - Research Symposium on Water, Environment and Sustainability, 11-12 June 2015, Munich.
- **Leix, C.**, Weißbach, M., Koch, K., Drewes, J., Berger, C., Thaler, K., Haisch, C. & Nießner, R. 2015 PANOWA: Enabling energy savings and recovery in contemporary wastewater treatment facilities through photoacoustic-based N_2O monitoring and control strategies. Annual International Graduate School of Science and Engineering (IGSSE) Forum, 1-3 July 2015, Burghausen.
- Thaler, K., Berger, C., Nießner, R., Haisch, C., **Leix, C.**, Weißbach, M., Koch, K. & Drewes, J. 2016 N_2O monitoring by photoacoustic spectroscopy during wastewater treatment. Annual International Graduate School of Science and Engineering (IGSSE) Forum, 1-3 June 2016, Burghausen.
- **Leix, C.**, Weißbach, M., Koch, K., Drewes, J., Berger, C., Thaler, K., Haisch, C. & Nießner, R. 2015 Innovative Vermeidungs- und Produktionsstrategien für N_2O in der Abwasseraufbereitung. 44. Abwassertechnisches Seminar (ATS), 14 July 2016, Garching.

Abbreviations

AbwV	Abwasserverordnung (Wastewater Directive)
Aer	Aeration strategy
AM	Anammoxosome membrane
AMO	Ammonia monooxygenase
Anammox	Anoxic ammonium oxidation
AnAOB	Anoxic ammonium oxidizing bacteria
ANOVA	Analysis of variance
AOA	Ammonium oxidizing archaea
AOB	Ammonium oxidizing bacteria
ATH	Allylthiourea
ATP	Adenosine triphosphate
BC	Biofilm on carriers
BNR	Biological nitrogen removal
BOD	Biochemical oxygen demand
C_0	Center point
CANDO	Coupled aerobic–anoxic nitrous decomposition operation
CLSM	Confocal laser scanning microscopy
COD	Chemical oxygen demand
Comammox	Complete ammonia oxidation
Cond.	Conductivity
DFG	Deutsche Forschungsgemeinschaft (German Research Foundation)
DNRA	Dissimilatory nitrate reduction to ammonia
DO	Dissolved oxygen
DoE	Design of experiment
EDTA	Ethylenediaminetetraacetic acid
FA	Free ammonia (or formamide as used in Paper I)
FI	Flow indication
FISH	Fluorescence *in situ* hybridization
FNA	Free nitrous acid
FTIR	Fourier transform infrared
GC	Gas chromatography
GHG	Greenhouse gas
GWP	Global warming potential
HAO	Hydroxylamine oxidoreductase
H^{cp}	Henry's law constant [mol/(m^3·Pa)]
HH	Hydrazine hydrolase
HRT	Hydraulic retention time
HZO	Hydrazine oxidoreductase
ICM	Intracytoplasmic membrane
IFAS	Integrated fixed-film activated sludge
IGSSE	International Graduate School of Science and Engineering
IPCC	Intergovernmental Panel on Climate Change

IR	Infrared
k_La	Mass transfer coefficient
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LOD	Limit of detection
MBBR	Moving bed biofilm reactor
MLR	Multiple linear regression
MLSS	Mixed liquor suspended solids
n	Amount of experiments/influencing factors
N_2OR	Nitrous oxide reductase
NAR	Nitrate reductase
NDIR	Nondispersive infrared
NIR	Nitrite reductase
NOB	Nitrite oxidizing bacteria
NOD	Nitric oxide dismutase
NOR	Nitric oxide reductase
NOS	Nitrite oxidoreductase
ORP	Oxidation-reduction potential
P & ID	Piping and instrumentation diagram
PA	Photoacoustic
PBS	Phosphate buffered saline
PE	Population equivalent
PFA	Paraformaldehyde
PN	Partial nitritation
PN/A	Partial nitritation coupled with the anammox process
Q^2	Goodness of prediction
Q_{feed}	Volumetric flow rate of the feed
R^2	Coefficient of determination
RBC	Rotating biological contactors
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RSM	Response surface methodology
SB	Suspended biomass
SBR	Sequencing batch reactor
SCADA	Supervisory control and data acquisition
SDS	Sodium dodecyl sulfate
SI	Supplementary information
SPE	Solid-phase extraction
SRT	Sludge retention time
TOrC	Trace organic compounds
Tris	Tris(hydroxymethyl)aminomethane
TSS	Total suspended solids
TUM	Technical University of Munich
VSS	Volatile suspended solids
WWTP	Wastewater treatment plant

X_1	pH value
X_2	Feeding strategy
X_3	Aeration strategy
X_j	Influencing factor j
Y_1	Nitrogen removal rate
Y_2	N_2O emission
Y_i	Response variable i
β	Coefficient
$\Delta G^{0'}$	Biological standard Gibbs energy change (at pH 7 and 25 °C)
ε	Error of the model
ϑ	Temperature

Chemicals

CH_4	Methane
CO_2	Carbon dioxide
$H_2N_2O_2$	Hyponitrous acid
H_2S	Hydrogen sulfide
HCl	Hydrogen chloride
HNO	Nitroxyl
HNO_2	(Free) nitrous acid
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate
N_2	Nitrogen gas
N_2H_4	Hydrazine
N_2O	Nitrous oxide
Na_2HPO_4	Disodium phosphate
NaCl	Sodium chloride
NaN_3	Sodium azide
NH_2OH	Hydroxylamine
NH_3	Free ammonia
NH_4-N	Ammonium nitrogen
NO	Nitric oxide
NO_2^-	Nitrite
NO_2-N	Nitrite nitrogen
NO_3^-	Nitrate
NO_3-N	Nitrate nitrogen
NO_x^-	Nitrogen oxides
N_{total}	Total nitrogen
O_2	Oxygen

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1 Introduction

Nitrogen removal is an important aim of wastewater treatment to prevent an adverse impact of nitrogen compounds on the receiving aquatic environment. With increasingly strict legal requirements for effluent discharge, wastewater treatment plants (WWTP) might reach their nitrogen removal design capacities. Beyond that, WWTPs are encouraged to constantly reduce their costs and energy consumption for a more cost-effective operation as wastewater treatment accounts for approximately 3 % of the electrical energy load of developed countries (McCarty *et al.* 2011).

An interesting opportunity to tackle these challenges is an implementation of a side-stream deammonification process comprised of a partial nitritation and an anoxic ammonium oxidation (anammox). Even if the internal wastewater flow – especially influenced by ammonium-rich reject water from dewatering of anaerobically digested sludge – represents only a low proportion of the total hydraulic load of approximately 1 % (van Dongen *et al.* 2001), it can account for 10-30 % of the total nitrogen load of a WWTP (van Loosdrecht and Salem 2006; Gilbert *et al.* 2015). A separate treatment of this wastewater stream offers the opportunity to severely reduce the nitrogen load in mainstream and thus, to improve the total nitrogen removal capacity of a WWTP (Fux *et al.* 2002). Furthermore, nitrogen removal by the deammonification process can reduce the operational costs of up to 90 % (Jetten *et al.* 2001) as it features several advantages compared to conventional nitrification/denitrification. It is an autotrophic nitrogen removal process, which is why the carbon-source demand can not only be reduced by 100 % (Fux *et al.* 2002), but alternatively be used for a conversion of soluble organic matter into biomass for enhanced methane (CH_4) production during digestion (Kartal *et al.* 2010; Ma *et al.* 2016). Additionally, the oxygen demand and the excess sludge production can be reduced by 60 % (Ma *et al.* 2016) and 80 % (Fux *et al.* 2002; Cao *et al.* 2017), respectively. Nevertheless, the deammonification process is vulnerable to process instabilities that can lead to a temporal process failure (Joss *et al.* 2011; Lackner *et al.* 2014), or as a worst case scenario even to an ultimate breakdown.

Even though the deammonification process offers the opportunity to improve the nitrogen removal capacity of a WWTP, it has also been reported to emit nitrous oxide (N_2O) as an undesired byproduct, yet with a high variation of concentrations (Kampschreur *et al.* 2008b). N_2O not only contributes to global warming as it is a strong greenhouse gas with a global warming potential 298 times higher than that of carbon dioxide (CO_2) based on a 100-year time horizon, but also to stratospheric ozone depletion (Intergovernmental Panel on Climate Change (IPCC) 2013). Currently, N_2O is the most heavily emitted ozone-depleting substance worldwide (Ravishankara *et al.* 2009). With deammonification being an energy efficient process, undesired emissions of N_2O with its high negative environmental impact would be extremely counterproductive from an environmental perspective. In December 2015, the participating parties at the United Nations Conference on Climate Change in Paris consented to the common,

generalized goal to limit the world's temperature increase to 1.5 °C above the pre-industrial value (United Nations Framework Convention on Climate Change 2015). However, no explicit restrictions for N_2O emissions were determined. Without legislative regulations, WWTP operators are neither forced to monitor nor to reduce their N_2O emissions. Thus, operational settings for both simultaneously increased nitrogen removal rates and reduced N_2O emissions would be one incentive for WWTP operators to implement voluntarily an environmentally friendly deammonification process. This would also avoid that the benefits of the deammonification's reduced energy consumption and with that its reduced carbon footprint would be compromised by these emissions.

For these reasons, this dissertation was aiming for two key research objectives: development of effective strategies for i) optimized nitrogen turnover rates of the deammonification process and ii) simultaneously reduced N_2O emissions. With a growing number of full scale deammonification installations worldwide (Lackner *et al.* 2014) and especially in times of climate change, such strategies for deammonification plants are crucial to improve their operation and minimize negative long-term effects on the environment induced by their N_2O emissions. In this respect, several experiments for one-stage and two-stage deammonification systems employing both suspended sludge and biofilm carriers at laboratory and pilot scale have been carried out. **Paper I** demonstrated how the nitrogen removal efficiency of a deammonifying moving bed biofilm reactor (MBBR) is positively influenced by even small quantities of residual suspended sludge. Results regarding the microbial segregation in the biofilm and the flocs based on chemical analyses were supplemented by fluorescence *in situ* hybridization (FISH) imaging. In **Paper II**, the reactor performances of two two-stage deammonification systems with suspended sludge and biofilm carriers in the second stage were compared. Additionally, dissolved N_2O concentrations were measured in all reactors employing different microbial reactions. Based on these results, possibilities for enhanced nitrogen removal and mitigation strategies for reduced N_2O formation and emissions were discussed. **Paper III** methodically investigated the simultaneous improvement of the nitrogen removal rate and the reduction of N_2O emissions under varying boundary conditions by the development of two models based on a design of experiment (DoE) method. As a side-aspect during this study, the reduction potential of trace organic chemicals (TOrCs) – including pharmaceuticals, personal care products, and household chemicals – by the one-stage deammonification process was elucidated.

2 Background

2.1 Biological Nitrogen Removal Processes

Nitrogen is one of the constituents that is required to be removed from wastewater to protect the environment form its undesired discharge which can lead to eutrophication, oxygen depletion, or fish die-off. For that reason, German legislation has restricted the effluent concentrations of the chemical oxygen demand (COD), the biochemical oxygen demand after five days of incubation (BOD_5), ammonium, total nitrogen, and total phosphorus by defining effluent standards for municipal wastewater treatment plants as listed in Table 1 (AbwV 2004). Nonetheless, neither N_2O emissions nor TOrCs effluent concentrations are regulated so far, even though they can have a severe effect on the environment (Intergovernmental Panel on Climate Change (IPCC) 2013) as well as on human and ecosystem health (Alidina *et al.* 2014). Therefore, it is important to develop N_2O mitigation strategies and to investigate which share of unintended, but desirable TOrC removal as an advantageous side-effect can be achieved by nitrogen removal processes.

Table 1. Effluent standards for municipal WWTPs (AbwV 2004)

WWTP Size Category		**COD [mg/L]**	**BOD_5 [mg/L]**	**NH_4-N [mg/L]**	**N_{total} [mg/L]**	**P_{total} [mg/L]**
I:	**BOD_5 < 60 kg/d**	150	40	-	-	-
II:	**60 kg/d ≤ BOD_5 ≤ 300 kg/d**	110	25	-	-	-
III:	**300 kg/d < BOD_5 ≤ 600 kg/d**	90	20	10	-	-
IV:	**600 kg/d < BOD_5 ≤ 6000 kg/d**	90	20	10	18	2
V:	**BOD_5 > 6000 kg/d**	75	15	10	13	1

Biological nitrogen removal (BNR) can generally be divided into two sub-processes. First, ammonium (NH_4^+) is oxidized to nitrogen oxides (NO_x^-) under aerobic conditions, followed by a NO_x^--reduction to nitrogen gas (N_2) under anoxic conditions, catalyzed by specific enzymes (Figure 1). In most WWTPs, the conventional treatment scheme of autotrophic nitrification combined with heterotrophic denitrification is applied. During nitrification, NH_4^+ is oxidized over hydroxylamine (NH_2OH) to nitrite (NO_2^-) and further to nitrate (NO_3^-) by use of molecular oxygen as electron acceptor supplied by aeration and by carbon dioxide serving as carbon source; during denitrification, NO_3^- is subsequently reduced to N_2 over NO_2^-, nitric oxide (NO), and nitrous oxide (N_2O) under anoxic conditions with organic matter serving as carbon source (Ahn 2006).

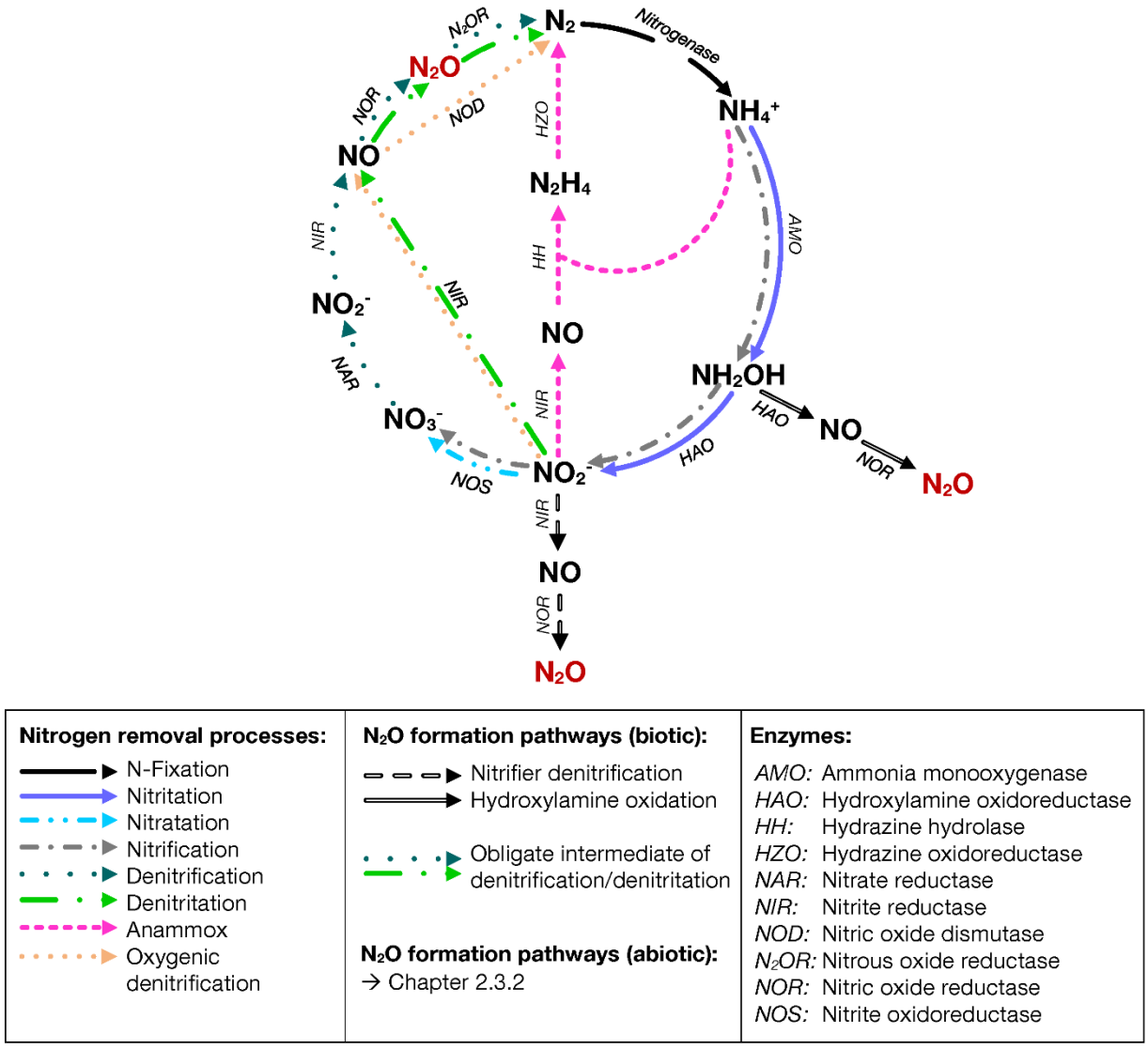

Figure 1. Nitrogen cycle demonstrating different biological nitrogen removal processes and N_2O formation pathways (after descriptions by (Schmidt *et al.* 2003; Jetten *et al.* 2009; Richardson *et al.* 2009; Chandran *et al.* 2011; Kartal *et al.* 2011; Stein 2011; Schreiber *et al.* 2012; Zhu *et al.* 2016)

Incentives for a more sustainable wastewater treatment have revealed novel and more economically beneficial BNR processes in the past years, which enable both the reduction of the aeration energy and carbon consumption. These approaches include a combination of nitritation with denitritation, which is a shortcut of the conventional nitrification/denitrification by omitting the process of nitratation (oxidation of NO_2^- to NO_3^-) rendering the subsequent reduction back to NO_2^- superfluously. For these reasons, 25 % of the aeration demand and 40 % of the carbon demand can be saved when a combination of nitritation with denitritation is applied (Fux *et al.* 2006).

Another possibility and an example for a complete autotrophic BNR method offers the deammonification process, which couples partial nitritation (PN) with the so-called anoxic ammonium oxidation (anammox), abbreviated as PN/A. Here, the term ‘anoxic’ instead of the traditionally utilized ‘anaerobic’ ammonium oxidation is applied, as electron acceptors in form of

oxidized nitrogen species, such as nitrite, are available. In the anammox process, anoxic ammonium oxidizing/anammox bacteria (AnAOB) convert residual ammonium via the intermediate hydrazine (N_2H_4) to N_2 with NO_2^- as electron acceptor and CO_2 as carbon source (Strous *et al.* 1998; Kartal *et al.* 2010; Kartal *et al.* 2013).

Comparing these nitrogen removal processes economically, an implementation of the deammonification process can offer the greatest aeration and carbon savings, yet a stable operation in the long term can still be challenging. Therefore, this biological process, as well as favorable process conditions, and diverse system configurations and operations are described in detail in Chapter 2.2. Furthermore, the potent greenhouse gas N_2O can occur as an undesired byproduct of nitrogen removal and is part as an obligate intermediate during denitrification (Figure 1), which can severely impact a WWTP's carbon footprint when it is emitted into the atmosphere (Pijuan *et al.* 2014). Therefore, Chapter 2.3 is dedicated to a detailed description of N_2O emissions during wastewater treatment, different N_2O formation pathways, favorable conditions for N_2O formation, as well as analytical methods for the measurement of dissolved and gaseous N_2O.

2.2 The Deammonification Process in Detail

2.2.1 Microbiological Reactions

The existence of AnAOBs as part of the deammonification process has already been predicted in 1977 based on thermodynamic calculations (Broda 1977). Nevertheless, it was not before the mid-1990s that this proposed nitrogen removal process was discovered in a denitrifying fluidized bed reactor giving first evidence for AnAOB existence (Mulder *et al.* 1995). Further research proposed the stoichiometry of the unique catabolic pathway of AnAOBs that allows them to gain their energy for microbial growth over the ammonium oxidization combined with nitrite reduction into nitrogen gas under anoxic conditions (Strous *et al.* 1998). This discovery not only offered new wastewater treatment possibilities, but also revolutionized the entire nitrogen cycle. Until now, AnAOBs have been determined to be present in both marine and freshwater ecosystems, including oceans, estuaries, lakes, rivers, marshes (Jetten *et al.* 2009) and are even believed to be ubiquitous in any nitrogen containing environment with an anoxic zone or a chemocline (Francis *et al.* 2007). Consequently, 30-50 % of the N_2 production by the oceans might be attributed to the anammox reaction (Devol 2003; Arrigo 2005). The different biological reactions that are related to the deammonification process and which are visualized in Figure 1 are described in detail in the following section.

To provide the required substrates for the anammox process, ammonium partially needs to be oxidized to nitrite in a first step called nitritation, which is performed by a wide range of microorganisms. Most of these are ammonium oxidizing bacteria (AOB) that can be assigned to two monophyletic groups of *beta*- and *gamma-Proteobacteria*. The genera *Nitrosomonas* and

Nitrosospira belong to the *beta*-ammonia oxidizers, whereas *Nitrosococcus* is part of the *gamma*-ammonia oxidizers (Schmidt *et al.* 2003). Besides those, some ammonium oxidizing archaea (AOA) have been found to be capable of ammonium oxidation under low oxygen concentration in marine environments (Könneke *et al.* 2005) and wastewater treatment plants (Park *et al.* 2006; Yang *et al.* 2012; Liu *et al.* 2017). Nevertheless, bacteria were always dominant within the microbial community of wastewater treatment systems (Yang *et al.* 2012; Liu *et al.* 2017).

Nitritation is an exergonic reaction, as the biological standard Gibbs energy change[1] $\Delta G^{0'}$ is below zero, and takes place according to the following stoichiometry (Schmidt *et al.* 2003):

$$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + 2H^+ + H_2O \qquad (\Delta G^{0'} = -275 \frac{kJ}{mol}) \qquad (1)$$

However, rather ammonia (NH_3) than ammonium is believed to be the actual substrate for the microorganisms (Van Hulle *et al.* 2007), which is first oxidized to the intermediate NH_2OH and subsequently to NO_2^-, catalyzed by the ammonia monooxygenase (AMO) and the hydroxylamine oxidoreductase (HAO), respectively, according to equation (2) and (3) (Schmidt *et al.* 2003):

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \qquad (\Delta G^{0'} = -120 \frac{kJ}{mol}) \qquad (2)$$

$$NH_2OH + H_2O \rightarrow HNO_2 + 4H^+ + 4e^- \qquad (\Delta G^{0'} = -289 \frac{kJ}{mol}) \qquad (3)$$

Nitratation is an undesired process during deammonification, as nitrite oxidizing bacteria (NOB) and AnAOBs compete for the substrate nitrite. Therefore, conditions should be adjusted in a way that they lead to a suppression of NOBs. Strategies for their inhibition in the short and their repression in the long term are described in Chapter 2.2.2. From a microbial perspective, NOBs can be assigned to different genera, such as *Nitrobacter* and *Nitrococcus* (*alpha-Proteobacteria*) and *Nitrospira* (forms a phylogenetical distinct division); they gain their energy for microbial growth from the oxidation of nitrite to nitrate catalyzed by the nitrite oxidoreductase (NOS) (Schmidt *et al.* 2003):

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^- \qquad (\Delta G^{0'} = -74 \frac{kJ}{mol}) \qquad (4)$$

Thus, nitrification so far was believed to be catalyzed by two physiologically distinct clades of microorganisms. However, complete ammonia oxidation to nitrate abbreviated “comammox” catalyzed by a single microorganism was demonstrated to be feasible recently (Daims *et al.* 2015; van Kessel *et al.* 2015). Two *Nitrospira* species were found to be capable of this process and tentatively named “*Candidatus* Nitrospira nitrosa” and “*Candidatus* Nitrospira nitrificans” (van Kessel *et al.* 2015).

[1] At standard conditions of pH 7 and 25 °C (Mavrovouniotis (1991).

The anammox reaction is a chemolithoautotrophic process performed by AnAOBs that convert ammonium with nitrite as an electron acceptor at a molar-ratio of 1:1.32 into nitrogen gas and a production of approximately 11 % nitrate to generate reducing equivalents for CO_2 fixation (and biomass represented by $CH_2O_{0.5}N_{0.15}$) under anoxic conditions according to equation (5) (Strous *et al.* 1998; Schmidt *et al.* 2003; Kuenen 2008; Jetten *et al.* 2009):

$$NH_4^+ + 1.32\,NO_2^- + 0.066\,HCO_3^- + 0.13\,H^+ \rightarrow 1.02\,N_2 + 0.26\,NO_3^- + 0.066\,CH_2O_{0.5}N_{0.15} + 2.03\,H_2O \qquad (\Delta G^{0'} = -357\,\frac{kJ}{mol}) \qquad (5)$$

Models based on genomics and experimental results suggest, that nitrite is first reduced to nitric oxide (NO), which is further transformed together with ammonium to toxic and energy-rich hydrazine (N_2H_4) and subsequently oxidized to N_2, catalyzed by the nitrite reductase (NIR), hydrazine hydrolase (HH), and hydrazine oxidoreductase (HZO), respectively (Jetten *et al.* 2009). Interestingly, 'Kuenenia stuttgartiensis' as one species of AnAOBs were found to be able of dissimilatory nitrate reduction to ammonia (DNRA), thus having the capacity to reduce nitrate to ammonium via nitrite as the intermediate followed by the usual anammox pathway as an additional nitrogen removal (Kartal *et al.* 2007a).

AnAOBs combine a number of unique features, including the use of N_2H_4 to form gaseous N_2, an intracytoplasmic cell compartment called "anammoxosome", where this reaction takes place, as well as tightly packed ladderane lipids surrounding the anammoxosome (Francis *et al.* 2007; van Niftrik *et al.* 2008; Jetten *et al.* 2009). This unique cell structure of AnAOBs has been studied by several researchers (Lindsay *et al.* 2001; van Niftrik *et al.* 2004; Jetten *et al.* 2009). It can be divided into three cytoplasmic parts – the paryphoplasm, the riboplasm, and the anammoxosome – all separated by specific membranes (Figure 2). The outer compartment consists of the paryphoplasm separated from the outside with a cell wall and a cytoplasmic membrane and from the inside with an intracytoplasmic membrane. The intermediate layer is composed of the riboplasm, which contains a fibrillar nucleoid and ribosome-like particles, thus all RNA. Most of the DNA within the fibrillary nucleoid appears in the riboplasm, but some DNA can be also found in the anammoxosome, which represents the internal ribosome-free cell compartment of the anammox cell bounded by the anammoxosome membrane and accounts for 50-70 % of the total cell volume. Dense packed ladderane membrane lipids surrounding the anammoxosome, which are unique in nature and have been found exclusively in anammox bacteria, is believed to help in keeping the toxic and mutagenic intermediates of hydrazine and nitric oxide within the anammoxosome. The anammoxosome membrane furthermore enables the generation and maintenance of a proton motive force for synthesis of adenosine triphosphate (ATP). The difference to other gram-negative bacteria is the lack of a peptidoglycan cell wall, which is instead a proteinaceous one and the fact that the cell wall is not enclosed in two membranes on the inside and outside, but both membranes – the cytoplasmic and intracytoplasmic membrane – are on the inside. AnAOBs are coccoid microorganisms, approximately 1 µm in diameter.

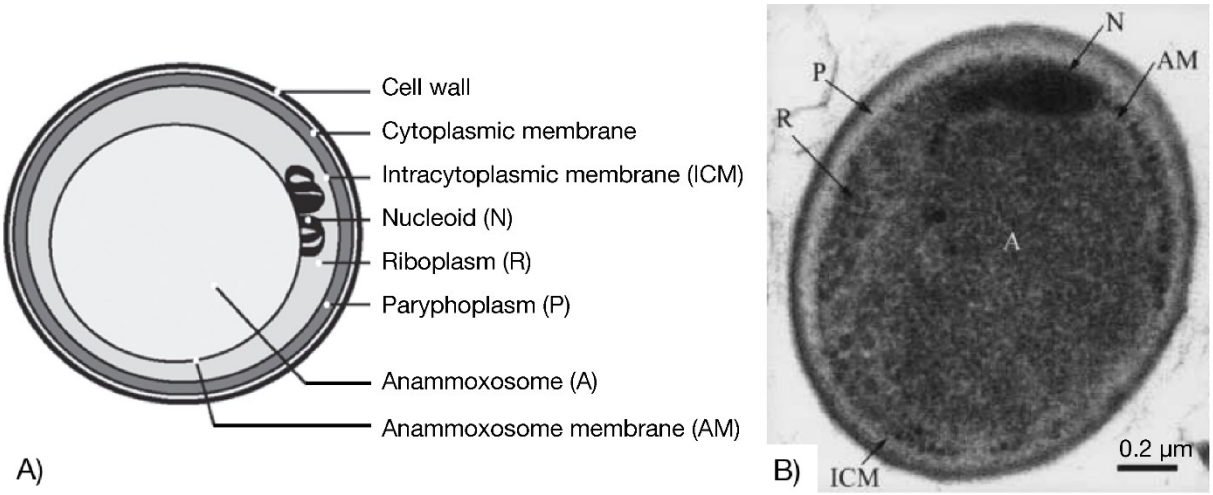

Figure 2. A) Schematic cell structure of AnAOBs (Jetten *et al.* 2009); B) Microscopic image of "Brocadia anammoxidans" (Lindsay *et al.* 2001)

Based on phylogenetic analysis, AnAOBs have been identified to belong to the order *Brocadiales* with the phylum *Planctomycetales* (Strous *et al.* 1999a; Schmid *et al.* 2005). Six anammox genera (*Candidatus* 'Kuenenia', *Ca.* 'Brocadia', *Ca.* 'Anammoxoglobus', *Ca.* 'Jettenia', *Ca.* 'Anammoximicrobium', and *Ca.* 'Scalindua') with 23 anammox species have been identified so far, including 'Kuenenia stuttgartiensis' (Schmid *et al.* 2000; Strous *et al.* 2006), 'Brocadia anammoxidans' (Jetten *et al.* 2001), 'Brocadia fulgida' (Kartal *et al.* 2008), 'Brocadia sp. 40' (van der Star *et al.* 2008a), 'Brocadia sinicia' (Hu *et al.* 2010), and 'Brocadia caroliniensis' (Rothrock *et al.* 2011), 'Anammoxoglobus propionicus' (Kartal *et al.* 2007b), and 'Anammoxoglobus sulfate' (Liu *et al.* 2008), 'Jettenia asiatica' (Quan *et al.* 2008) and 'Jettenia caeni' (Ali *et al.* 2015), as well as 'Anammoximicrobium moscowii' (Khramenkov *et al.* 2013). The sixth genera with 'Scalindua sorokinii' (Kuypers *et al.* 2003), 'Scalindua wagneri' (Schmid *et al.* 2003), 'Scalindua brodae' (Schmid *et al.* 2003), 'Scalindua arabica' (Woebken *et al.* 2008), 'Scalindua sinooilfield' (Li *et al.* 2010), 'Scalindua zhenghei' (Hong *et al.* 2011), 'Scalindua richardsii' (Fuchsman *et al.* 2012), 'Scalindua profunda' (van de Vossenberg *et al.* 2013), 'Scalindua marina' and 'Scalindua pacifica' (Dang *et al.* 2013), 'Scalindua sp.' (Awata *et al.* 2013), and 'Scalindua japonica' (Ali and Okabe 2015) has often been found in a marine environment and oxygen minimum zones. For the detection and visualization of the different AnAOB species, different FISH probes have been developed in the past. This technique has been applied in **Paper I** to demonstrate the microbial segregation of AOBs and AnAOBs in suspended sludge and biofilm. On a larger scale, biomass of AnAOBs exhibit a brown-reddish color, which is probably related to their high cytochrome content (Jetten *et al.* 1999).

Compared to conventional nitrification/denitrification, partial nitritation combined with the anammox process has several advantages. As it is an autotrophic nitrogen removal process, the carbon-source demand can be decreased by 100 % (Fux *et al.* 2002). Additionally, ammonium only needs to be oxidized partially to nitrite and a further oxidation of nitrite to nitrate is omitted

which is why the oxygen demand can be reduced by 60 % (Ma *et al.* 2016). Due to a low biomass yield of AnAOBs, the excess sludge can be decreased by 80 % (Fux *et al.* 2002; Cao *et al.* 2017). Thus, implementing the deammonification process can reduce the operation costs of up to 90 % (Jetten *et al.* 2001).

2.2.2 Favorable Process Conditions

For a stable and efficient operation of the deammonification process, favorable process conditions for both AOBs and AnAOBs are essential, while NOBs need to be suppressed effectively to prevent nitrate production. Therefore, several control strategies can be applied, which include influencing the aeration strategy and the dissolved oxygen (DO) concentration, the free ammonia (FA) and free nitrous acid (FNA) concentration, the pH and temperature range, the sludge retention time (SRT), as well as the nitrite concentration amongst others as described hereinafter. These approaches can not only be applied to favor AOBs and AnAOBs, and enable an inhibition of the NOB activity in the short-term, but were also successfully in causing a low NOB abundance in the long-term (Yang *et al.* 2007; Lemaire *et al.* 2008; Wang *et al.* 2014; Ma *et al.* 2015).

- Aeration strategy and DO concentration:
  An alternating aeration strategy of oxic and anoxic phases can take advantage of the NOBs' longer lag-phase in comparison to the AOBs' (Yoo *et al.* 1999; Kornaros *et al.* 2010; Gilbert *et al.* 2014). According to (Gilbert *et al.* 2014), the duration of the NOB's lag phase is biomass specific, however in their study, it was in a range of 5-15 min; additionally, an anoxic phase of at least 15-20 min should be supplied. Furthermore, this intermittent aeration strategy also provides anoxic phases which are crucial for AnAOBs, as they are already inhibited by DO concentrations as low as 0.2 mg/L (Joss *et al.* 2011); however, oxygen inhibition is reversible (Strous *et al.* 1997; Egli *et al.* 2001; Joss *et al.* 2011). This reversibility enables the deammonification process to be conducted in one reactor as one-stage treatment of wastewater (for the different system configurations and operations, see Chapter 2.2.3). Nevertheless, also continuous aeration was proven to support simultaneous PN/A under low DO concentrations; this is possible as the measured DO concentrations do not necessarily coincide with those in a floc/granule/biofilm due to a DO gradient that develops over the depth with anoxic conditions in deeper layers (Maslon and Tomaszek 2007; Joss *et al.* 2009; Almstrand *et al.* 2014). Continuous aeration has been successfully applied in full scale applications, for instance in a sequencing batch reactor (SBR) at the WWTP Zürich at a targeted DO concentration below 0.05 mg/L (Lackner *et al.* 2014). According to (Joss *et al.* 2009), continuous aeration is advantageous regarding its simplicity, as the aeration aggregates do not need to be turned on and off alternatingly; it also simplifies the monitoring of the online-signals as the signal-to-noise can be reduced and it was hypothesized to lead

eventually to an higher deammonification performance. Furthermore, providing low DO concentrations favor AOBs in comparison to NOBs as they have a higher oxygen affinity (Guisasola *et al.* 2005; Ciudad *et al.* 2006; Blackburne *et al.* 2008). Thus, the activity of NOBs is more affected by low DO concentrations than that of AOBs. (Hanaki *et al.* 1990) demonstrated that nitrite oxidation did not take place at DO concentrations below 0.5 mg/L which was later also supported by a model-based approach for optimized nitrogen removal by (Sin *et al.* 2006).

- FA concentration:
  Even though NH_3 (free ammonia[2], FA) is believed to serve as the substrate for AOBs, it has also an inhibitory effect on AOBs, NOBs, and AnAOBs at different threshold concentrations. As the FA concentration correlates positively with an increasing ammonium concentration, temperature, and pH value (Anthonisen *et al.* 1976), adjusting the process conditions offers the possibility to supply FA concentrations that inhibit nitratation, but still enable the nitritation and anammox process. Generally, AOBs were reported to endure higher FA concentrations than NOBs (Anthonisen *et al.* 1976). An inhibitory effect on *Nitrobacter* as a representative of NOBs has been reported to begin at FA concentrations as low as 0.1 mg_N/L and cause a complete inhibition at 1.0 mg_N/L, while values for *Nitrosomonas* as a representative of AOBs were remarkably higher at 10 mg_N/L and 150 mg_N/L (Anthonisen *et al.* 1976). (Vadivelu *et al.* 2006a) revealed in their study that FA concentrations of up to 16 mg_N/L did not inhibit *Nitrosomonas* in any way, yet this threshold might be even higher as more elevated FA concentrations were not investigated. (Turk and Mavinic 1989) demonstrated that the microbial community also could get adapted to elevated NH_3 concentrations, as AOBs and NOBs were not influenced by FA concentrations as high as 40 mg_N/L in their system. (Fernandez *et al.* 2012) observed that the activity of AnAOBs was not negatively affected by FA concentrations of 20 mg_N/L in the long-term, however, elevated concentrations between 35 and 40 mg_N/L caused an unstable operation. (Tang *et al.* 2009) reported a process instability at even higher FA concentrations of approximately 70 mg_N/L. Thus, inhibitory FA concentrations might be different for every WWTP due to different operational conditions, microbial communities, and adaptational durations, but generally, NOBs are the most sensitive bacteria of the deammonifying microbial community towards FA concentrations.

- FNA concentration:
  Besides the inhibitory effect of FA, also free nitrous acid[3] (FNA; HNO_2) can negatively influence the microorganism's metabolism. The concentration of FNA increases with increasing nitrite concentrations, decreasing pH values and temperature. Again, AOBs

[2] Calculation of the FA concentration is listed in the Appendix.
[3] Calculation of the FNA concentration is listed in the Appendix.

were found to be less sensitive to elevated FNA concentrations than NOBs (Anthonisen *et al.* 1976; Vadivelu *et al.* 2006a; Vadivelu *et al.* 2006b; Kim *et al.* 2012). The inhibitory threshold FNA concentrations differ between AOBs and NOBs by approximately one order of magnitude (Zhou *et al.* 2011). For instance, FNA concentrations of 0.40 mg_N/L and 0.02 mg_N/L were determined to completely inhibit the anabolic metabolism of *Nitrosomonas* and *Nitrobacter*, respectively (Vadivelu *et al.* 2006a; Vadivelu *et al.* 2006b). (Wang *et al.* 2014) transferred this knowledge successfully into practice and could achieve nitritation by treating a portion of recirculated sludge with FNA in a side-stream unit. AnAOBs are even more sensitive to FNA than NOBs. (Fernandez *et al.* 2012) reported a destabilized system and efficiency loss for long term operation at FNA concentrations around 1.5 $\mu g_N/L$. In short-term tests, they could show that the activity decreased to 30 % of its initial value for flocculent AnAOBs at FNA concentrations of 4.4 $\mu g_N/L$ with complete inhibition at 11.0 $\mu g_N/L$. AnAOBs protected by their biofilm structure were more resistant, as no inhibition was observed at concentrations of up to 6.6 $\mu g_N/L$, but their activity was halved at 11.0 $\mu g_N/L$. Therefore, the technique for NOB inhibition by elevated FNA concentrations seems to be restricted to two-stage deammonification systems, where nitritation is locally separated from the anammox process.

- pH value, temperature, and SRT:
  Apart of influencing the FA and FNA concentrations, the pH value and temperature also effect directly the microbial activity. Generally, the favorable pH range for AOBs is between pH 6.5 and pH 8.0 (Van Hulle *et al.* 2007), which applies comparably for NOBs (Van Hulle *et al.* 2010). In pure culture, *Nitrosomonas* exhibited its optimum at pH 8.1 and 35 °C, whereas the optimal pH and temperature for *Nitrobacter* were at pH 7.9 and 38 °C, respectively (Grunditz and Dalhammar 2001). AnAOBs prefer a slightly higher pH range of pH 6.7-pH 8.3 with an optimum at pH 8.0 (Jetten *et al.* 1999). Their physiological temperature range varies between 20 and 43 °C (Strous *et al.* 1999b) with an optimum reported at 37 °C (Egli *et al.* 2001). However, with AnAOBs being widespread in diverse environments, they were also reported to endure temperatures as low as -2°C, such as in the ice sea in Greenland (Jetten *et al.* 2009). With the temperature influencing the microbial activity, increasing the temperatures above 25 °C and reducing the SRT to 1.0-1.5 d promote a faster growth of AOBs than NOBs and therefore, favor the retention of AOBs and wash-out of NOBs (Van Hulle *et al.* 2010). However, the application of this strategy is limited for a combined partial nitritation/anammox process in one reactor due to a lower growth rate of AnAOBs and is not applicable for biofilm growth systems (Lackner *et al.* 2014). AnAOBs have low maximum specific growth rates compared to AOBs and NOBs, with reported values of 0.006 h^{-1} (Tang *et al.* 2011) and 0.0027 h^{-1} (Strous *et al.* 1998), resulting in a doubling time in the range of 5-11 d, respectively. Based on a maximal conversion capacity, (van der Star *et al.* 2008b) estimated minimum doubling times of AnAOBs to be 5.5-7.5 d, but possibly also as low as 3 d. Those for

AOBs and NOBs were reported to be 7-8 h and 10-13 h, respectively (Bock *et al.* 1987; Van Hulle *et al.* 2010).

- Nitrite concentration:
  AnAOBs are insensitive to elevated ammonium concentrations as no inhibition could be observed by concentrations of at least 1,000 mg_N/L (Strous *et al.* 1999b). However, elevated nitrite concentrations were reported to influence the activity of AnAOBs negatively, although nitrite also serves as a substrate and thus, stimulates the anammox process (Szatkowska *et al.* 2007). Nevertheless, conflicting information exists for inhibitory threshold nitrite concentrations as a large range of nitrite concentrations of as low as 5 mg_N/L (Wett *et al.* 2007) up to 750 mg_N/L (Kimura *et al.* 2010) has been reported to decrease the AnAOB activity. This might not only be related to microbial adaptation, but also to diffusive limitation of inhibitors, as AnAOBs tend to grow in aggregates (Trigo *et al.* 2006), which is why inner layers are less exposed.

- Other influencing factors:
  Generally, exposure to light should be avoided as it has an inhibitory effect on AOBs, NOBs, and AnAOBs (van de Graaf *et al.* 1996; Van Hulle *et al.* 2010). Additionally, shock loads of suspended solids ought to be prevented (e.g. with an upstream sedimentation as implemented in **Paper II**), as previous studies have demonstrated that they can adversely influence the deammonification's performance (Lackner *et al.* 2014).

2.2.3 System Configurations and Operations

The deammonification process can be implemented either as a mainstream or side-stream nitrogen removal process. So far, PN/A is mainly applied as side-stream treatment in WWTPs due to specific favorable characteristics of the reject/process water from digested sludge dewatering, which has in general high ammonium concentrations (500-1,500 mg_N/L), low COD/N-ratios (mostly <1), and elevated temperatures (>30°C) (Lackner *et al.* 2014). Internal wastewater flows, which are especially influenced by this reject water stream, can amount to 10-30 % of the total nitrogen load of a WWTP (van Loosdrecht and Salem 2006; Gilbert *et al.* 2015). However, it generally represents only a low proportion of the total hydraulic load of approximately 1 % (van Dongen *et al.* 2001). An additional side-stream treatment of this wastewater stream offers the opportunity to reduce the nitrogen load in mainstream significantly (Fux *et al.* 2002). Thus, such a process implementation can upgrade a WWTP which might need to improve its nitrogen removal capacity due to increasingly strict nitrogen effluent concentrations. However, there is also a growing interest to apply the deammonification process as the new, resource-saving technology of nitrogen removal in the mainstream instead of conventional nitrification/denitrification to bring a WWTP closer to an energy-positive operation. Yet, mainstream deammonification treatment faces the challenges of low temperatures

(influences microbial growth rates and activities, which might lead to an imbalance of the microbial community), high COD/N-ratios (favors undesired growth of heterotrophic bacteria), low ammonium concentrations (impedes NOB suppression by FA), and the need to comply with strict effluent limits (Ma *et al.* 2011; Winkler *et al.* 2012; Gilbert *et al.* 2015; Laureni *et al.* 2016; Cao *et al.* 2017).

Different technologies as well as control strategies and reactor designs have been developed and successfully established in full scale side-stream applications in the past. A fundamental difference between the reactor designs is an implementation either as a single/one-stage or a two-stage process. The latter brings along the advantage of a potentially better control of both sub-processes of partial nitritation/anammox, but has the disadvantage of higher construction costs and space requirements (Lackner *et al.* 2014). Thus, favorable conditions can be either supplied by changing the process conditions spatially (applies for the two-stage deammonification and one-stage deammonification process as plug-flow reactor or by utilizing prevailing spatial substrate gradients in biofilms, granules, or flocs) or time-dependently (applies for the one-stage deammonification process) as presented in Figure 3.

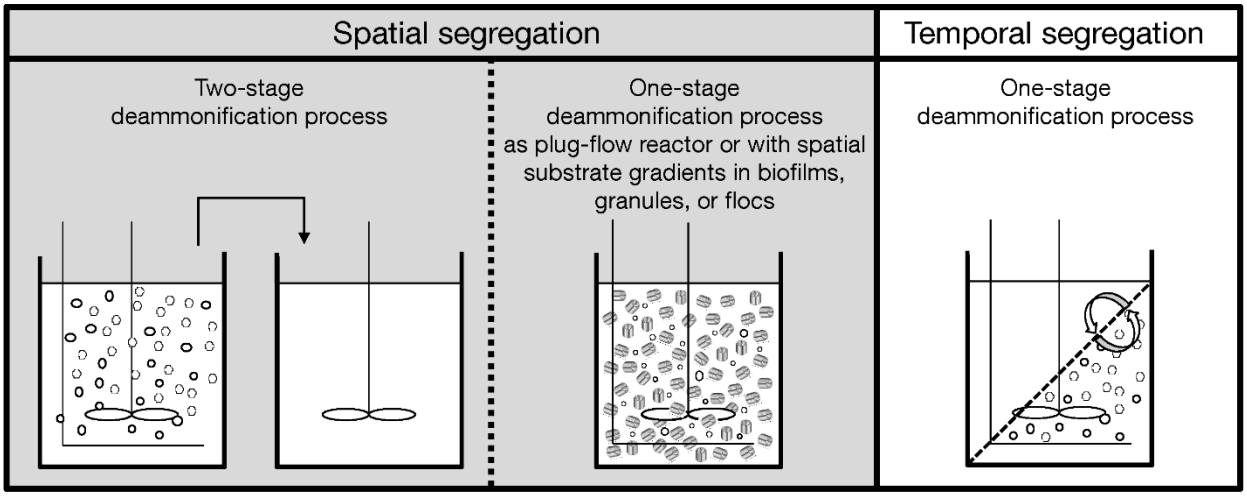

Figure 3. Operational schemes to provide favorable conditions for the deammonification process by either spatial or temporal segregation

Apart from the number of reactors, the deammonification process can be performed by a variety of microbial assemblages, e.g. as suspended sludge, granules, or biofilm, being implemented as SBRs, granular reactors, or MBBRs, respectively (Lackner *et al.* 2014). The SBR approach, which was applied in **Paper II** and **Paper III,** employs a discontinuous operation. Therefore, all necessary processes of feeding, reaction phase including anoxic and oxic phases, sedimentation, and idle phase can be performed in one single reactor (Figure 4). Thus, a flexible process operation is possible, as interval durations for feeding, aeration, mixing without aeration, settling, and idling can be adjusted according to the composition of the wastewater, the performance and settleability of the microbial community, the aeration intensity and oxygen

transfer, desired effluent concentrations and feeding volumes, etc. This enables e.g. an individually adapted implementation of single/intermittent/continuous feeding and intermittent/continuous aeration in adjustable sequences. The MBBR approach (applied in **Paper I** and **Paper II**) is comparable, however, it employs biofilm carriers and does not require a settling phase, as the microorganisms are attached to moving growth bodies, which are kept in the system e.g. by a sieve. As an MBBR enables higher biomass concentrations, both constructional costs and occupied space can be decreased due to a reduced required reactor volume (Xue *et al.* 2009). The carriers are used to maximize the active biofilm surface and for keeping the microorganisms within the system, even at a short hydraulic retention time (HRT). This is especially required for slowly growing bacteria, such as AnAOBs (Strous *et al.* 1998; Tang *et al.* 2011). Additionally, the biofilm provides protective and diverse favorable conditions for the abundant microorganisms. Driven by substrate gradients (e.g. DO, ammonium, and nitrite concentrations) over the biofilm depth, AOBs grow predominantly in the outer layers where they consume oxygen, thus, provide both anoxic conditions and nitrite for AnAOBs in the inner layers (Maslon and Tomaszek 2007; Almstrand *et al.* 2014).

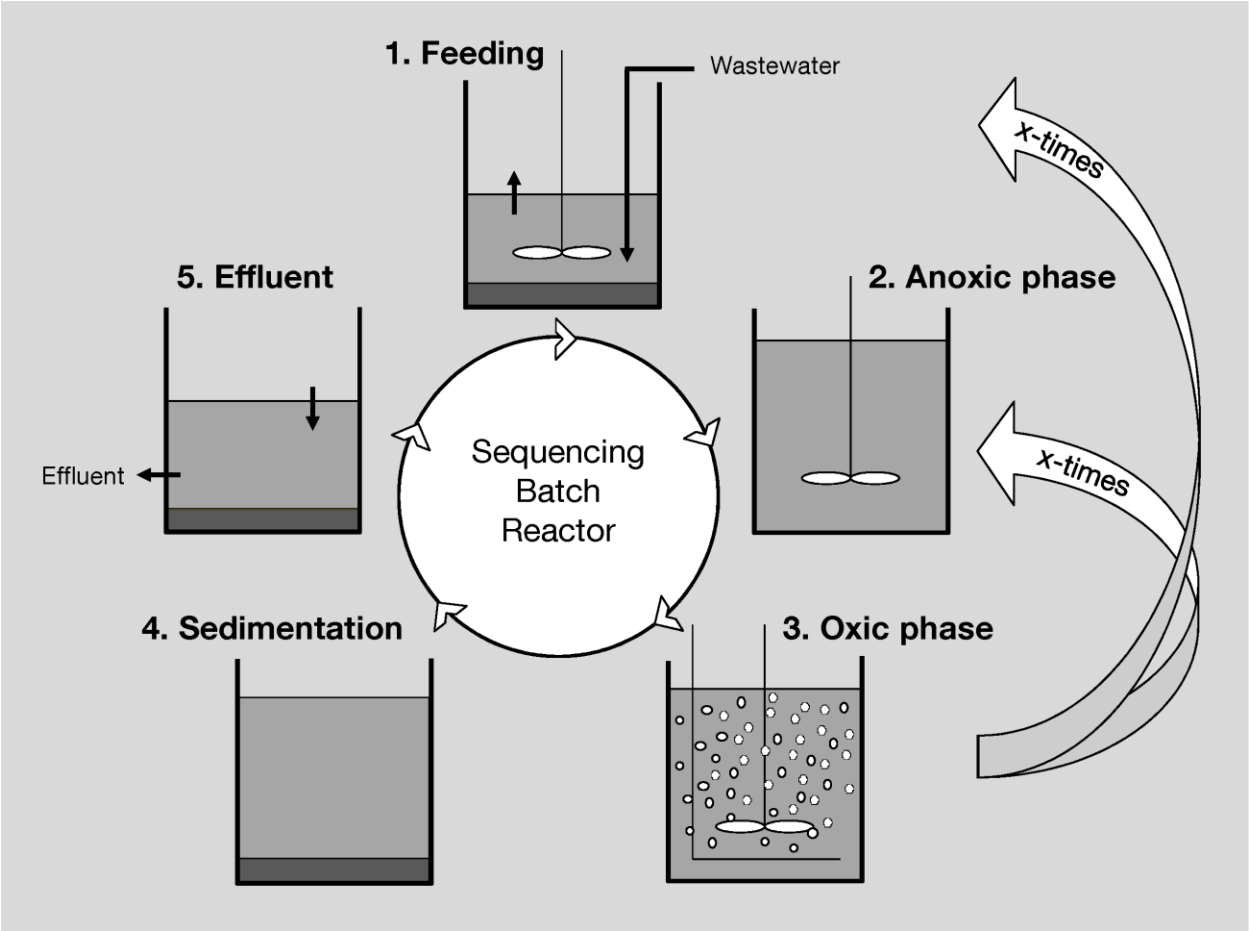

Figure 4. Cycle strategy of a SBR

Apart from SBRs and MBBRs, other, however rather rarely implemented systems are established as well, such as rotating biological contactors (RBC) or integrated fixed-film activated sludge (IFAS) systems. These IFAS systems combine by design both attached and suspended growth with typical mixed liquor suspended solid (MLSS) concentrations of 2.5-5.0 g/L (Zhao *et al.* 2014). (Veuillet *et al.* 2014) reported a three to four fold higher nitrogen removal efficiency in comparison to MBBR systems due to the positive effect of the substantial concentrations of suspended microorganisms. Furthermore, they investigated the microbial allocation in these IFAS systems and found AOBs mostly to be present in suspension (93 %), whereas AnAOBs were mainly located in the biofilm (96 %). MBBR systems also contain suspended biomass, however at very low concentrations (**Paper I**). Its contribution to the deammonification performance and the microbial composition of both the residual quantities of suspended microorganisms and biofilm in these MBBR systems has not been investigated before. For this reason, these research objectives were examined and discussed in **Paper I**. Furthermore, WWTP operators need to take the decision, which microbial system to implement in the deammonifying process. Therefore, the performances of a SBR with suspended sludge and a MBBR with biofilm carriers were compared and practical experiences were discussed in **Paper II**.

For a successful operation of the deammonification process, several process control strategies have been established. Some of them are patented, including e.g. DEMON® (DEMON GmbH, Gommiswald, Switzerland) and ANITA™Mox (Veolia Water Technologies AB, Lund, Sweden). The DEMON® process is a SBR, controlled by time, the pH value, and the DO concentration (Wett 2006; Wett *et al.* 2007). It has a fixed cycle duration of 8 h with a reaction phase of 6 h. Its intermittent aeration is controlled by a tight pH and feeding control. Continuous feeding stops, when an upper pH set-point is reached, which activates the aeration with a DO set-point close to 0.3 mg/L. Aeration stops, when the lower pH set-point is reached. Both pH and DO control avoid nitrite built-up and nitratation. Furthermore, the application of a hydrocyclone enables to adjust the SRT for AOBs and AnAOBs separately for a specific accumulation of microbial groups (Wett *et al.* 2010). The patented method applied by ANITA™Mox controls the DO concentrations dependently on the ratio of the influent and effluent ammonium concentration and nitrate production (Lackner *et al.* 2014). Others proposed the online measured ammonium concentrations (or alternatively the conductivity) as a surrogate to detect and therefore prevent NO_2^- accumulation by adjusting the air supply (Joss *et al.* 2011). Generally, the deammonification process can be run with continuous or intermittent aeration, single, intermittent, or continuous feeding, suspended or attached biomass, fixed or variable cycle duration, different HRT, SRT, and total suspended solids (TSS) concentrations, etc. An upper ammonium threshold concentration and pH value, or a lower DO limit can be employed to activate the aeration. In contrast, aeration should be turned off when the ammonium concentration or the pH value fall below a lower limit or if an upper DO threshold concentration is exceeded. Feeding of reject water can e.g. be controlled by threshold concentrations for ammonium, conductivity, and the pH value. Summing up, endless options exist for the operation of a non-patented deammonification process, but require close monitoring to prevent process failures.

A survey by (Lackner *et al.* 2014) revealed that the majority of full scale deammonifying installations worldwide (>100 plants) applied SBR technology (>50 %), followed by granular, and MBBR systems. A total of 88 % of these installations were employing one-stage processes, thus only 12 % applied a deammonification system in two reactors. In most cases, municipal wastewater was treated (75 %), while the remainder treated industrial wastewater. To investigate not only the most commonly used, but also as many system configurations as possible, this dissertation focused on the investigation of both one-stage deammonification (**Paper I** and **Paper III**) and two-stage deammonification systems (**Paper II**) in side-stream employing suspended sludge (**Paper II** and **Paper III**) and biofilm carriers (**Paper I** and **II**). Figure 5 in Chapter 3 gives a structured overview of all investigated process configurations.

2.3 Nitrous Oxide (N_2O) Emissions

2.3.1 N_2O Emissions during Wastewater Treatment

N_2O is formed during BNR processes, both as an undesired byproduct and an obligatory intermediate during heterotrophic denitrification (cf. Figure 1). It is not only a potent greenhouse gas (GHG) with a global warming potential (GWP) 298 times higher than that of carbon dioxide based on a 100-year time horizon, but also causes stratospheric ozone depletion (Intergovernmental Panel on Climate Change (IPCC) 2013). As a persistent GHG with a lifetime of approximately 120 years, less than 1 % of N_2O is removed from the atmosphere per year, induced in particular by oxidative reactions and photolysis (Montzka *et al.* 2011). In the last 30 years, atmospheric N_2O concentrations have increased at a mean rate of 0.7 ppb/year (Montzka *et al.* 2011), with an atmospheric concentration of 328 ppb measured in April 2016 (Carbon Dioxide Information Analysis Center (CDIAC) 2016). N_2O is currently the most heavily emitted ozone-depleting GHG worldwide and expected to remain so for the remainder of the 21st century (Ravishankara *et al.* 2009).

N_2O emissions arise from both natural and anthropogenic sources. Approximately 40 % of all N_2O emissions are caused by human activities, mainly from agricultural and industrial processes (Montzka *et al.* 2011). WWTPs also contribute to the total estimated anthropogenic N_2O emissions with an estimated share of 2.8 % (Intergovernmental Panel on Climate Change (IPCC) 2007). Although this proportion might seem to be little, even those small amounts of N_2O emissions need to be reduced to the greatest possible extent to mitigate the highly adverse effect of N_2O on the environment.

N_2O formation and emissions between different WWTPs for BNR were reported to vary substantially (Ahn *et al.* 2010; Foley *et al.* 2010; Law *et al.* 2012b). 0-95 % and 0-15 % of the nitrogen load were found to be emitted as N_2O in laboratory scale and full scale studies, respectively (Kampschreur *et al.* 2009b). Another survey regarding full scale installations

reported even higher N_2O emission factors[4] (again related to the nitrogen load) of up to 25 % (Law *et al.* 2012b). (Yang *et al.* 2009) stated that these high variations are triggered by different wastewater qualities, operational parameters, and environmental conditions. Due to these fluctuations and the high GHG potential of N_2O, a WWTP's carbon footprint can severely be influenced by N_2O emissions. In a full scale study by (Daelman *et al.* 2013), the carbon footprint was dominated by N_2O emissions with a share of 75 %, yet with seasonal changes. In laboratory scale experiments, even 81 % of the carbon footprint were accounted for by N_2O emissions (Pijuan *et al.* 2014). However, in other WWTPs' carbon footprints, N_2O emissions have been reported to play only a minor role (Daelman *et al.* 2013). Thus, there is not only a high variation of N_2O emissions between different WWTPs, but also a seasonal, as well as a temporal and spatial variation due to highly dynamic N_2O formation (Ahn *et al.* 2010; Foley *et al.* 2010; Houweling *et al.* 2011; Law *et al.* 2012b; Schreiber *et al.* 2012). This demonstrates that the estimation of N_2O emitted by WWTPs based on a default emission factor as proposed by the (Intergovernmental Panel on Climate Change (IPCC) 2007) cannot be expected to provide a representative inventory, as it is afflicted by high uncertainties. Thus, N_2O emissions should rather be assessed individually for every WWTP.

It is necessary to note that N_2O formation does not equal N_2O emission. Thus, dissolved N_2O, which has been produced during wastewater treatment, is not necessarily (entirely) emitted into the atmosphere. The transfer of dissolved N_2O over the liquid-gas interface is dependent on different driving forces. Generally, dissolved N_2O can be further reduced to nitrogen gas during denitrification/denitritation if a carbon source is available (cf. Figure 1). Consequently, it can no longer contribute to N_2O emissions. N_2O reduction catalyzed by the enzyme nitrous oxide reductase (N_2OR) is the only N_2O consuming process known so far which counteracts a potential release of N_2O into the atmosphere (Richardson *et al.* 2009). Furthermore, emissions are dependent on the solubility of a gas. According to Henry's law, the amount of dissolved N_2O under equilibrium conditions is proportional to its partial pressure in the gaseous phase. The solubility additionally depends on the temperature as well as on the composition of the water matrix (Sander 2015). Hence, the solubility of a gas decreases with an increasing temperature and salinity as well as with a decreasing partial pressure. N_2O is a well soluble gas in water with a Henry's law solubility constant H^{cp} of $1.8 \cdot 10^{-4}$ to $2.5 \cdot 10^{-4}$ mol/(m³·Pa) as in comparison to oxygen with H^{cp} of $1.2 \cdot 10^{-5}$ to $1.3 \cdot 10^{-5}$ mol/(m³·Pa) at 298.15 K (Sander 2015). Thus, N_2O is likely to accumulate during unaerated phases (Law *et al.* 2012b) and N_2O emission is mainly driven by the concentration gradient at the gas-liquid interface. During aerated phases, however, N_2O emissions are additionally enhanced by active stripping (Law *et al.* 2012b). In a study by (Ahn *et al.* 2010) comparing 12 different WWTPs, N_2O emissions during aerated phases were

[4] The N_2O emission factor [%] is represented as the mass of daily emitted N_2O-N [kg_N/d] related to either the daily nitrogen load [kg_N/d] or daily removed amount of nitrogen [kg_N/d] (Law *et al.* (2012b). However, the latter expression is more meaningful and representative, as the N_2O emissions are in the light of the plant's performance. In contrast, the N_2O emission factor related to the daily nitrogen load would approach zero under high nitrogen loading rates even if no degradation of nitrogen would take place (worst case scenario).

investigated to be up to three orders of magnitude higher in comparison to unaerated phases. The flux of dissolved N_2O from the liquid into the gas depends on a large number of influencing factors, including reactor geometry (especially the aeration submergence depth), air bubble size, aeration flow rate, liquid viscosity, superficial gas velocity, etc. (Gillot *et al.* 2005; Foley *et al.* 2010). Thus, not only the process conditions and regulation influence the N_2O formation and subsequent emission (e.g. accumulation of dissolved N_2O during unaerated phases with a peak of N_2O emissions after aeration started; for favorable conditions of N_2O formation, see Chapter 2.3.3). Also the design of the reactor (e.g. depth of aerators, presence or absence of coverage possibly influencing partial pressure and thus, the solubility of dissolved N_2O, etc.) and the equipment (e.g. progressing clogging of aeration membranes influences air bubble size, etc.) have an influence on the N_2O emissions of the BNR process.

The deammonification process for the treatment of wastewater is economically advantageous compared to conventional nitrification/denitrification due to its savings regarding aeration energy, carbon, as well as sludge disposal (Jetten *et al.* 2001). However, favorable conditions for its performance, such as low DO concentrations and presence of nitrite (cf. Chapter 2.2.2), have also been reported to influence the N_2O formation (Kampschreur *et al.* 2009b), as described in detail in Chapter 2.3.3. Thus, effective mitigation strategies need to be developed and employed in WWTPs with deammonification processes (further details are presented in **Paper II** and **Paper III**) to not only minimize deleterious effects of emitted N_2O on the environment, but also to avoid that the CO_2 balance turns positive despite the deammonification's reduced energy consumption.

2.3.2 N_2O Formation Pathways

N_2O can be produced by complex mechanisms during nitrification/nitritation, denitrification/denitritation[5], and due to chemical reactions (cf. Figure 1). While N_2O is an obligatory intermediate of denitrification, it is an unintended and undesired product of nitrification and chemical reactions. N_2O formation can be divided into four main pathways, three of which occur biologically, namely nitrifier denitrification (also called aerobic nitrous denitritation as proposed by (Weißbach *et al.* 2017a)) and hydroxylamine oxidation during nitrification as well as N_2O production via heterotrophic denitrification. In contrast, N_2O does not seem to play a role in the metabolic pathway of AnAOBs (Kartal *et al.* 2007a). The forth N_2O production pathway summarizes abiotic processes, represented by diverse chemical reactions. The different N_2O formation pathways are discussed in detail hereinafter.

[5] Due to simplicity, nitritation and denitritation as part of nitrification and denitrification are not going to be named explicitly in the following, but are included in the meaning of the generic terms.

- Nitrifier denitrification (biotic):
  AOBs, AOAs, and NOBs are able to reduce nitrite to nitric oxide using nitrite reductase, followed by a further reduction to N_2O by means of nitric oxide reductase under oxygen limitation; nevertheless, AOBs and AOAs are believed to be the dominant source of N_2O formation by nitrifier denitrification (Law *et al.* 2012b; Schreiber *et al.* 2012). Some studies also reported that two isolates of AOBs (*Nitrosomonas europaea* and *Nitrosomonas eutropha*) were capable to reduce NO_2^- to N_2 (Poth 1986; Schmidt and Bock 1997; Schmidt *et al.* 2004; Schmidt 2009). Nevertheless, this complete autotrophic denitrification is rather uncommon (Colliver and Stephenson 2000). Ammonia, hydrogen, and hydroxylamine can serve as electron donors for nitrifier denitrification (Law *et al.* 2012b; Schreiber *et al.* 2012; Wunderlin *et al.* 2012). The contribution of nitrifier denitrification to N_2O formation by oxidizing bacteria increases with decreasing DO concentrations (Peng *et al.* 2014; Peng *et al.* 2015) and can be the dominant production pathway during suboxic and anoxic conditions (Law *et al.* 2012b). Furthermore, nitrifier denitrification is favored upon increasing nitrite concentrations (Wunderlin *et al.* 2012; Peng *et al.* 2015) and lower ammonia concentrations (Wunderlin *et al.* 2012). It has been hypothesized that the microorganisms perform the pathway of nitrifier denitrification as a detoxifying process to avoid an accumulation of nitrite to toxic concentrations (Beaumont *et al.* 2004a; Beaumont *et al.* 2004b).

- Hydroxylamine oxidation (biotic):
  During biotic hydroxylamine oxidation performed by AOBs, NH_2OH is oxidized to the intermediate NO, which is subsequently reduced to N_2O, catalyzed by the enzymes HAO and NOR, respectively; yet, a direct formation of N_2O from HAO or other processes might also be discovered in the future (Schreiber *et al.* 2012). In contrast to nitrifier denitrification, hydroxylamine oxidation contribution to N_2O formation is favored at elevated ammonia and low nitrite concentrations at a high metabolic AOB activity induced by DO concentrations of 2 to 3 mg/L (Wunderlin *et al.* 2012). (Peng *et al.* 2014; Peng *et al.* 2015) also observed a positive correlation between the hydroxylamine oxidation and the DO concentration.

- Heterotrophic denitrification (biotic):
  N_2O is an obligate intermediate of heterotrophic denitrification, however, it can completely be reduced to gaseous nitrogen by use of the nitrous oxide reductase (N_2OR) under anoxic conditions and an available carbon source (cf. Figure 1). Yet, inhibition of N_2OR occurs under specific conditions (cf. Chapter 2.3.3). Consequently, this can lead to an accumulation of dissolved N_2O potentially causing subsequent N_2O emission. Besides, N_2O can be the end product for some denitrifying microorganisms, as there is only an energy loss of approximately 20 % if the last step of denitrification producing N_2 is not completed (Richardson *et al.* 2009).

- Chemical reactions (abiotic):
  Diverse chemical reactions can be responsible for the formation of N_2O. (Schreiber *et al.* 2012) hypothesized that deammonification systems treating reject water trend to favor chemical formation of N_2O due to high oxidation rates of ammonia, high concentrations of the intermediate hydroxylamine, accumulation of nitrite in some instances, and ubiquitous presence of catalyzing iron; nevertheless, the proportion of produced N_2O induced by chemical reactions in relation to the overall N_2O formation is difficult to estimate. Again, NO_2^- (or its acidic form HNO_2) and NH_2OH are the main precursors of N_2O formation. Besides, N_2O can also be produced abiotically by nitroxyl (HNO) via hyponitrous acid ($H_2N_2O_2$) according to equation (6) (Bonner *et al.* 1978; Bonner and Hughes 1988; Alluisetti *et al.* 2004):

$$2HNO \rightarrow H_2N_2O_2 \rightarrow N_2O + H_2O \quad (6)$$

  HNO was postulated to occur as a transient intermediate compound of hydroxylamine oxidation by (Anderson 1964), which could later be demonstrated by (Igarashi *et al.* 1997).

  Iron in its oxidation states Fe^{2+} and Fe^{3+} can trigger N_2O formation via NO_2^- (with reduction to NO as an intermediate) and NH_2OH, respectively. (Kampschreur *et al.* 2011) could demonstrate that reduced iron at the presence of NO_2^- could significantly contribute to NO and N_2O formation according to equation (7) and (8):

$$NO_2^- + Fe^{2+} + 2H^+ \rightarrow Fe^{3+} + NO + H_2O \quad (7)$$

$$NO + Fe^{2+} + H^+ \rightarrow Fe^{3+} + 0.5N_2O + 0.5H_2O \quad (8)$$

  As iron salts are often used for the precipitation of phosphate during wastewater treatment, elevated concentrations of Fe^{2+} induced by the reducing conditions of digestion are not uncommon in process water; furthermore, Fe^{3+}, which oxidizes NH_2OH to N_2O according to equation (9), is also commonly present, as it is ubiquitous in wastewater treatment systems (Schreiber *et al.* 2012):

$$2NH_2OH + 4Fe^{3+} \rightarrow N_2O + 4Fe^{2+} + H_2O + 4H^+ \quad (9)$$

  Besides a N_2O formation at the presence of iron, also a copper-catalyzed oxidation of hydroxylamine according to equation (10) was observed (Anderson 1964):

$$2\mathrm{NH_2OH} + 4\mathrm{Cu^{2+}} \rightarrow \mathrm{N_2O} + 4\mathrm{Cu^+} + \mathrm{H_2O} + 4\mathrm{H^+} \quad (10)$$

  After digestion, rather reduced copper is assumed to be present in the reject water, but might as well be oxidized to Cu^{2+} during partial nitritation, and therefore could be available for equation (10). Generally, copper concentrations are believed to be more elevated in urban than in rural areas due to a higher density of applied copper materials, such as copper roofs. Therefore, the abiotic formation of N_2O is hypothesized to be higher in urban than in rural areas, however, this still needs to be elucidated.

According to equation (11) and (12), N_2O formation can also take place by chemical oxidation of hydroxylamine with nitrous acid (Döring and Gehlen 1961; Bonner *et al.* 1978; Harper *et al.* 2015; Terada *et al.* 2017) or with oxygen (Anderson 1964), respectively:

$$NH_2OH + HNO_2 \rightarrow N_2O + 2H_2O \quad (11)$$

$$2NH_2OH + O_2 \rightarrow N_2O + 3H_2O \quad (12)$$

As hydroxylamine is a highly reactive compound, only low concentrations (i.e. ppb or ppm) are expected to occur during nitritation (Harper *et al.* 2015). Nevertheless, (Soler-Jofra *et al.* 2016) found elevated concentrations in a range of 0.03-0.11 mg_N/L in a nitritation reactor, thus, the proportion of abiotic N_2O formation might have been underestimated so far (Harper *et al.* 2015). From a thermodynamical point of view, the comproportionation between hydroxylamine and nitrous acid according to equation (11) is favorable over biotic hydroxylamine oxidation (Harper *et al.* 2015) and can even be the dominant production pathway in comparison to nitrifier denitrification and biotic hydroxylamine oxidation (Terada *et al.* 2017). Furthermore, equation (11) is likely enhanced under slightly acidic conditions due to a higher availability of FNA.

Apart of abiotic hydroxylamine oxidation, hydroxylamine is also the precursor of N_2O via its chemical decomposition (disproportionation/dismutation) according to equation (13) (Alluisetti *et al.* 2004):

$$4NH_2OH \rightarrow N_2O + 2NH_3 + 3H_2O \quad (13)$$

Apart from that, oxygenic dismutation of nitric oxide catalyzed by a putative NO dismutase (NOD) was recently presented (Zhu *et al.* 2016). This new pathway in the microbial nitrogen cycle named 'oxygenic denitrification' bypasses the reduction of NO to N_2O by directly yielding N_2 and O_2 (cf. Figure 1). (Zhu *et al.* 2016) found that *nod* genes were remarkably abundant and unexpectedly diverse in different kinds of wastewater treatment systems, such as conventional denitrification as well as one- and two-stage deammonification systems employing suspended, granulated, and biofilm biomass. These findings reveal a potential to reduce N_2O production by supplying conditions that favor oxygenic denitrification.

2.3.3 Conditions Triggering N_2O Formation

Conditions triggering the complex mechanisms of N_2O formation during nitrification, denitrification, and due to chemical reactions, can be manifold. However, biological N_2O formation generally seems to be related to suboptimal growth conditions (Kampschreur *et al.* 2008a). According to literature, key factors influencing the biological N_2O production are the DO concentration, the nitrite concentration, the pH value, as well as FNA and ammonium

concentrations. Furthermore, the COD/N ratio during denitrification and the SRT amongst other factors have been reported to influence the formation of N_2O. These key factors are discussed in detail hereinafter.

- DO concentration:
  WWTPs can be operated at different aeration regimes ranging from continuous to intermittent aeration with different aeration intensities causing a wide range of DO concentrations and a transition from oxic to anoxic conditions and vice versa. Dissolved oxygen influences the metabolic pathways of microorganisms under both oxic and anoxic conditions directly, such as the incompleteness of nitrification, which in turn can lead to nitrite accumulation or inhibition of denitrification influencing N_2O formation as discussed below. Different aeration strategies have been carried out in **Paper III** to investigate their role on N_2O emissions.
  For the deammonification process including partial nitritation combined with the anammox process, low DO concentration or changes from oxic to anoxic conditions are necessary to suppress NOBs and provide suitable conditions for AnAOBs (cf. Chapter 2.2.2). However, these requirements pose the risk of elevated N_2O emissions, as N_2O formation was reported to increase at low DO concentrations (generally below 1 mg/L) (Kampschreur *et al.* 2008b; Kampschreur *et al.* 2009b) and upon transition between anoxic and oxic conditions (Ahn *et al.* 2010; Yu *et al.* 2010; Law *et al.* 2012a; Castro-Barros *et al.* 2015).
  During nitrification, (Zheng *et al.* 1994) investigated the influence of different DO concentrations (0.1, 0.2, 0.5, 1.7, and 6.8 mg/L) on N_2O formation and found the highest gaseous N_2O concentration at 0.2 mg/L; however, N_2O was detected at all tested DO concentration which is why the authors postulated that N_2O formation is not avoidable at any DO concentration. (Kampschreur *et al.* 2008b) observed an accumulation of dissolved N_2O upon DO depletion (from 1 down to 0 mg/L, when air was replaced by N_2, thus the stripping effects remained the same). (Peng *et al.* 2015) reported a maximal specific N_2O production rate[6] at a DO concentration of 0.85 mg/L, while maximal N_2O emissions occurred at a slightly higher DO concentration of 1 mg/L in a study by (Tallec *et al.* 2006); for DO concentrations above 2 mg/L, N_2O emissions were relatively low. Also (Kimochi *et al.* 1998) claimed that nitrification should be strictly operated at aerobic conditions for negligible N_2O formation. However, this is hardly applicable for partial nitritation systems to prevent NOB growth and nitrate production.
  Surprisingly, also the contrary case with decreasing DO concentrations causing decreasing N_2O formation has been reported in several studies (Kampschreur *et al.* 2009a; Law *et al.* 2012a; Ni *et al.* 2013; Peng *et al.* 2014; Rathnayake *et al.* 2015). An explanation could be given by the fact that the ammonia oxidation and the N_2O

[6] 'Specific' indicates that the N_2O production rate is related to the biomass concentration, e.g. $g_{N2O\text{-}N}/(d \cdot g_{VSS})$

production rate correlated positively (Law *et al.* 2012a; Domingo-Félez *et al.* 2014). Based on these observations, (Law *et al.* 2012a) postulated that increasing ammonium oxidation produces more intermediate HNO, which could lead to an increased N_2O formation by chemical reactions (cf. Chapter 2.3.2). However, not only the intermediate HNO, but also hydroxylamine for augmented N_2O formation is conceivable. Also (Kampschreur *et al.* 2009a) reported that lowering the DO concentration below 2 mg/L down to 0 mg/L in their PN/A system caused decreasing gaseous N_2O concentrations. However, at DO concentrations above 4 mg/L, the gaseous N_2O concentrations was constant which led to the conclusion that an efficient aeration control is important to limit N_2O emissions, as an higher aeration flow rate would lead to increased N_2O emissions.

Reduction of dissolved N_2O to gaseous nitrogen is the only known sink for N_2O emissions (Richardson *et al.* 2009). However, this process is highly sensitive to the presence of dissolved oxygen. DO concentrations of as low as 0.09 mg/L have been reported to inhibit denitrification severely (Oh and Silverstein 1999). Also (Tallec *et al.* 2006) reported a decreasing heterotrophic denitrification as DO concentration increased from 0.1 up to 2.0 mg/L. Due to a higher sensitivity of N_2OR to dissolved oxygen in comparison to NAR, NIR, and NOR causing an imbalance of the enzyme activity (Schulthess *et al.* 1994; Morley *et al.* 2008), N_2O can accumulate. Thus, the N_2O production rate can significantly increase at the presence of low dissolved oxygen concentrations, as reported by (Wunderlin *et al.* 2012). Nevertheless, denitrification proceeds after oxygen depletion, as N_2OR activity can recover from inhibition (Morley *et al.* 2008).

- Nitrite concentration:

  Nitrite is simultaneously a substrate, a product, as well as an inhibitor for a variety of microorganisms under both oxic and anoxic conditions (cf. Figure 1 and Chapter 2.2.2). Therefore, it is hard to elucidate its definite role at the dynamic conditions of biological nitrogen removal processes, but might as well be manifold at a time.

  At an early stage, nitrite was observed to influence N_2O formation, both during nitrification (Zheng *et al.* 1994) as well as denitrification (Firestone *et al.* 1979b). (Hanaki *et al.* 1992) proposed the nitrite concentration to be used as an indicator for N_2O formation during denitrification. (Zheng *et al.* 1994) described a simultaneous occurrence of high N_2O formation at incomplete nitrification and therefore concluded that elevated nitrite concentrations might inhibit the oxidation pathway. Also (Noda *et al.* 2003) observed an accelerated N_2O formation at insufficient nitrification, thus at accumulated nitrite concentrations.

  Even though (Betlach and Tiedje 1981) could show that an addition of nitrite did not induce N_2O accumulation in their system of three different denitrifiers, it is nevertheless widely accepted that elevated nitrite concentrations or nitrite pulses generally result in N_2O formation (Itokawa *et al.* 2001; Burgess *et al.* 2002; Tallec *et al.* 2006; Kampschreur *et al.* 2008b; Kampschreur *et al.* 2009a; Yang *et al.* 2009; Foley *et al.* 2010; Okabe *et al.* 2011; Wunderlin *et al.* 2012; Peng *et al.* 2015). In contrast to incentives for reduced N_2O

formation, (Scherson *et al.* 2013) make use of nitrite pulse feeding for an intentional production and recovery of N_2O in their proposed *C*oupled *A*erobic–anoxic *N*itrous *D*ecomposition *O*peration (CANDO) process for energy recovery by methane combustion with N_2O.

The reason for enhanced N_2O formation by elevated nitrite concentrations is believed to be twofold with nitrite having a toxic and thus, an inhibitory effect on N_2O reduction during denitrification (Itokawa *et al.* 2001). It also serves as electron acceptor under oxygen-limited conditions promoting nitrifier denitrification during nitritation (Poth and Focht 1985; Bock *et al.* 1995) for a hypothesized detoxification (Beaumont *et al.* 2004a; Beaumont *et al.* 2004b).

- pH value:

  It was demonstrated in various studies that the pH value has an effect on the N_2O formation (Hynes and Knowles 1984; Hanaki *et al.* 1992; Thörn and Sörensson 1996; Baggs *et al.* 2010; Law *et al.* 2011; Rathnayake *et al.* 2015). Its influence is rather indirect, as the pH value influences the FNA concentration, the activity of microorganisms, etc. (cf. Chapter 2.2.2), which in turn can impact N_2O formation. The processes of nitrification and denitrification exhibit a different behavior towards N_2O formation influenced by the pH value, with an advantageous pH value for nitrification rather in a slightly acidic milieu, and for denitrification rather in a slightly alkaline pH range for the purpose of reduced N_2O formation.

  (Hynes and Knowles 1984) investigated the production of N_2O by *Nitrosomonas europaea* with a minimal and maximal N_2O formation at pH 6.0 and pH 8.5, respectively. Also (Law *et al.* 2011) found lowest N_2O formation of a partial nitritation system in a range between pH 6.0 and pH 7.0, which increased with increasing pH and reached its maximum at pH 8.0. They also observed a positive, linear correlation between the ammonium oxidation rate and the N_2O production rate. Thus, it was hypothesized that increased ammonium oxidation activity might promote N_2O formation by nitrification, induced either by a higher electron availability for the nitrifier denitrification pathway or higher production of intermediates (e.g. NH_2OH and NOH) for hydroxylamine oxidation and chemical reactions (cf. Chapter 2.3.2). Results by (Rathnayake *et al.* 2015) deviated slightly, as they found the N_2O production rate in their partial nitrifying system to peak at pH 7.5, with lower rates below and above this value.

  In denitrifying systems, slightly acidic conditions seem to favor N_2O formation. (Thörn and Sörensson 1996) observed N_2O formation only at pH values below pH 6.8. (Hanaki *et al.* 1992) demonstrated that N_2O formation significantly increased if the pH value was lowered down to pH 6.5, while there was no severe change between pH 7.5 and pH 8.5, yet with a little lower N_2O formation at pH 8.5. This is most likely influenced by the activity of the nitrous oxide reductase which was observed to be relatively constant in the range between pH 7.5 and pH 8.5 (Zhou *et al.* 2008), while another study reported a maximum between pH 8.0 and pH 9.0 (Gorelsky *et al.* 2006). However, at pH values below pH 7.0,

N_2OR were observed to be less active as NAR, NIR, and NOR inducing N_2O accumulation (Richardson *et al.* 2009). As deammonification is generally operated above this set-point (Lackner *et al.* 2014), a severe decreased contribution of N_2O reduction is not expected. While the influence of the pH value on N_2O formation in these studies was investigated separately for the discussed processes, the deammonification process combines a complex interaction of different reactions and microorganism. Therefore, the influence of the pH value on the N_2O formation in these systems was investigated in **Paper III** to enable more general conclusions.

- FNA concentration:
  (Zhou *et al.* 2008) suggested that FNA[7] rather than nitrite or the pH value alone was the true inhibitor of N_2OR. A complete, however, reversible inhibition of N_2OR was observed at a FNA concentration of 4 $\mu g_N/L$ and therefore is even below those values for complete AnAOBs inhibition as described in Chapter 2.2.2. The inhibition was not strongly dependent on the time of exposure, but on the FNA concentration microorganisms were exposed to, with higher FNA concentrations requiring a longer recovery period. Inhibition of N_2OR results in accumulation of N_2O and thus, a reduced contribution of denitrification towards N_2O reduction causing potentially higher N_2O emissions.

- Ammonium concentration:
  Ammonium concentrations are influenced by both the feeding strategy and the ammonium oxidation rate. (Burgess *et al.* 2002) reported a positive correlation between ammonia shock loads and N_2O concentrations in the off-gas up to an ammonia load of 1.6 mg_N/g_{TSS}; at higher ammonia loads, however, N_2O formation by nitrifying microorganisms seemed to have reached its maximum. Also (Kampschreur *et al.* 2008a) observed an increase of N_2O formation during nitrification at highly dynamic variations of the ammonium concentration induced by pulse feeding. These observations might be related to higher ammonium concentrations or/and rapidly changing process conditions influencing N_2O formation as discussed below. (Yang *et al.* 2009) suggested a direct relation between the N_2O formation and the feed ammonium concentration in their nitritation system, as both increased jointly. (Domingo-Félez *et al.* 2014) reported that decreasing N_2O peaks and production rates corresponded to decreasing ammonium concentrations during the PN/A process. In contrast, (Kampschreur *et al.* 2009a) did not observe an impact of the ammonium concentration on the N_2O formation in their PN/A system. Different feeding strategies directly influencing the ammonium concentration have been carried out in **Paper III** to investigate their role on N_2O emissions.

[7] The FNA concentration depends on the nitrite concentration, the pH value, as well as the temperature Anthonisen *et al.* (1976). Calculation of the FNA concentration is listed in the Appendix.

- COD/N ratio:
  The combination of partial nitritation with anammox is an autotrophic process and as such not dependent of the COD/N ratio. Nevertheless, denitrifying microorganisms can co-exist in such systems (Lackner and Horn 2012; Agrawal *et al.* 2017). (Itokawa *et al.* 2001) reported incomplete denitrification at a COD/N ratio of 3.5 with a conversion to N_2O related to the influent nitrogen of 20-30 %, while N_2O emissions were comparably low (less than 1 %) at elevated COD/N ratios of 5.0 and 5.5. (Law *et al.* 2012b) concluded that for complete heterotrophic denitrification, a COD/N ratio above 4 is required. The negative correlation between the COD/N ratio and N_2O formation even increases with decreasing SRTs, as discussed below (Hanaki *et al.* 1992). If the carbon availability is limited, the denitrifying enzymes NAR, NIR, NOR, and N_2OR compete for electrons, which can lead to an incomplete denitrification and consequently, an accumulation of N_2O; however, addition of an carbon source resulted in a decreased N_2O formation (Schalk-Otte *et al.* 2000). The N_2O removal is limited to subsequent reduction by both complete autotrophic denitrification – even if rather uncommon (Colliver and Stephenson 2000) – and heterotrophic denitrification. However, for the application of the side-stream deammonification process fed with reject water, COD/N ratios are mostly lower than one (Lackner *et al.* 2014), thus, well below the required value for complete denitrification. Furthermore, the COD that is still available, can be assumed to be hardly degradable due to the previous fermentation. Nevertheless, denitrification was detected to be responsible for the steep decline of dissolved N_2O during non-aerated phases in such systems in **Paper II**, which is also supported by previous findings (Domingo-Félez *et al.* 2014). This might be related to an adaptation of the denitrifying microorganisms or an additional carbon supply by biomass decay providing a readily degradable carbon source as proposed in **Paper II**.
  If an additional supply of external carbon is employed as a countermeasure against N_2O emissions during deammonification, methanol – often applied in wastewater treatment for downstream denitrification – should be strictly avoided. Several studies have proven that methanol caused (irreversible) inhibition of AnAOBs, however, at different threshold concentrations starting from 0.5 mmol/L (Güven *et al.* 2005; Isaka *et al.* 2008; Oshiki *et al.* 2011). (Jin *et al.* 2012) hypothesized that these differences might be related to various examined AnAOBs species. Furthermore, rather formaldehyde than methanol was suggested to be the actual inhibitory substance (Güven *et al.* 2005; Isaka *et al.* 2008). Formaldehyde can be a product of intracellular conversion of methanol by AnAOBs (Güven *et al.* 2005), which inactivates enzymes by cross-linking the peptide chains (Metz *et al.* 2004). Besides methanol, also dosing of ethanol should be avoided, as it causes likewise inhibition, however, not as severe as methanol (Isaka *et al.* 2008). A suitable carbon source could be for instance primary effluent, however, its applicability still needs to be evaluated (cf. Chapter 7.4).

- SRT:
  A negative correlation was determined to exist between the SRT and the N_2O formation (Hanaki *et al.* 1992; Zheng *et al.* 1994; Noda *et al.* 2003). Thus, the lower the SRT, the higher the N_2O formation was determined to be (also see **Paper III**). Nevertheless, (Zheng *et al.* 1994) detected N_2O formation from nitrification throughout any SRT tested, even at a SRT of 20 d. Furthermore, they concluded that the SRT had a higher influence on N_2O formation than the DO concentration. Even though the correlation of the SRT and the N_2O formation is not completely clarified, it was hypothesized that it is related to incomplete nitrification, thus to elevated nitrite concentrations. Therefore, the influence of the SRT might combine several influencing factors discussed above.

- Other influencing factors:
  The deammonification process can be subjected to rapidly changing process conditions due to its process control. Several studies have revealed that N_2O emissions increased upon these alternating environmental conditions, such as changes in the nitrite concentration (Tallec *et al.* 2006) or ammonium shock loads (Burgess *et al.* 2002). Changes in process conditions most probably cause stress for the microorganisms, which can result in peak formation/emission of N_2O; however, a microbial adaptation to continuously varying conditions is possible (Kampschreur *et al.* 2009b), which highlights that a gradual decrease of N_2O emissions over the long-term might be possible.
  Furthermore, formaldehyde was found to be toxic to denitrification leading to increased N_2O formation; nevertheless, again an adaptation of microorganisms was observed to be possible if exposed successively to higher concentrations of formaldehyde (Garrido *et al.* 1998).
  As N_2OR is a copper enzyme, its biosynthesis will be incomplete, if the copper supply is insufficient (Richardson *et al.* 2009). Thus, under copper-limited conditions (< 3 $nmol_{Cu}$/L), N_2O was reported to accumulate, while an addition of 10 $nmol_{Cu}$/L led to a complete consumption of N_2O during denitrification (Granger and Ward 2003). Nevertheless, copper can also catalyze the abiotic oxidation of hydroxylamine as presented in Chapter 2.3.2 (Anderson 1964).
  The presence of NO has an inhibitory effect on N_2OR, also causing an accumulation of N_2O; a complete inhibition of N_2O reduction was reported for N_2O-grown denitrifying cells of *Pseudomonas perfectomarina* at a concentration of 20 µmol/L, while other denitrifying bacteria were determined to be less sensitive (Frunzke and Zumft 1986).
  Besides NO, also sulfide was described to inhibit N_2O reduction leading to increased dissolved N_2O concentrations at a tested hydrogen sulfide (H_2S) concentration of 0.3 µmol/L (Sørensen *et al.* 1980; Knowles 1982).

2.3.4 Analytical Methods for N_2O Measurement

Analytical methods for the measurement of both dissolved and gaseous N_2O are available. Generally, the appropriate selection of the technique with its respective limit of detection (LOD), measurement range, and temporal resolution should be carefully considered and adjusted to the measurement purpose and the expected N_2O concentrations.

For the measurement of gaseous N_2O, offline (grab) samples can be collected and analyzed, for instance by conventional gas chromatography (GC) with an electron capture detection (Zheng *et al.* 1994; Kimochi *et al.* 1998; Noda *et al.* 2003; Kampschreur *et al.* 2008b). However, this technique does not allow for the detection of dynamic N_2O emissions, as the measurement is discontinuously. As N_2O emissions were reported to be subjected to a high temporal variation (cf. Chapter 2.3.1), the application of this approach might lead to a false estimation of the overall N_2O emissions if results are extrapolated based on several grab samples. In contrast, online measurements do not only offer an advantageous high temporal resolution showing N_2O emission dynamics, but also enable an accurate quantification of N_2O emissions. Different types of online measurement devices are commercially available and have been applied in WWTP successfully, such as gas analyzers based on infrared (IR) (Burgess *et al.* 2002; Ahn *et al.* 2010), Fourier transform infrared (FTIR) (Joss *et al.* 2009; Wunderlin *et al.* 2012), nondispersive infrared (NDIR) (Butler *et al.* 2009), chemiluminescence (Kampschreur *et al.* 2008a), as well as mass spectrometry (Otte *et al.* 1996; Zeng *et al.* 2003). Recently, a novel microelectrode-based system for the online measurement of gaseous N_2O has been proposed (Marques *et al.* 2014) and also successfully applied in a full scale WWTP (Marques *et al.* 2016). In comparison to the other analyzers, this approach has the advantage that neither a preconditioning of the gas (dehumidification and removal of particles), nor a minimal gas flow is needed. The temporal resolution, LODs, and detection ranges for all measurement devices vary between different manufacturers, but may be adjusted individually according to desired requirements. For the study presented in **Paper III**, N_2O emissions were analyzed via a gas analyzer using a NDIR sensor (X-STREAM XEGP, Emerson Process Management GmbH & Co. OHG, St. Louis, USA).

Besides commercially available analyzers for the online detection of gaseous N_2O, the Chair of Analytical Chemistry of TUM has developed a photoacoustic (PA) spectroscopy for WWTP applications (Thaler *et al.* 2017). This technology uses the thermal expansion characteristics of gases initiated by a chopped light source (optical energy), which leads to an acoustic signal that is finally transferred into the gaseous N_2O concentration (Haisch 2012, 2012). During a field-study at a full scale deammonification plant, we could successfully prove the applicability of such a PA system employing a semi-conductor laser at 2.9 µm excitation wavelength; with a LOD of 0.8 ppm_v, a detection range of more than four orders of magnitude (up to 1 % N_2O), and no cross sensitivity towards humidity or any other gaseous component in the off-gas, the PA system could fulfill all necessary requirements for the reliable and robust measurement of N_2O in the specific environment of a deammonification plant's off-gas (Thaler *et al.* 2017). Thus, a

preconditioning of the off-gas was not necessary enabling an even smaller and highly mobile setup.

Besides gaseous measurements, dissolved N_2O can also be measured online in the aqueous phase using a Clark-type microsensor or its industrial version as applied in **Paper II** for laboratory scale and full scale applications, respectively (Unisense Environment A/S, Denmark). The commercial electrochemical microsensor's response was reported to be linear in the range of 0-1.2 mmol/L with a detection limit of less than 1 µmol/L and a 90 % response time of 40-50 s (Andersen *et al.* 2001). However, regular calibration is necessary due to a drift of the sensor within days; furthermore, cross-sensitivity towards nitric oxide and temperature fluctuations need to be eliminated by correction functions (Jenni *et al.* 2012). Again, the manufacturer offers the possibility of customized sensors regarding its detection range and limit as well as its response time which directly influences the lifetime of the sensor (short response time correlates with shorter lifetime). Generally, the manufacturer guarantees a lifetime of four months for the industrial electrode, however with an expected lifetime of more than six months. Nevertheless, the ongoing expenses for the replacement of the sensors and the necessity of periodical calibration are not negligible for long-term application.

Measurements of dissolved N_2O coupled with liquid-gas transfer mass estimations (based on the mass transfer coefficient k_La, gas flow rate, liquid volume, and Henry coefficient according to (Schulthess and Gujer 1996); cf. Chapter 2.3.1) also enable the assessment of N_2O emissions (or vice versa by measuring the gaseous N_2O concentrations). Nevertheless, the determination of the k_La is not straightforward for full scale WWTPs (Foley *et al.* 2010). Thus, off-gas measurements are preferably applied for the reliable quantification of N_2O emissions while the liquid-phase measurements are useful for better process understanding of N_2O production and emission; consequently, combining the measurement of both gaseous and aqueous N_2O enables an increased reliability of the data (Law *et al.* 2012b).

Due to the highly temporal, spatial, and even seasonal variation of N_2O emissions (cf. Chapter 2.3.1), the entire off-gas of a full scale WWTP reactor should ideally be collected and analyzed over a longer period for the assessment of the N_2O emissions. This can easily be accomplished for fully covered (ideally completely gas-tight) reactors by measuring the off-gas N_2O concentration and the total gas flow rate. For uncovered reactors however, a complete catchment of the off-gas is hardly feasible. Nevertheless, multiple gas catchments simultaneously collecting the off-gas at different locations would be advantageous to create a N_2O inventory as reliable as possible; for such an approach, generally floating chambers are applied (Law *et al.* 2012b). However, no standardized procedure (regarding measurement duration, quantity and location of gas catchments, logging interval, etc.) for the off-gas measurement exists so far, which would render the specific N_2O emissions of different deammonification plants comparable and representative.

3 Research Significance, Research Questions, and Hypotheses

The deammonification process is an energy and cost saving technology compared to conventional nitrification/denitrification for the nitrogen removal of wastewater streams (Jetten *et al.* 2001; Fux *et al.* 2002; Ma *et al.* 2016; Cao *et al.* 2017). However, it is also capable to produce the undesired byproduct N_2O which can potentially be emitted into the atmosphere, negatively effecting the environment in the long term. Therefore, it is important to investigate the impact of influencing factors on the biological production of N_2O during the treatment of wastewater in order to reduce the N_2O emissions to a minimum by developing mitigation strategies. As no legislative regulations limit N_2O emissions so far, the motivation of a WWTP operator for a voluntarily reduction could be directly linked to simultaneous improvement of the deammonification performance. For this reason, this doctoral thesis focused on possibilities for both enhanced nitrogen removal rates and reduced N_2O emissions. To address these aspects, the following research questions with their respective hypotheses are elucidated in detail in Chapters 4-6, relating to **Paper I - Paper III**. Moreover, Figure 5 provides an overview of this dissertation's structure, additionally including the investigated processes, applied methods, and research objectives.

Paper I: "The role of residual quantities of suspended sludge on nitrogen removal efficiency in a deammonifying moving bed biofilm reactor"

Research Question I:
Are the key microorganisms contributing to the nitrogen removal in an one-stage deammonifying moving bed biofilm reactor (MBBR) attached to the carriers within the biofilm or do the suspended microorganisms, which represent a minor percentage of the population in biofilm reactors, also play an important role?

Hypothesis I:
Residual quantities of suspended microorganisms in an one-stage deammonifying MBBR play a key role in the biological removal of nitrogen.

Paper II: "Performance and N_2O formation of the deammonification process by suspended sludge and biofilm systems—A pilot-scale study"

Research Question II.1:
Is the microbial system of either suspended sludge or of biofilm carriers more advantageous with respect to the nitrogen removal rate?

Hypothesis II.1:
The microbial system of biofilm carriers has a higher nitrogen removal rate compared to the microbial system of suspended growth.

Research Question II.2:
Different process conditions are applied in the different reactors of a two-stage deammonification system. Do these influence the reactors' specific N_2O production potentials?

Hypothesis II.2:
The reactors' specific process conditions cause different dissolved N_2O concentrations.

Paper III: "Strategies for enhanced deammonification performance and reduced nitrous oxide emissions"

Research Question III.1:
There are many different ways to control the deammonification process, such as intermittent vs. single-feeding, continuous vs. intermittent aeration, high pH vs. low pH, but which combination of these process conditions leads to highest nitrogen removal rates and lowest N_2O emissions in an one-stage deammonification system?

Hypothesis III.1:
Process conditions for maximized nitrogen removal rates and minimized N_2O emissions can be determined for the one-stage deammonification process.

Research Question III.2:
Is there a correlation between the total nitrogen removal rate and the N_2O emissions? Can operational conditions be defined that lead to minimized N_2O emissions, while the nitrogen removal rate is maximized?

Hypothesis III.2:
A negative correlation exists between the nitrogen removal rate and the emission of nitrous oxide for the one-stage deammonification process, thus, a simultaneous enhancement of the deammonification performance and a minimization of N_2O emissions is possible.

Research Question III.3:
Is it possible to reduce trace organic compounds (TOrCs) besides ammonium with the side-stream one-stage deammonification process?

Hypothesis III.3:

The one-stage deammonification process as side-stream treatment of ammonium-rich wastewater is able to reduce TOrCs.

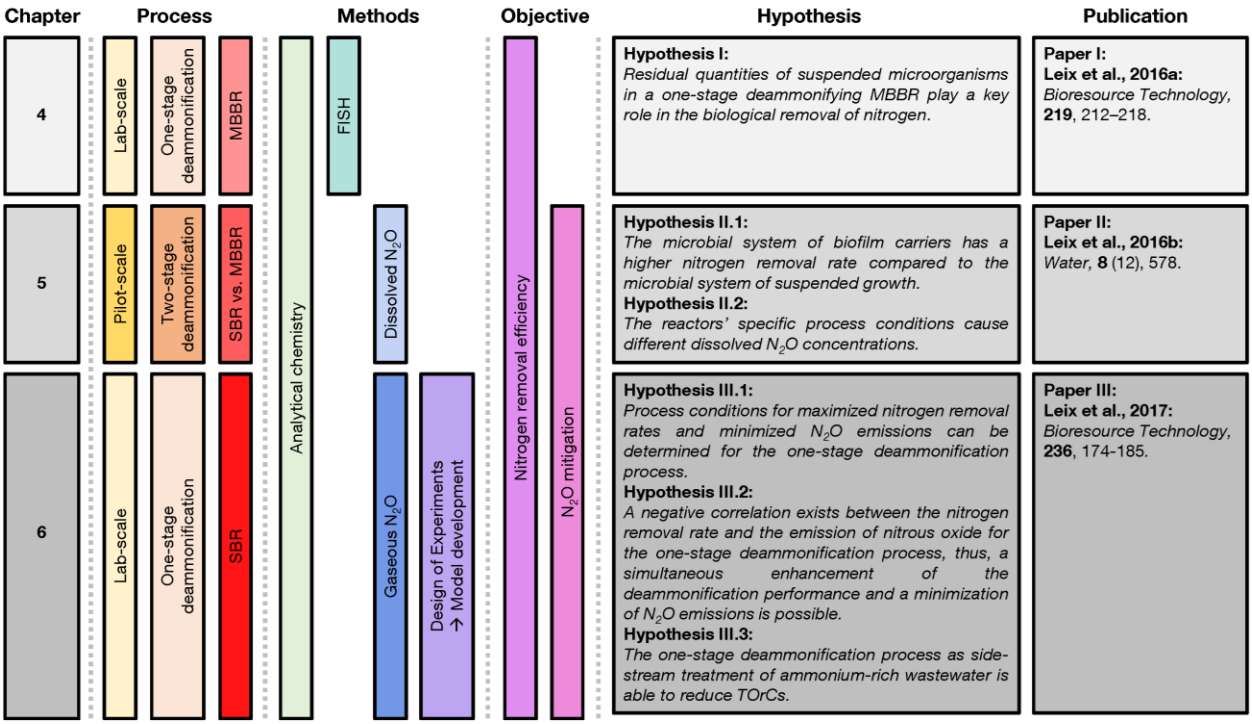

Figure 5. Overview of PhD thesis

4 Paper I: The Role of Residual Quantities of Suspended Sludge on Nitrogen Removal Efficiency in a Deammonifying Moving Bed Biofilm Reactor[8]

For the performance of the deammonification process, different designs executed as one-stage or two-stage process and microbial systems as suspended sludge, granules, moving bed biofilm reactors (MBBR), or integrated fixed-film activated sludge (IFAS) systems - a hybrid of attached and suspended growth systems by design - can be applied. All systems have the common goal to reach nitrogen removal efficiencies as high as possible. In this study, we focused on one-stage deammonifying MBBR systems to investigate the role of small quantities of suspended sludge (with a volatile suspended solids (VSS) concentration of only 0.09±0.03 g/L) coexisting with the biofilm attached to carriers for the performance of these systems. Previous studies focusing on IFAS systems have demonstrated a positive impact of the high amount of suspended sludge in addition to the biofilm for nitrogen removal (Veuillet *et al.* 2014). However, investigations regarding the role of rather small amounts of suspended microorganisms in MBBR systems for nitrogen removal are lacking in the peer-reviewed literature so far. We hypothesized, that *residual quantities of suspended microorganisms in an one-stage deammonifying MBBR play a key role in the biological removal of nitrogen* (hypothesis I).

To test this hypothesis, we performed three experiments in triplicate at laboratory scale investigating the nitrogen conversion rates of the original biomass being comprised of both biofilm carriers and small quantities of suspended sludge as well as their individual contributions as suspended sludge and biofilm carriers only. The results revealed that these minor quantities of suspended sludge severely influenced the nitrogen removal efficiency of the deammonifying MBBR system, which is why hypothesis I could be accepted. Separating the residual suspended sludge from the biofilm carriers resulted in a 3.5-fold lower average removal of nitrogen in comparison to the original system. Nevertheless, the deammonification system was able to recover, when the suspended sludge was not entirely removed. The time of recovery correlated positively with the amount of removed suspended sludge. While biofilm carriers only were capable of a balanced deammonification process, the residual suspended sludge alone was found to accumulate nitrite without efficient nitrogen removal. These results suggested a high abundance of ammonium oxidizing bacteria (AOB) in suspended sludge and a co-existence of AOBs and anoxic ammonium oxidizing (anammox) bacteria in the biofilm. These conclusions based on chemical analyses regarding the microbial community were supported by microbial investigations using fluorescence *in situ* hybridization (FISH). The VSS concentration and the nitrogen removal rate correlated positively, which is why the nitrogen removal efficiency of a deammonifying biofilm system could be further increased by re-adjusting the withdrawal of effluent to sustain a higher concentration of suspended sludge in the system.

[8] Leix *et al.* (2016a); Supplementary information is attached in the Appendix.

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The role of residual quantities of suspended sludge on nitrogen removal efficiency in a deammonifying moving bed biofilm reactor

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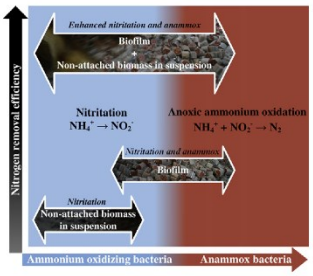
HIGHLIGHTS

- Suspended sludge can severely influence nitrogen removal in MBBR deammonification.
- Removal of suspended sludge resulted in drop of nitrogen removal rate.
- Biofilm without suspended sludge was capable of balanced deammonification.
- Suspended sludge only led to nitrite accumulation.
- Recovery of system was possible after partially removing suspended sludge.

GRAPHICAL ABSTRACT



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ABSTRACT

In a moving bed biofilm reactor (MBBR) system, the vast majority of biomass is immobilized as biofilm besides small amounts of suspension. In this study, the influence of the individual biomass components of a deammonifying MBBR, the biofilm on carriers (BC), residual suspended biomass (SB) with a volatile suspended solids concentration of 0.09 ± 0.03 g/L, and its combination (BC + SB) on nitrogen removal efficiency was investigated. While the performance was highest for BC + SB (0.42 kg N/(m^3·d)), it was reduced by a factor of 3.5 for BC solely. SB itself was only capable of nitrite accumulation. This suggests a high abundance of AOBs within suspension besides the coexistence of AOBs and anammox bacteria in the biofilm, which could be supported by results using fluorescence *in situ* hybridization (FISH). Thus, small amounts of suspended microorganisms can positively influence the deammonification's efficiency. If this fraction is partially washed out, the system recovers nevertheless within hours.

1. Introduction

Deammonification represents an attractive process for autotrophic nitrogen removal as a sidestream treatment of high-strength wastewater such as process water from anaerobically digested sludge. It is comprised of partial nitritation carried out by aerobic ammonium oxidizing bacteria (AOBs) within the phylum *Betaproteobacteria* (Head et al., 1993) followed by the subsequent conversion of nitrite and ammonium to gaseous nitrogen by anaerobic ammonium oxidizing bacteria within the phylum *Planctomycetes* (Strous et al., 1999). These anaerobic ammonium oxidation (anammox) bacteria were discovered in 1995 in a denitrifying fluidized-bed reactor (Mulder et al., 1995) and since then the process has been further developed and successfully implemented in over 100 full-scale wastewater treatment facilities with a growing number of installations worldwide (Lackner et al., 2014).

Besides its potential of reducing the nitrogen load in mainstream processes by 15–25% (Gilbert et al., 2015), the production of excess sludge is mitigated due to the slow-growing anammox

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bacteria (Strous et al., 1998; Tang et al., 2011). Being an autotrophic process and including a partial oxidation of ammonium to nitrite instead to nitrate, the demand for an external carbon source and aeration can be decreased by 100% and more than 50%, respectively, compared to conventional nitrification/denitrification processes (Jetten et al., 2001; Fux et al., 2002). Thus, deammonification can result in reduced operating costs of up to 90% (Jetten et al., 2001).

Implementing deammonification as a single-stage biofilm system offers the added benefit of improving the volumetric nitrogen removal capacity (Lackner et al., 2014) and retaining the microorganisms in one system effortlessly. This is especially advantageous for the slow growing anammox bacteria. However, on the downside usually extended initial start-up phases are required for the establishment of a biofilm reactor in which nitrogen removal takes place by a complex interaction of immobilized microorganism.

Driven by the predominant boundary conditions and the highly diffusion limited processes within a biofilm, AOBs are generally developing in the outer aerobic layer generating essential nitrite for the anammox bacteria prevailing in the deeper oxygen-limited layer (Helmer et al., 2001; Picioreanu et al., 2004). The efficiency of the anammox process does not only depend on the activity of AOBs, but also on the diffusion of substrate and dissolved oxygen (DO) into the inner layers of the biofilm as anammox bacteria are reversibly inhibited by oxygen, even at low concentrations (Strous et al., 1997).

In a MBBR system, also a small amount of non-attached microorganisms can be present in the bulk phase. The influence of this residual suspended sludge on the performance of a biofilm system, even at low concentrations, is often neglected but might be more important than expected as recently proposed by Hubaux et al. (2015) in their numeric model for mixed liquor flocs and granules. In contrast, integrated fixed-film activated sludge (IFAS) systems are by design a hybrid of attached and suspended growth systems operated at substantial concentrations of suspended sludge. Typical mixed liquor suspended solid (MLSS) concentrations are in the range of 2.5–5.0 g/L (Zhao et al., 2014) and therefore significantly higher than in MBBR systems. The effect of additional suspended microorganisms on the nitrogen removal efficiency and the microbial distribution in IFAS systems have been investigated before. Veuillet et al. (2014) reported a three to four fold higher nitrogen removal rate in comparison to a MBBR system with 96% of total anammox bacteria located in the biofilm, while 93% of total AOBs were found in suspension. Zhang et al. (2015) also confirmed highly abundant AOBs in the activated sludge and predominant anammox bacteria in the biofilm, but less than 3% of anammox bacteria in suspension and AOBs in the biofilm for an IFAS system. To the best of our knowledge for MBBR systems, the contribution of non-attached biomass present at very low quantities on the plant's performance has not been investigated before. Therefore, for these systems nitrogen conversion have been assumed to be exclusively attributed to biofilm activity.

We hypothesized that even small amounts of suspended sludge can have a significant impact on the nitrogen removal efficiency of a deammonifying MBBR system. Therefore, ammonium, nitrite and nitrate conversion rates were determined individually for each fraction of a MBBR comprised of the combined biofilm carriers and suspended biomass, the suspended biomass separated from carriers, and the biofilm carriers after removing residual suspended biomass flocs. These investigations allowed to elucidate their individual contributions to the deammonification process and to draw conclusions regarding the microbial community structure within the biofilm and the residual suspended sludge and the overall system performance.

2. Materials and methods

2.1. Experimental setup

All experiments were carried out in three sequencing batch reactors (SBR) with a total volume of 13 L at the laboratories of the Technical University of Munich (TUM). The reactors were identical in design and equipped with a stirrer with adjustable speed of rotation (IKA®-Werke GmbH & CO. KG, Staufena, Germany), two peristaltic pumps (Watson Marlow GmbH, Rommerskirchen, Germany) for feeding of process water and dosing of sodium hydroxide for pH adjustment, an aeration unit manually controllable by a rotameter (KROHNE Messtechnik GmbH, Duisburg, Germany), and a circulation thermostat (Peter Huber Kältemaschinenbau GmbH, Offenburg, Germany) for temperature control.

Online measurement devices were implemented for dissolved oxygen (optical sensor), ammonium and nitrate (ion-selective probe), pH and oxidation-reduction potential (ORP; combination electrode), temperature, conductivity, and pressure (Endress+Hauser Messtechnik GmbH + Co. KG, Reinach, Switzerland). Each reactor was controlled by a supervisory control and data acquisition (SCADA) system (Schneider Electric GmbH, Seligenstadt, Germany).

2.2. Inoculation

Experiments were conducted with inoculum from a MBBR of a two-stage deammonification pilot-plant located in Kempten (Abwasserverband Kempten Allgäu, Germany). This inoculum used from second stage was comprised of both biofilm carriers and suspended biomass with a low volatile suspended solids concentration of 0.09 ± 0.03 g/L during the observation period. Biofilm of the deammonification process has been cultivated on carriers of the type AnoxKaldnes™ K2 (Veolia Water Technologies AB – AnoxKaldnes, Lund, Sweden). The ratio of carriers in the second stage of the pilot facility to the total volume was 40%. While the experiments for this study were executed using laboratory-scale reactors at TUM, the MBBR in Kempten was intermittently aerated and fed with the effluent of the nitritation tank consisting of 993 ± 114 mg NH_4-N/L and 235 ± 106 mg NO_2-N/L. During this time, a medium effluent quality of 111 ± 23 mg NH_4-N/L and 58 ± 37 mg NO_2-N/L was achieved. Thus, the required NH_4-N/NO_2-N ratio of 1:1.32 was not maintained according to the underlying stoichiometry (Strous et al., 1998), which is why the second stage served to 84 ± 12% as a single-stage deammonification reactor after consumption of the inflowing nitrite to reach its full performance. Inoculum from this pilot-scale reactor was transferred to lab-scale reactors at TUM, which were subsequently operated as a single-stage deammonification process. With the inoculum being adapted to single-stage deammonification, the experiments at TUM could already proceed after several days of reactor operation.

2.3. Measurement campaign

2.3.1. Experiments

A series of three experiments was performed, each carried out in triplicate (I, II, and III; $n_{total} = 50$) and always freshly inoculated with biofilm carriers and/or suspended biomass from the same pilot-plant reactor. One laboratory-scale reactor was inoculated with the original MBBR medium of the deammonification plant in Kempten consisting of biofilm carriers and suspended sludge (BC + SB), another reactor used its fraction of suspended sludge only (SB). The third experiment was comprised of biofilm carriers only (BC) after suspended sludge had been removed. With this approach, comparable starting conditions could be guaranteed for the different microbial fractions of a MBBR coming from one

single source and having the same history. For all three fractions of biomass, their individual ammonium, nitrate, nitrite and overall nitrogen conversion rates were determined. Here, the term conversion does not only describe the transformation of a nitrogen species into another, but is also suggesting removal, when the nitrogen balance of the system resulted in an elimination of total nitrogen. By determining nitrogen conversion rates individually for each biomass fraction, insight regarding their specific role in the deammonification process could be provided. Whenever we refer to a 'balanced deammonification', steady nitrogen concentrations in the effluent without significant build-up of nitrite or nitrate were observed.

The operating volume for all SBRs was set to 10 L of fluid medium and a total of 600 carriers for experiments BC + SB and BC representing a filling ratio of 15%. To provide comparable conditions throughout all experiments while minimizing potential matrix effects, the fluid medium in experiment BC was reused from experiment BC + SB after any residual, non-adherent suspended sludge had been removed. To facilitate the elimination of the residual suspended sludge present as tiny flocs with a poor settling capability, the entire fluid without carriers was pretreated by centrifuging at 10,000 rpm for 5 min (Centrifuge 4K15, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) followed by filtration (filter paper No. 388, pore size 10–15 µm, Sartorius AG, Göttingen, Germany). In order to not alter the biofilm structure, the carriers were carefully immersed at least five times into the supernatant to remove suspended sludge still attached to the biofilm (Deighton et al., 2001; Stepanovic et al., 2007). The effluent was repeatedly purified after each washing step.

Two additional and independent experiments, comparable to experiment BC but only using a two- and a four-fold washing step, were conducted to investigate the time-dependent recovery of the system.

2.3.2. Cycle strategy

The SBRs were operated as a single-stage deammonification process at an operating temperature of 33.0 ± 1.0 °C with a cycle strategy of single feed, reaction phase with alternating anoxic and oxic periods, and discharge phase lasting in total 600 ± 300 min. Withdrawal of effluent from the lab-scale reactors was performed without a prior settling phase identical to the mode of operation of the pilot-plant in Kempten. The first set of experiments during repetition I consisted of 78% of aerated phases and 22% of non-aerated phases per cycle (anoxic phase of 240 s and an oxic phase of 840 s per interval). Due to accumulating nitrite during the first repetition of experiment BC + SB representing the baseline for applicable conditions in experiments SB and BC, the anoxic phase was extended up to 360 s throughout repetitions II and III leading to 70% of aerated phases per cycle. During aerated phases, a constant air volume flow of 50 L/h was supplied. Aeration was automatically terminated at a DO concentration above 0.3 mg/L and restarted at a DO set point of 0.1 mg/L during aerated phases (Hubaux et al., 2015). Adjustment of pH was automatically performed at a minimal set point of pH 7.5 by dosage of 2% sodium hydroxide solution via a peristaltic pump. Intermittent dosage of 2 s followed by a pause of 30 s allowed for time-delayed adjustment of the feedback-pH-signal. Process water of the municipal wastewater treatment plant Garching/Munich was used as feed. Influent concentrations were characterized as 1,271 ± 103 mg NH_4-N/L, 798 ± 186 mg/L as COD, pH of 8.3 ± 0.1, and an electrical conductivity of 8.5 ± 0.1 mS/cm.

2.4. Analytical methods

Nitrogen species for the calculation of nitrogen conversion rates were analyzed throughout every repetition of experiments using photochemical testing kits (Hach Lange GmbH, Düsseldorf, Germany). Grab samples of both influent and effluent were directly processed for analysis. To eliminate the impact of disruptive nitrite concentrations as interfering ions when analyzing nitrate, samples were either diluted or sulfamic acid was added as described previously (Wu et al., 1997).

Additionally, online ion-selective sensors (CAS40D, Endress +Hauser Messtechnik GmbH, Reinach, Switzerland) continuously monitored the concentration of ammonium and nitrate. Regular *in situ* calibration was performed using Hach Lange photochemical test kits. For monitoring and controlling of pH and oxygen concentration, online sensors were utilized (Memosens CPS16D, Oxymax COS61D, Endress+Hauser Messtechnik GmbH, Reinach, Switzerland). Total suspended solids (TSS) as well as volatile suspended solids (VSS) were frequently analyzed according to standard methods (APHA, 2012).

2.5. Microbial investigations

Fluorescence *in situ* hybridization using confocal laser scanning microscopy (CLSM) for qualitative identification and imaging of the microbial community was applied in support of findings determined by chemical methods. The main goal of applying FISH was to obtain an overview of ammonium oxidizers and anammox bacteria being abundant in suspended sludge and biofilm. However, the aim was neither to quantitatively characterize the entire microbial community present nor to define their local distribution within the biofilm which is why both next generation sequencing and cryosection were not employed in this study.

Due to a low biomass concentration in the mixed liquor, samples of suspended sludge were concentrated via centrifugation at 3,000 rpm for 10 min (Centrifuge 5417 R, Eppendorf AG, Hamburg, Germany) and resuspended in 1× phosphate buffered saline (PBS) solution of pH 7.4 for subsequent cell fixation. Biofilm was scraped of the carrier and also resuspended in 1× PBS. Due to a high density of the biofilm, homogenization was achieved via a Teflon Potter-Elvehjem homogenizor. Hereafter, samples were pretreated following a protocol by Nielsen et al. (2009) as described in the supplementary information (SI).

The fluorescent probes EUB 338 targeting *bacteria*, NSO 1225 detecting ammonium oxidizing β-*Proteobacteria*, and Bfu 613 specifically targeting *Brocadia fulgida* were simultaneously applied in one sample for image acquisition. The latter probe was chosen as Candidatus *Brocadia fulgida* was previously found in different microbial systems of deammonification processes to be the dominant anammox bacteria species. While also other dominant anammox bacteria were reported in literature (Chen et al., 2016; Zhang et al., 2016), the probe Bfu 613 was applied as to characterize a typical representative of anammox bacteria to support chemical findings regarding the microbial distribution. Each probe was labeled with a different fluorochrome (FLUOS, Cy 3, Cy 5) and processed at a formamide (FA) concentration of 30% to best match all requirements. Images were taken using the CLSM ECLIPSE Ti with a 20×/0.75 Plan-Apochromat oil immersion lens applying the software NIS-Elements AR (Nikon Corporation, Tokyo, Japan). Fluorescent probes used with their specifications are summarized in Table 1.

3. Results and discussion

3.1. Reactor performance

Nitrogen conversion rates were determined for three biomass fractions (BC + SB, SB, and BC) to elucidate their individual contribution within the deammonification process using a MBBR. The

Table 1
Ribosomal ribonucleic acid (rRNA) targeted oligonucleotide probes for the detection of all bacteria, AOBs, and anammox bacteria.

Probe	Specificity	Sequences (5́-3́)	FA [%]	Reference
EUB 338	*Bacteria*	GCT GCC TCC CGT AGG AGT	0–60 (*30)	Amann et al. (1990)
NSO 1225	Ammonia-oxidizing β-*Proteobacteria*	CGC CAT TGT ATT ACG TGT GA	35 (*30)	Mobarry et al. (1996)
Bfu 613	*Brocadia fulgida*	GGA TGC CGT TCT TCC GTT AAG CGG	30	Kartal et al. (2008)

* Applied formamide concentration (FA).

ammonium and the total nitrogen conversion rates varied significantly ($p \leqslant 0.005$; normal distribution successfully tested with Kolmogorov-Smirnov test at $\alpha = 0.01$) between the three different biomass fractions, besides for one exception (Fig. 1; further information regarding the statistical analysis is provided in the SI, Fig. II-1). Experiments with the BC + SB fraction resulted by far in the highest conversion rate, however, this fraction also exhibited the highest standard deviation across all three repetitions. Ammonium and total nitrogen conversions were significantly lower for the SB and the BC system. Even though, the SB fraction was capable of higher ammonium conversion rates in comparison to the BC fraction, the latter proved to have a higher and more balanced nitrogen removal without build-up of nitrite or nitrate. In comparison, nitrogen removal for the SB fraction was close to zero due to nitrite accumulation.

The results revealed that the BC + SB fraction as well as the BC fraction were both capable of providing a balanced deammonification, however, the performance of the BC fraction was on average reduced by a factor of 3.5. In contrast, the SB fraction alone was not capable of performing a balanced deammonification.

3.2. Oxygen concentrations

During all repetitions of experiments using the BC + SB and SB fractions, dissolved oxygen concentrations did not exceed the threshold concentration of the DO control (0.3 mg/L), which let to complete aeration during oxic phases. In comparison, oxygen consumption by the BC fraction was low leading to prompt increase of dissolved oxygen concentrations and slow oxygen depletion. To comply with the limiting DO value, the time of aeration needed to be shortened by an average of 77% in comparison to experiments using the BC + SB and SB fractions. This did not cause prolonged DO-limiting conditions for the BC fraction, which could have led to extended nitrite limitation for anammox bacteria and therefore reduced nitrogen turnover rates. Quite the contrary was the case, shortened aeration times were necessary to guarantee anoxic periods for the anammox process.

Average, maximal, and minimal dissolved oxygen concentrations of the three fractions are illustrated in Fig. 2. With regard to whole cycles, the average dissolved oxygen concentration was lowest for fraction BC + SB, slightly higher for fraction SB, and highest for fraction BC. The same regime could be observed for maximal concentrations. Thus, reduced times of aeration for the BC fraction are unlikely to be responsible for lower nitrogen turnover rates as sufficient dissolved oxygen concentration for the ammonium oxidation was provided. Minimal DO concentrations were low for all experiments promoting anoxic anammox reactions.

3.3. Impact of microbial community on nitrogen conversion rates

3.3.1. Distribution of microbial community in suspended sludge and in biofilm derived through chemical analysis

Chemical analysis revealed that a consistent and complete deammonification was achieved during the experiment with fraction BC + SB suggesting the presence of an abundant microbial community comprised of AOBs and anammox bacteria. However, it was not possible to distinguish the individual contribution of suspended sludge and biofilm on nitrogen conversion rates with this particular experimental approach. For this reason, separated

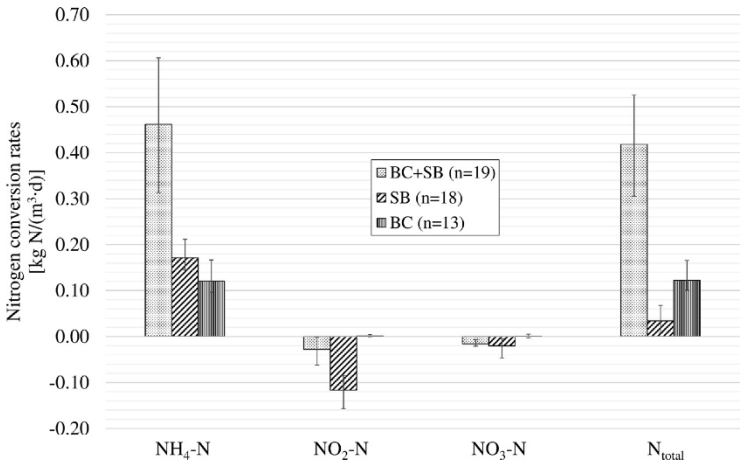

Fig. 1. Minimal, mean, and maximal conversion rates of ammonium, nitrite, nitrate, and total nitrogen for experiments of biofilm carriers and suspended sludge (BC + SB), suspended sludge (SB), and biofilm carriers (BC).

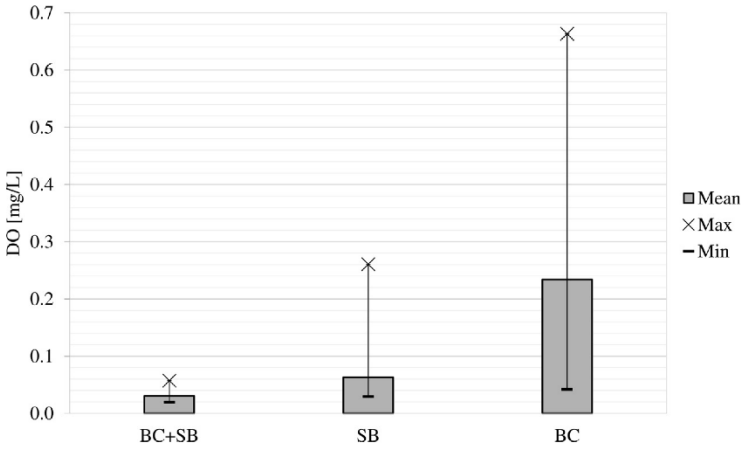

Fig. 2. Minimal, mean, and maximal oxygen concentrations over all cycles for experiments of biofilm carriers and suspended sludge (BC + SB), suspended sludge (SB), and biofilm carriers (BC).

experiments with suspended sludge only and biofilm only were conducted.

During experiment with fraction SB, a nitrite built-up was observed nearly as high as ammonium degradation. The ammonium conversion rate of the SB fraction was still 37% of the rate achieved during the experiment with the BC + SB fraction. Due to a slight built-up of nitrate and the low average conversion rate of total nitrogen, a subordinate activity of nitrite oxidizing bacteria (NOB) and anammox bacteria can be assumed resulting in nitrite accumulation. Low activity of NOBs goes well together with the low average oxygen concentrations observed throughout the experiment with fraction SB, as NOBs have an oxygen affinity constant that is higher than that of AOBs (Guisasola et al., 2005; Ciudad et al., 2006). Thus, either NOBs were suppressed due to the prevailing operating conditions or exhibited a low abundance in the microbial community. For the anoxic ammonium oxidation, ideal settings with simultaneously available nitrite and ammonium as substrate combined with anoxic conditions would have been available. However, the overall nitrogen balance proved a nitrogen removal close to zero. Hence, experiment with fraction SB clearly indicated a high abundance of AOBs with an insignificant quantity of anammox bacteria within the microbial community of suspended sludge. Minor anammox activity is hypothesized to be attributed to little detachment of biofilm from the carriers.

Biofilm carriers without suspended sludge as tested in experiment with fraction BC were able to perform a balanced deammonification under the given DO control which suggests that AOBs and anammox bacteria are likely both abundant within the biofilm. This is in accordance with the multi-layer biofilm model of MBBRs (Helmer et al., 2001; Picioreanu et al., 2004), but does not reflect the microbial distribution of biofilms studied in IFAS systems (Veuillet et al., 2014; Zhang et al., 2015), most probably because of the shift of AOBs from the biofilm into the substantial amounts of suspended sludge. Due to the removal of suspended microorganisms in experiment with fraction BC, average and maximal oxygen concentrations were considerably higher compared to experiments with fractions BC + SB and SB. This is yet another piece of evidence that suspended sludge is mainly represented by AOBs, because oxygen consumption was severely decreased in fraction BC. Removal of suspended sludge therefore shifted the balanced microbial community of the original inoculum to disequilibrium with a too low abundance of AOBs in experiment with fraction BC only. Even though sufficient oxygen was provided throughout aerated phases, supply of nitrite was reduced which likely influenced the nitrogen removal efficiency (Szatkowska et al., 2007). For that reason, biofilm carriers not associated with suspended biomass were not able to reach nitrogen removal capacities as high as observed in experiment with the BC + SB fraction.

3.3.2. Microbial investigations regarding community of suspended sludge and biofilm

The findings of the microbial composition of biofilm and suspended sludge studied by chemical analyses were further augmented by additional microbial investigations. FISH investigations could reveal the coexistence of AOBs as well as anammox bacteria represented by *Brocadia fulgida* within the biofilm of the lab-scale reactors. Fig. I-1a and I-1b in the SI display the microbial composition of the BC fraction in detail. Even though samples were homogenized before cell fixation, still considerable clusters of AOBs and anammox bacteria were detectable. This is again in good agreement with the proposed biofilm layer model (Helmer et al., 2001; Picioreanu et al., 2004). In contrast, anammox bacteria were absent within the suspended flocs, whereas AOBs could be detected (Fig. I-1c in SI).

With *Brocadia fulgida* previously being reported to be the dominant anammox species in different deammonification systems (Winkler et al., 2012; Gilbert et al., 2013; Laureni et al., 2015), the FISH images are supposed to give a representative assessment of the microbial community, yet with a slight underestimation of anammox bacteria.

3.3.3. Influence of the volatile suspended solid concentration on the nitrogen conversion rates

Nitrogen conversion rates during experiment with fraction BC + SB varied throughout the three repetitions (Fig. 1 and Fig. II-1 in SI). The reason is likely due to varying starting conditions regarding the amount of suspended sludge in the bulk phase for each repetition. The results obtained clearly suggest a positive correlation between the VSS concentration of the fluid medium and the conversion rates of ammonium, nitrite and total nitrogen (Fig. 3). This link again matches well with the fact that AOBs were the most abundant bacteria within the suspended sludge samples. Increasing the amount of suspended sludge leads to increasing abundance of AOBs resulting in a higher ammonium oxidation rate to nitrite. In a IFAS system, improved nitrogen removal by accumulating suspended microorganisms to a MLSS concentration up to 5 g/L was also reported by Veuillet et al. (2014). However, we could show, that the impact of suspended sludge on the plant's

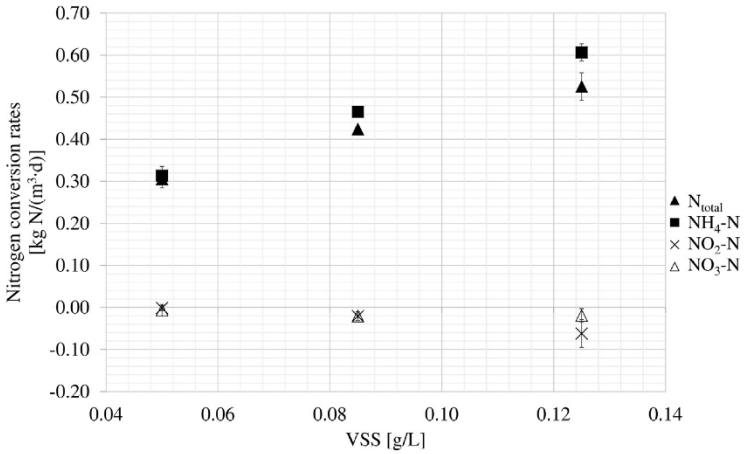

Fig. 3. Correlation between the VSS concentration and the nitrogen conversion rates for the experiment of biofilm carriers and suspended sludge (BC + SB).

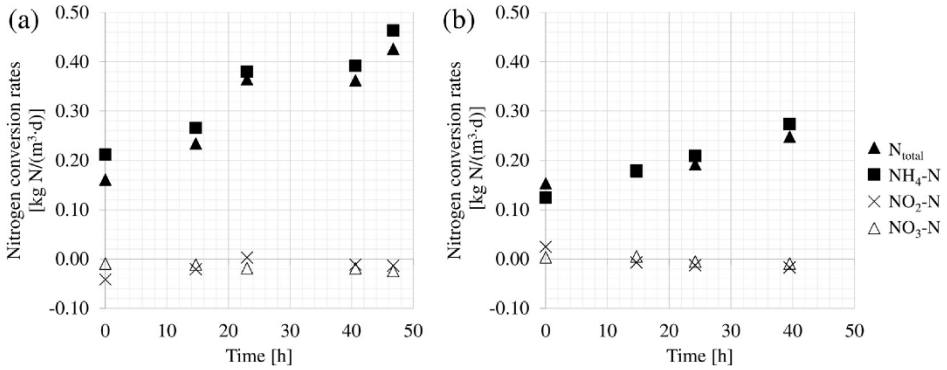

Fig. 4. Recovery of the system after a double washing step of biofilm carriers (a) and a fourfold washing step (b).

performance in a biofilm system already begins at a much lower concentration. Thus, the nitrogen removal efficiency in a MBBR system could be increased by accumulating suspended sludge with an additional settling phase. However, a balanced ratio between AOBs and anammox bacteria has still to be guaranteed.

3.4. Recovery of system

Even though the nitrogen removal efficiency suffered by more than 50% from a withdrawal of suspended sludge with total nitrogen conversion rates only slightly higher than those of the BC fraction at the beginning of the experiments, recovery of the system could be observed as long as suspended sludge was not entirely removed. This was confirmed by two additional experiments, where the washing step for the preparation of experiment with fraction BC was reduced to two (Fig. 4a) and four times (Fig. 4b). With increasing operating time, conversion rates of ammonium and total nitrogen increased again. The curve progressions revealed that the time of recovery was highly dependent on the amount of suspension removed with a higher abundance of suspension resulting in faster recovery. These observations regarding a quick recovery of the MBBR's performance after decreasing the VSS concentration are in good agreement with the system recovery of a IFAS system by Veuillet et al. (2014), although operated at VSS levels several orders of magnitude higher.

An increase in the ammonium conversion rate and as a consequence an increasing nitrogen conversion rate in a time frame of hours was observed for both systems indicating a fast growing guild to be involved. Different maximum specific growth rates for anammox bacteria were reported with 0.006 h^{-1} (Tang et al., 2011) to 0.0027 h^{-1} (Strous et al., 1998) resulting in a doubling time of approximately 5–11 d. With anammox bacteria being rather slow growing microorganisms, the observed prompt increase in nitrogen removal efficiency is unlikely to be attributed to additional growth of anammox bacteria. However, AOBs, such as *Nitrosomonas europaea*, grow much faster than anammox bacteria. Maximal growth rates of 0.017–0.088 h^{-1} have been reported, which is equivalent to a doubling time of 0.3–1.7 days (Prosser, 1989). Growth rates of AOBs contributing to the ammonium oxidation and with its production of nitrite to total nitrogen conversion rates correlate well with the time dependency of the system's recovery. This is another piece of evidence that suspended sludge is mainly represented by AOBs and recovery of the system was determined by their growth rate.

4. Conclusions

It was demonstrated that small amounts of suspended microorganisms coexisting in a MBBR system can positively influence the nitrogen conversion rate. If deammonification is exclusively per-

formed by the biofilm community, the efficiency was reduced by a factor of 3.5. Thus, performance could be increased by accumulating suspended bacteria. AOBs were highly abundant in the residual suspension, whereas anammox bacteria were absent. However, both were found to cluster within the biofilm. If suspended microorganisms get partially lost, the system is able to recover. Time of recovery correlates positively with the extent of lost bacteria and is in the range of hours.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2016.07.134.

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5 Paper II: Performance and N_2O Formation of the Deammonification Process by Suspended Sludge and Biofilm Systems—A Pilot-Scale Study[9]

The number of full scale deammonification installations is growing worldwide, yet various designs (one-stage vs. two-stage processes; suspended sludge vs. biofilm, etc.) are available (Lackner *et al.* 2014) potentially influencing the deammonification performance and N_2O emissions. While **Paper I** focused on biofilm systems and investigated how their performance is influenced by residual quantities of suspended sludge, **Paper II** elucidated differences between reactor performances using different microbial systems and between dissolved N_2O concentrations of different microbial processes. Therefore, two two-stage deammonifying systems at pilot scale employing a suspended sludge (entitled SBR) and a biofilm system (entitled MBBR) in the second stage were studied over a 1.5-year period to compare reactor performances and N_2O formation potentials. We hypothesized that *the microbial system of biofilm carriers has a higher nitrogen removal rate compared to the microbial system of suspended growth* (hypothesis II.1) and that *the reactors' specific process conditions cause different dissolved N_2O concentrations* (hypothesis II.2).

Due to only moderate nitritation performance, the WWTP operators decided to implement an uncommon supplementary aeration in both second stages (originally designed for the anammox process) to enable an additional one-stage deammonification side-process for performance enhancement. Owing to this countermeasure, both the SBR and the MBBR achieved the required nitrogen removal rates and degradation rates of above 500 $g_N/(m^3 \cdot d)$ and 80 % set by the WWTP operators. Comparing the average nitrogen removal rates of the SBR and MBBR individually, the SBR's performance exceeded the MBBR's by 19 %, yet being in advantage over the MBBR due to inflowing nitrite concentrations that were comparably elevated. To avoid this imbalance for the evaluation of hypothesis II.1, the calculated nitrogen conversion rates of the one-stage deammonification side-process were taken as a basis. In this respect, the MBBR had a higher performance as the SBR, which is why hypothesis II.1 could be accepted. Dissolved N_2O concentrations varied considerably for the different reactors with the buffer tank interconnecting the first and second stages exhibiting the highest concentrations. Thus, hypothesis II.2 could likewise be accepted. Based on the reactors' operational conditions, individual N_2O production pathways and emissions were discussed and N_2O mitigation strategies were developed, such as a HRT decrease and frequent removal of settled biomass in the buffer tank and a re-arrangement of the aeration strategy in the second stage to enable N_2O reduction by denitrification during an initial unaerated phase to reduce subsequent stripping effects. Taking all operational conditions for both the SBR and the MBBR into account, neither suspended biomass nor biofilm seemed to have an advantage regarding a lower N_2O formation.

[9] Leix *et al.* (2016b); Supplementary information is attached in the Appendix.

water

MDPI

Article

Performance and N_2O Formation of the Deammonification Process by Suspended Sludge and Biofilm Systems—A Pilot-Scale Study

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Abstract: A two-stage deammonification pilot plant with two different second-stage reactors, namely a sequencing batch reactor (SBR) with suspended sludge and a moving bed biofilm reactor (MBBR) with biofilm carriers, was investigated over a 1.5-year period to compare reactor performances. Additionally, dissolved nitrous oxide (N_2O) was measured to determine the reactors' N_2O formation potential. Although the nitritation performance was moderate (NO_2-N/NH_4-N effluent ratio of 0.32 ± 0.15 in combination with SBR and 0.25 ± 0.14 with MBBR), nitrogen turnover and degradation rates exceeding 500 g N/(m^3·day) and 80%, respectively, were achieved in both second stages, yet requiring additional aeration. The SBR's average nitrogen removal was 19% higher than the MBBR's; however, the SBR's nitrite influent concentration was comparably elevated. Concerning N_2O formation, the nitritation reactor exhibited the lowest N_2O concentrations, while the buffer tank, interconnecting the first and second stages, exhibited the highest N_2O concentrations of all reactors. Given these high concentrations, a transfer of N_2O into the second stage was observed, where anoxic phases enabled N_2O reduction. Frequent biomass removal and a decreased hydraulic retention time in the buffer tank would likely minimize N_2O formation. For the second stage, enabling anoxic periods in the intermittent aeration cycles right after feeding to support N_2O reduction and thus minimize the stripping effects or the implementation of a complete anoxic ammonium oxidation will mitigate N_2O emissions.

Keywords: anammox; dairy industry; emissions; greenhouse gases; MBBR; nitritation; nitrogen removal; nitrous oxide; two-stage deammonification

1. Introduction

One of the key objectives of the biological nutrient removal processes is to remove nitrogen from wastewater effluents in order to achieve load reductions for receiving water bodies. Increasingly strict legal requirements for effluent threshold concentrations are one reason that many wastewater treatment plants (WWTP) must increase their nitrogen removal capacity. A possible method of decreasing the nitrogen load during conventional mainstream biological wastewater treatment is to establish an additional side-stream treatment step for ammonium-rich process water from onsite anaerobically digested sludge treatment. During this deammonification process, ammonium is partly oxidized to nitrite by ammonium-oxidizing bacteria (AOBs). This nitritation step is followed by anoxic ammonium oxidation (anammox), in which autotrophic anoxic ammonium-oxidizing bacteria (AnAOBs) convert ammonium and nitrite to gaseous nitrogen [1]. Therefore, the deammonification

process can be considered an energy- and resource-saving strategy in comparison to conventional nitrification/denitrification because the oxygen supply is decreased and no additional carbon source is needed [2]. Here, we adopt the term anoxic instead of anaerobic ammonium oxidation/oxidizing bacteria following the new standardized nomenclature for biological nitrogen removal processes proposed by Weißbach et al. [3].

A crucial requirement to establish a stable deammonification process is to provide conditions that favor both partial nitritation and anammox. These two subprocesses can be implemented in either single- or two-stage systems using, for instance, suspended sludge, granules, or biofilm carriers [4]. However, a two-stage deammonification is advantageous over the single-stage process in terms of operational stability because the strategies for the suppression of undesired side processes can be implemented more easily in independent reactors [4]. As AnAOBs and nitrite-oxidizing bacteria (NOBs) compete for the substrate nitrite, the suppression of NOBs is desired to prevent further oxidation to nitrate after partial nitritation. Favorable conditions for AOBs versus NOBs were reported for frequently alternating aerobic and anoxic conditions, causing a lag phase for NOBs [5], and low dissolved oxygen (DO) concentrations due to the lower oxygen affinity constant of AOBs relative to NOBs [6]. Additionally, an appropriate sludge retention time (SRT) of 1.0–1.5 days [7] and elevated temperatures (≥30 °C) [8] were reported to favor the wash-out of NOBs. Both NOBs and AOBs are inhibited by free ammonia (NH_3), which positively correlates with an increasing pH value and ammonium concentrations. NOBs are already inhibited by low NH_3 concentrations, whereas AOBs endure higher NH_3 concentrations [9]. Unlike nitritation, the anammox process requires strictly anoxic conditions; however, AnAOBs are reversibly inhibited by oxygen [10,11]. Although nitrite serves as a substrate for AnAOBs, it can also have inhibitory effects. A large range of 5 mg NO_2-N/L up to 750 mg NO_2-N/L has been reported to decrease the anammox activity [12,13]. Even though the second stage of the two-stage deammonification process is commonly fed with high nitrite concentrations, the anammox process is feasible anyway because the influent is diluted by the reactor content generating lower nitrite concentrations. Additionally, AnAOBs tend to grow in aggregates, thus the inner layers are more protected from potential inhibitors due to diffusive limitation [14].

On the downside, biological nitrogen removal processes have been reported to emit N_2O, albeit over a wide range of concentrations. Kampschreur et al. reported 0%–95% of the nitrogen load being emitted as N_2O in laboratory-scale studies and 0%–15% in full-scale studies [15]. The Intergovernmental Panel on Climate Change (IPCC) estimated the N_2O emissions from WWTPs to account for about 2.8% of the total estimated anthropogenic N_2O emissions [16]. At the United Nations Conference on Climate Change in Paris in December 2015, the participating parties agreed on a common goal of limiting the temperature increase to 1.5 °C above pre-industrial levels by equalizing the anthropogenic emissions and sinks in the second half of the 21st century [17]. However, no explicit limit for nitrous oxide emissions was set during the Paris summit, even though it is presently the most heavily emitted ozone-depleting substance in the world and is expected to remain so for the remainder of this century [18]. N_2O causes stratospheric ozone depletion and has a global warming potential that is 298 times higher than that of carbon dioxide based on a 100-year time horizon [16]; thus, nitrous oxide severely affects the climate. Therefore, a minimization of N_2O emissions is necessary to mitigate its negative effects on the environment. Numerous N_2O production pathways have been identified, two of which occur during nitritation due to the oxidation of hydroxylamine and reduction of nitrite to N_2O in the so-called nitrifier denitrification performed by AOBs [15]. Furthermore, N_2O is an obligatory intermediate of the catabolic respiratory pathway of heterotrophic denitrification [19]. Although process water contains little readily degradable organic matter, denitrifiers are still abundant in deammonifying systems and can therefore also contribute to N_2O production or reduction [20]. Due to non-stringent nomenclature for biological nitrogen removal processes, the new terms "aerobic nitrous denitritation" instead of nitrifier denitrification and "anoxic nitrous denitritation" for the reduction of nitrite to N_2O during denitrification were recently proposed [3]. Nevertheless, the established terms are going to be applied throughout this manuscript due to their deep-rooted connections to

these described processes. In contrast to AOBs, no production of N_2O is attributed to the metabolic pathway of AnAOBs [21]. The main factors influencing N_2O production have been identified as low DO concentrations (mainly due to nitrifier denitrification but also due to inhibition of nitrous oxide reductase during denitrification), high nitrite concentrations, and highly dynamic process conditions, such as ammonia shock loads [15]. Other important factors include toxic compounds, low temperatures, and high salinity, among others [15]. The N_2O produced during wastewater treatment is not necessarily emitted into the atmosphere; it can also be further reduced to gaseous nitrogen during denitrification if a carbon source is available. Generally, N_2O emissions are enhanced by stripping during phases of active aeration, while emissions during non-aerated phases are limited due to the high solubility of N_2O (having a Henry's law constant H^{cp} of 1.8×10^{-4} to 2.5×10^{-4} mol/(m^3·Pa) at 298.15 K) [22]. A more accurate assessment of the emitted N_2O is possible on the basis of gaseous measurements. The detection of dissolved N_2O by online sensors can be helpful for identifying conditions favorable for the production and reduction of N_2O to develop mitigation strategies.

This study was designed to evaluate and compare two two-stage deammonification processes at pilot-scale at the Kempten WWTP (Germany), employing a suspended sludge as well as a biofilm system in the second stage. In order to derive recommendations for a stable and optimized full-scale process, the performance across the entire deammonification plant was assessed during the period from February to December 2014 using suspended sludge in the second stage (excluding 2.5 months beginning in August 2014 due to whey tests, leading to a total of 308 days) and from January to June 2015 for the system employing biofilm carriers (in total 148 days). An additional objective of this study was to quantify the N_2O formation potentials of the reactors and develop mitigation strategies based on these results. To achieve this goal, the pilot-scale plant was investigated from July to August 2015 to determine reactor-specific dissolved N_2O concentrations using an online sensor (in total 29 days).

2. Materials and Methods

The wastewater treatment plant in Kempten was put into operation in 1986 with a capacity of 250,000 population equivalents (PE). With an increasing influent load, the WWTP was expanded several times, after which a two-stage deammonification process was implemented as a side-stream treatment of process water. This deammonification process further increased the WWTP's capacity to 465,000 PE and the full-scale process was put into operation in November 2015.

Prior to full-scale implementation, a pilot-scale facility with a volumetric dimension of 0.1% of the full-scale plant was built and put into operation at the end of 2013. The pilot plant was implemented for two reasons. Firstly, WWTP operators were keen in obtaining knowledge regarding the stable operation of the deammonification process with respect to the specific process water of the WWTP, which is strongly influenced by the local dairy industry providing wastewater high in ammonium and calcium carbonate concentrations. Secondly, WWTP operators wanted to determine whether a suspended sludge or biofilm system process configuration was more favorable for full-scale implementation. Therefore, the pilot plant employed a split into two second stages, one reactor equipped with suspended sludge and the other one equipped with biofilm carriers. Both second stages were initially inoculated with concentrated sludge from a full-scale single-stage deammonification plant at the WWTP Ingolstadt, Germany. A high abundance of AOBs as well as AnAOBs has been detected for these biofilm carriers in our previous study [23], providing evidence for deammonification being the main underlying process. Due to the identical inoculation as well as a comparable startup and operation regime for both second stages, results from this predominant nitrogen removal process are also applicable to the reactor with suspended sludge.

The pilot plant consisted of five reactors in total: one for sedimentation, one for nitritation, an intermediate buffer tank, and two reactors for the anammox process in the second stage (Figure 1). The anammox reactor with suspended sludge was employed as a sequencing batch reactor (SBR), whereas a moving bed biofilm reactor (MBBR) utilized biofilm carriers. Therefore, in the following we refer to these reactors as SBR and MBBR depending on the process configuration of the second stage.

An impression of the plant and the reactor with suspended sludge and fixed biofilm is provided in the Supplementary Materials (Figure S1). All reactors except for the sedimentation tank were thermally insulated to minimize temperature losses.

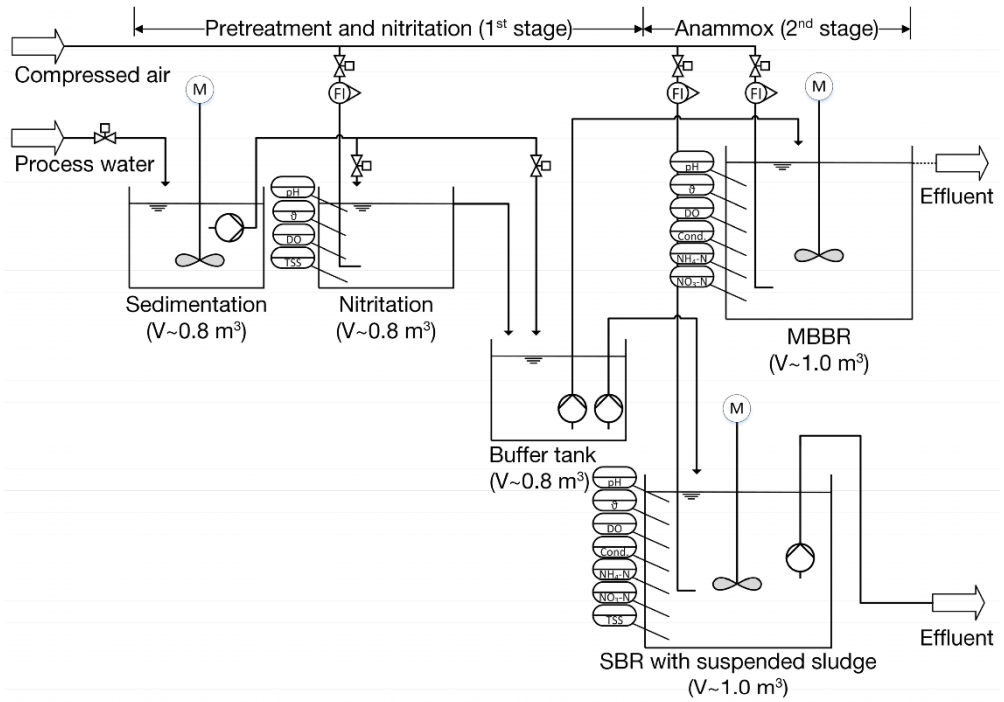

Figure 1. Piping and instrumentation diagram (P & ID) of the deammonification pilot plant.

The sedimentation, nitritation, and buffer tank pre-treatment was the same for both SBR and MBBR. As previous studies have demonstrated that shock loads of total suspended solids (TSS) can negatively influence deammonification performance [4], process water was pre-treated in a 0.8 m^3 sedimentation tank. In this way, the transfer of elevated concentrations of solids into the subsequent biological system was significantly decreased. The subsequent 0.8 m^3 nitritation tank was continuously fed. The effluent was withdrawn by an overflow pipe installed at the water surface without biomass retention. The nitritation tank was initially inoculated with mixed liquor from a trickling filter previously used for nitrification treatment of the process water. To promote the activity of AOBs, the reactor temperature was maintained at approximately 31 °C with a heat exchanger. Various aeration systems (Oxyflex®-OM and Oxyflex®F650, SUPRATEC Gesellschaft für Umwelt-und Verfahrenstechnik mbH, Simmern, Germany; MESSNER Aeration Panel®, RMU Rudolf Messner Umwelttechnik AG, Adelsdorf, Germany) were tested in the nitritation tank because process water high in calcium carbonate led to rapid clogging of the aeration membranes due to calcification (Figure S2). Beginning in October 2014, the aeration systems were occasionally flushed with acetic and formic acid to counteract calcification and improve their lifetime. The aeration could be manually adjusted using a flow meter but was maintained at 2000 L/h. The reactor was equipped with a vertical stirrer and online sensors for dissolved oxygen, temperature, pH, and turbidity (Hach Lange GmbH, Düsseldorf, Germany). However, the pH and dissolved oxygen concentrations were not used for evaluation of the process, as the results were non-representative due to a calcium layer constantly present on the sensors interfering with the measurements. The effluent of the nitritation tank was diverted and stored in a 0.8 m^3 buffer tank before entering either the SBR or MBBR, both of which had a working volume of 1.0 m^3. The buffer tank with a hydraulic retention time (HRT) of approximately one day was insulated, but not heated, aerated, or stirred. As this tank only served as a buffer interconnecting the nitritation and the two second stages, it was not equipped with any online sensors.

Nevertheless, it was assumed that the wastewater characteristics did not change severely in this reactor in comparison to the nitritation effluent.

The SBR utilizing suspended sludge was fed intermittently (2.4 ± 0.5 h) and was time-controlled to establish aerated/unstirred and unaerated/stirred phases, with the aeration phases comprising 44%–78% of the total reaction time during the observation period in 2014 and varying cycle durations of 7.5 ± 1.1 h in total including feeding, reaction, and decant phase. Generally, an aeration of the second-stage anammox process is uncommon. However, due to a rather low performance of the nitritation step, the WWTP operator decided to implement an aeration option in this original anammox reactor to enable an additional single-stage deammonification side-process as a countermeasure and thus, to improve the overall process performance. The reactor was equipped with a vertical mixer and online sensors for ammonium, nitrate, pH, temperature, conductivity, oxygen, turbidity, and filling level (Hach Lange GmbH, Düsseldorf, Germany). Additionally, the stainless-steel tank was temperature-controlled. After a settling step (~0.5 h), the effluent was removed via a submersible pump at the medium height of the filling level.

The MBBR was also fed intermittently with 40%–78% of the entire time being intermittently aerated during the observation period in 2015, which was very similar to the SBR. Again, the supplementary aeration in second stage was implemented to compensate for the rather low performance of the nitritation step. The effluent was released by an overflow without a settling phase, creating a constant volume of 961 L. This reactor was equipped with a vertical mixer, the same heating system as used for the SBR, and online sensors for ammonium, nitrate, pH, temperature, conductivity, and dissolved oxygen. The MBBR utilized uncolonized AnoxKaldnes™ K2 carriers (Veolia Water Technologies AB—AnoxKaldnes, Lund, Sweden) at the startup of the reactor with a filling ratio of 40% of the total volume. One-third of the carriers were initially distributed over a trickling filter treating process water with recirculation to accelerate biofilm growth by fast-growing AOBs, providing a biofilm basis for the subsequent attachment of AnAOBs. Subsequently, these carriers were combined with the rest of the carriers in the MBBR. During the startup phase, the nitrite concentration was kept at 20–30 mg/L to supply AnAOBs with sufficient nitrite and prevent substrate limitation. A thin biofilm layer could be observed after approximately three to four months and grew over time.

The deammonification system was operated via a FlowChief process control system (FlowChief GmbH, Fürth, Germany) combined with a PLC Simatic S7 Modular Controller (Siemens GmbH, Munich, Germany). Therefore, the data were automatically recorded and available for analysis. The dissolved concentrations of ammonium, nitrite, and nitrate were additionally analyzed using photochemical testing kits (Hach Lange GmbH, Düsseldorf, Germany). TSS was analyzed according to standard methods [24]. For the online measurement of dissolved N_2O, an additional Clark-type sensor (Unisense Environment A/S, Aarhus, Denmark) was installed in the bulk phase of the various reactors.

3. Results and Discussion

3.1. Reactor Performance

The performance results of the two two-stage deammonification systems operated with suspended sludge as SBR and as a MBBR with biofilm carriers in the second stage are summarized in Table 1.

While these two studies with the SBR and MBBR in the second stage were conducted sequentially, the ammonium concentration of the process water remained almost constant in 2014 and 2015 and is depicted in Figures S3 and S4 in the Supplementary Materials.

Table 1. Reactor performance of two two-stage deammonification systems with suspended sludge (SBR) and biofilm carriers (MBBR) in the second stage.

Parameter	Unit	Nitritation	SBR	Nitritation	MBBR
		(2014)		(2015)	
HRT [1]	[day]	~1.3	2.3 ± 1.1	~1.3	2.2 ± 0.5
Temperature	[°C]	31.0 ± 0.3	31.2 ± 0.7	32.4 ± 0.4	32.0 ± 0.9
pH	-	Invalid [2]	7.84 ± 0.15	Invalid [2]	7.60 ± 0.26
DO	[mg/L]	Invalid [2]	0.10 ± 0.02	Invalid [2]	1.5 ± 1.3
TSS	[g/L]	0.2 ± 0.1	2.2 ± 0.7	n/a	n/a
NO_2-N/NH_4-N [3]	-	0.32 ± 0.15	0.32 ± 0.11	0.25 ± 0.14	0.23 ± 0.12
NH_4-N conversion rate	[g N/(m^3·day)]	447.5 ± 154.9	448.9 ± 134.4 332.5 ± 109.6 [4]	234.3 ± 82.7	445.8 ± 126.2 388.5 ± 150.8 [4]
N_{total} conversion rate	[g N/(m^3·day)]	95.9 ± 135.8	598.2 ± 200.8	12.8 ± 43.6	501.2 ± 100.0
N_{total} effluent	[mg N/L]	1321 ± 118	142 ± 33	1327 ± 61	234 ± 66
Degradation [5]	[%]	31.6 ± 6.5	90.1 ± 2.6	23.4 ± 9.2	81.9 ± 5.4

Notes: [1] Hydraulic retention time; [2] Measured pH and dissolved oxygen (DO) concentrations were invalid due to a calcium layer constantly present on the sensors; [3] In the nitritation effluent and in the second-stage influent; [4] Calculated as single-stage deammonification process; [5] NH_4-N oxidation for nitritation and N_{total} degradation for the second stage.

3.1.1. Performance of Nitritation Reactor

As the first step of the deammonification process, nitritation provides the substrate of nitrite and ammonium necessary for the anoxic ammonium oxidation in the second stage [1]. Although the average temperature for the nitritation in 2014 was lower than that in 2015, a higher NO_2-N/NH_4-N ratio and ammonium degradation were achieved in 2014. Specifically, the effluent of the nitritation reactor was composed of 955 ± 91 mg NH_4-N/L and 291 ± 117 mg NO_2-N/L in 2014 and 1028 ± 107 mg NH_4-N/L and 242 ± 112 mg NO_2-N/L in 2015. These results reveal that the performance of the partial nitritation was moderate and well below other nitritation processes reported with a NO_2-N/NH_4-N ratio up to one [4]. The development of the nitrogen concentrations over the study period is displayed in Figures S3 and S4 in the Supplementary Materials. The hydraulic retention time (HRT) remained stable at approximately 1.3 days and thus could not be held responsible for the enhanced conversion of ammonium to nitrite in 2014 even though prolonging the HRT has been shown to positively influence nitritation [25]. Although a positive correlation between TSS and nitrite concentrations would have been expected, this impact could not be conclusively clarified due to missing TSS values for 2015. However, the different aeration systems installed in the nitritation reactor might have had an influence on the NO_2-N/NH_4-N ratio. The installation of new aeration systems increased the performance of nitritation immediately after installation (Figures S3 and S4); however, the turnover rates decreased over time. This change could be due to calcification of the aeration membranes, which resulted in larger air bubbles and thereby decreased the oxygen transfer into the water. Recurrent acidification of the membranes could mitigate this effect and improve their lifespan. Although the oxygen transfer coefficients of all aeration systems given by the manufacturers were in a comparable range of ~25 g O_2/(m_n^3·m_{depth}), the higher values observed in practice and a triple replacement of the aeration systems in 2014 most likely positively influenced the nitritation, leading to higher nitrite effluent concentrations than in 2015. The nitrate effluent concentration was low for both systems over the entire study period (77 ± 30 mg NO_3-N/L in 2014 and 60 ± 27 mg NO_3-N/L in 2015), suggesting adequate conditions for the suppression of NOBs. However, this also indicates that the aeration intensity and the HRT should be prolonged to potentially improve the nitritation performance.

Although nitritation cannot remove nitrogen, positive values for the conversion rate of total nitrogen (N_{total}) could be observed, and these values were higher in 2014 than in 2015. Additionally, the nitritation effluent had a higher NO_2-N/NH_4-N ratio than the second-stage influent. These findings might generally be explained by the production and emission of N_2O and ammonia gas due

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to a shift of ammonium ion to ammonia gas with increasing pH. However, this correlation could not be conclusively proved because of the lack of measurements of these gases and inadequate pH measurements. Nonetheless, the formation of N_2O in solution could be verified using an online sensor.

3.1.2. SBR Performance

For the second stage, a total nitrogen conversion rate of 16 mg N/(L·h) or 384 g N/(m^3·day) was set as a minimum by the WWTP operator. Both the suspended sludge and the biofilm carrier systems could fulfill this requirement on average. The SBR almost constantly exceeded this value after a short startup phase (Figure 2). Additionally, a positive correlation between the development of the nitrogen conversion rate and the nitrite influent concentration is apparent. However, as per the reaction stoichiometry, 1 mol of ammonium and 1.32 mol of nitrite are necessary for a complete anammox process [1]. This condition was not fulfilled using a NO2-N/NH4-N influent ratio of 0.32 ± 0.11, but the average nitrogen degradation was still 90.1% ± 2.6%. With the aeration phases being longer than the non-aerated phases starting after approx. 25 days in the second stage, SBR was assumed to simultaneously perform a partial nitritation during the oxic periods, besides the anammox process. To confirm this hypothesis, the required single-stage ammonium conversion rate performed by the second stage was calculated to achieve total nitrogen conversion after the consumption of inflowing nitrite (Table 1). On average, 74% of the entire ammonium conversion of the SBR and 87% of the MBBR could be accounted for by this process. These results suggest that the second stage partly acted as a single-stage deammonification process with an increasing trend over the study period despite its original function as an anammox reactor without aeration. This is also the reason for the high performance at the end of 2014 despite the fact that the nitrite influent concentrations had been rather low (Figure 2). Beyond these calculations, high AOB and low NOB activities of the suspended sludge with an ex-situ conversion rate of 330 g N/(kg VSS·day) and 72 g N/(kg VSS·day), respectively, could be experimentally proven in controlled lab-scale respiratory batch tests (Table S1).

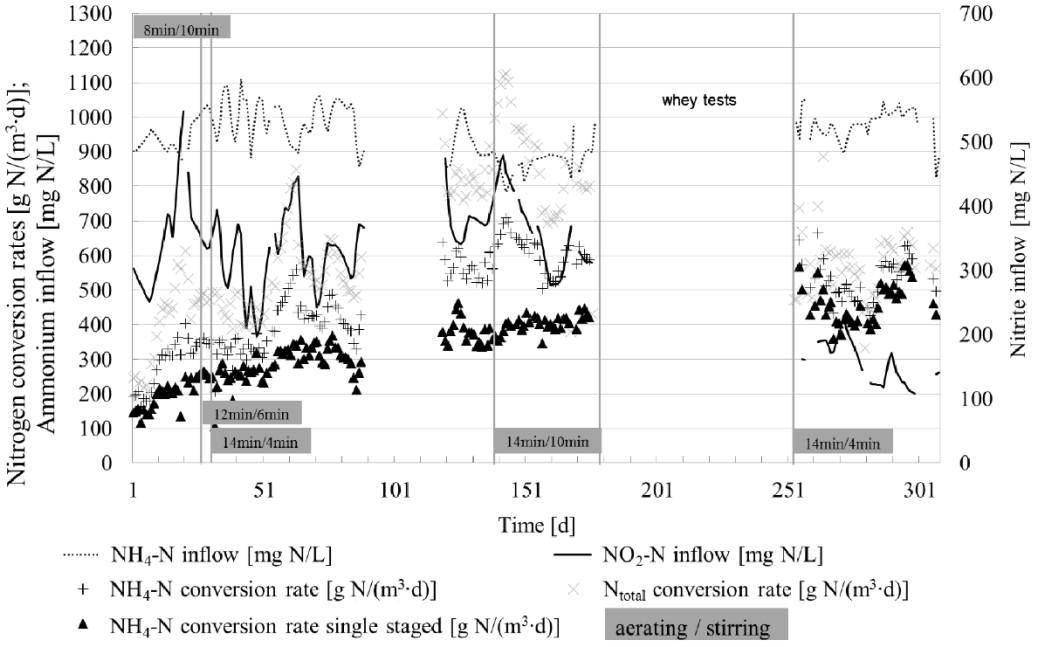

Figure 2. SBR conversion rates related to inflowing ammonium and nitrite concentrations (shaded boxes indicate duration of aerated/stirred phases).

Additionally, online data suggested an oxidation of ammonium during the aerated phases of SBR operation (Figures 3 and 6). This correlation is depicted by the decline of the ammonium-signal not

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only during unaerated phases, when the anammox process took place, but also during the aeration. The pH decline in Figure 6 during aeration is likely caused by nitritation. These findings are yet another piece of evidence that the second stage partly acted as a single-stage deammonification reactor.

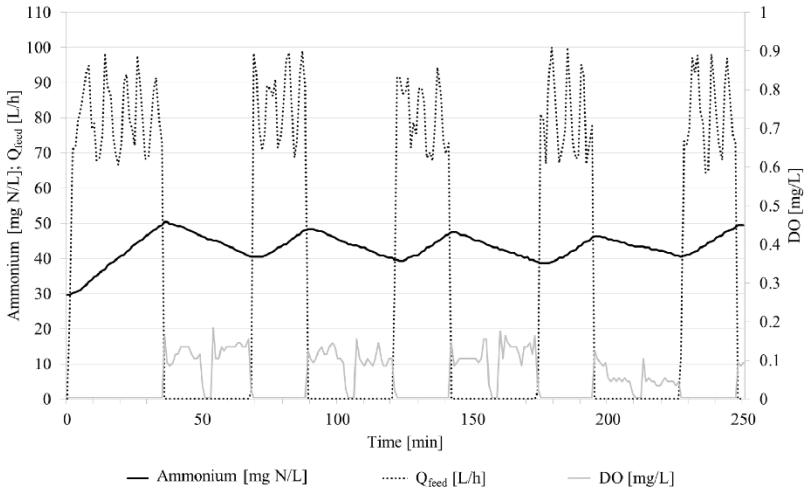

Figure 3. Online data for the second stage of the deammonification process by suspended sludge with intermittent feeding (Q_{feed}) and aeration.

3.1.3. MBBR Performance

After a startup phase lasting several months to achieve suitable biofilm thickness on the carriers, the MBBR was also able to perform above the demanded conversion threshold of 384 g N/(m^3·day) at almost all times in 2015 (Figure 4). Higher nitrite concentrations in the effluent of the MBBR than in the influent were responsible for the higher ammonium conversion rates in comparison to the overall nitrogen degradation in April 2015. This finding implies that the biofilm was likewise able to perform partial nitritation besides the anammox process due to the extensive aeration. Nitrite accumulation indicates insufficient anoxic phases even in the deep layers of the biofilm, as also indicated by the average DO concentration of 1.5 ± 1.3 mg/L in the bulk phase. The ammonium conversion rate was comparable for both the suspended sludge and the biofilm carriers. However, the calculated single-stage ammonium conversion rate for the MBBR exceeded that of the SBR and could therefore moderately compensate for the lower NO_2-N/NH_4-N inflow ratio. Still, the SBR performed better in terms of overall conversion rate and nitrogen degradation, presumably due to the higher nitrite influent concentration and longer HRT, respectively. Finally, the Kempten WWTP decided to implement a full-scale two-stage deammonification facility with suspended sludge due to its high performance and shorter startup phase of several weeks using inoculum from other deammonification plants in contrast to the time-consuming biofilm formation within a MBBR [26].

Interestingly, AnAOBs seemed to accumulate preferably at locations with a calcified layer. Thus, the high concentrations of calcium carbonate present in process water were hypothesized to be favorable for biofilm formation, possibly serving as a substrate or a primary, rough surface for enhanced biofilm attachment. This biological or physical correlation could not be proven, as it was beyond the scope of this study. However, the artificial calcification of carriers prior to inoculation is hypothesized to accelerate biofilm growth and could be an interesting topic for further research. These assumptions are supported by a previous study that has investigated a positive correlation between calcium concentrations and biofilm thickness and density and therefore, an enhancement of biofilm accumulation [27].

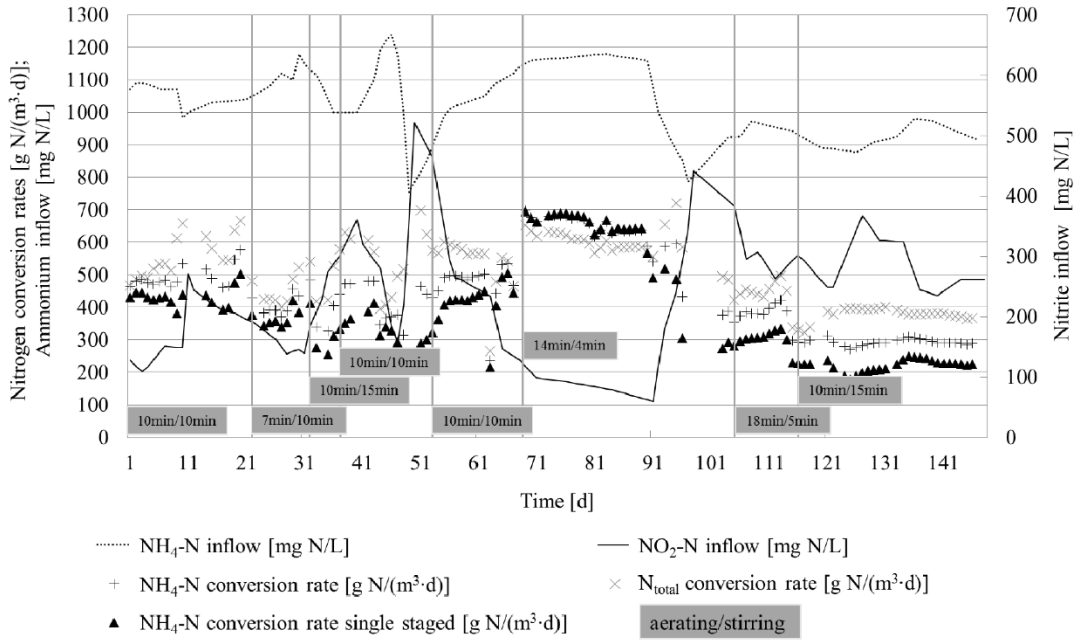

Figure 4. MBBR conversion rates related to inflowing ammonium and nitrite concentrations (shaded boxes indicate duration of aerated/stirred phases).

3.1.4. Operational Assessment

The results demonstrate that the observed degradation rates would not have been possible without the uncommon aeration in the second stage causing an additional single-stage deammonification process in this stage that was originally designed as an anammox reactor. Thus, a well-balanced mixture of ammonium and nitrite in a molar ratio as close as possible to 1:1.32 in the nitritation effluent is essential for the implementation of a second stage as a pure anammox process without aeration. Therefore, the dimensions as well as the physical parameters regarding aeration intensity, oxygen transfer coefficients, HRT, temperature, and pH of the nitritation reactor must be properly designed to stimulate the activity of AOBs while suppressing the growth of NOBs. In this way, appropriate conditions within both reactors of the two-stage deammonification process can be guaranteed, enabling the development of two distinct microbial communities in each reactor and permanently preventing the growth of undesired microorganisms. This advantage is not attained when the second stage must partly act as a single-stage deammonification process to achieve high degradation rates for the overall process. Although AnAOBs recover from oxic phases [10,11], a process adjustment with fully anoxic conditions in the second stage of the deammonification process is assumed to favor the anammox process and thus further increase and stabilize the plant performance.

In general, the deammonification process with suspended sludge is advantageous to a biofilm-based system with respect to the duration of the startup phase, as the biofilm growth process is time-consuming if pre-colonized biofilm carriers from an already established system are not available. Moreover, no additional investment for carriers is needed. Apart from that, the operator of the deammonification plant reported a lower workload for the MBBR as a positive aspect. With microorganisms being fixed in the biofilm, wash-out of the bacteria is easily prevented. This is especially crucial for slow-growing AnAOBs [1]. Their predominant clustering in biofilms [23] is advantageous for their stable abundance in the system, while poor settling of the suspended sludge is often the reason for loss of biomass. Additionally, no settling phase is needed for biofilm carriers, elongating the reaction phase in the MBBR. In the case of an unintended transfer of microorganisms from the first stage into the second stage or the growth of undesired microorganisms present as flocs in the suspension, such as NOBs, due to intensive aeration, the fluid matrix could be easily discarded and

replaced in MBBRs. This would enable a fast startup of the second stage even after such an incident. As a disadvantage, completely overgrown biofilm carriers exhibit a lower specific surface area, thus decreasing diffusive processes, which can decrease the turnover rates. However, thick biofilm rather provides permanent anoxic conditions for AnAOBs, even during aeration, which is why numerous geometries of biofilm carriers with different specific surface areas are used for various applications [28]. Considering the several advantages and disadvantages of the reactor systems with either utilizing suspended sludge or immobilized biofilms, the optimal decision is generally plant-specific.

3.2. Nitrous Oxide Formation

3.2.1. Measurements and Possible Production Pathways

The dissolved N_2O concentrations were measured online in the nitritation reactor, buffer tank, SBR, and MBBR to identify their potential for N_2O formation related to their specific operational conditions. The results are summarized in Figure 5, and further information regarding the development of the dissolved N_2O concentrations in the reactors over the entire monitoring period is provided in Figures S5–S8 in the Supplementary Materials. As the MBBR has been operated as a single-stage deammonification process when the monitoring campaign for dissolved N_2O was conducted, the results are hardly directly comparable with the SBR performing as the second stage of the deammonification process. However, results are also discussed and presented in Figure 5 for completeness.

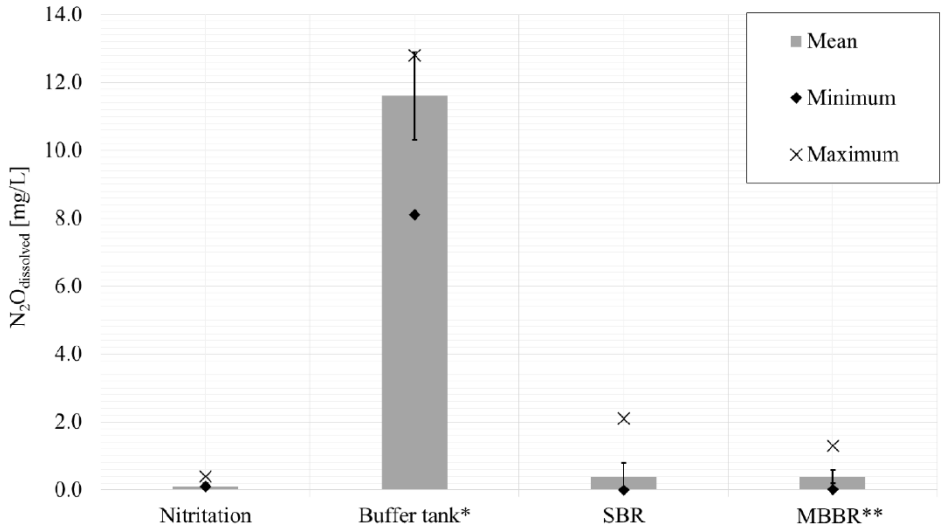

Figure 5. Mean, minimal, and maximal dissolved N_2O concentrations including standard deviations as error bars in the nitritation, buffer tank, SBR, and MBBR (* N_2O concentrations measured in the buffer tank exceeded the detection limit of the sensor many times, likely resulting in an underestimation of the mean and maximal N_2O concentrations reported (n = 3980); ** Dissolved N_2O concentrations in the MBBR operated as single-stage deammonification).

The average dissolved N_2O concentration of the nitritation tank (0.1 ± 0.1 mg/L; translates to 0.02% ± 0.01% relative to NH_4-$N_{oxidized}$ as the total N_2O emission potential in theory; however, this does not necessarily mean that it was emitted entirely) was lower than that of the other two reactors. Although nitrite serving as a substrate for (nitrifier) denitrification was constantly available, continuous aeration at the supplied aeration intensity seemed to produce a DO concentration that was sufficiently high to avoid conditions favorable for significant N_2O production. In comparative point measurements with a thoroughly decalcified sensor, the DO concentration reached 3 mg/L, which prohibited transient oxygen conditions that would have favored N_2O production via nitrifier denitrification. The measured

dissolved N_2O concentrations are in good agreement with the observations by Gabarró et al. [29], who reported comparable concentrations during the aerated phases of nitritation; however, they also observed that anoxic phases promoted N_2O production. Therefore, continuous aeration is assumed to be advantageous over intermittent aeration during nitritation with respect to the mitigation of N_2O production. Nevertheless, aeration results in stripping of N_2O, which directly influences the dissolved N_2O concentration. Additionally, the measured dissolved N_2O concentrations were constantly above the concentrations that would naturally be present when being in equilibrium with the atmospheric N_2O concentration. Thus, constant diffusion and stripping of N_2O into the atmosphere is expected to occur during this nitritation step, negatively influencing the carbon footprint of the overall plant. However, without simultaneous off-gas measurements, an evaluation regarding the effect of diffusion and stripping leading to N_2O emissions and therefore a decrease of the dissolved N_2O concentration could not be verified.

The buffer tank exhibited the highest N_2O concentrations of all reactors, due to a high N_2O formation. However, the mean and maximal values represent an underestimation of the real concentrations, as the detection limit of the sensor (12.8 mg/L) was exceeded many times. The high production rates of N_2O in the buffer tank could be identified to be partially associated with nitrifier denitrification by AOBs, as no aeration was supplied to the buffer tank, whereas microorganisms and effluent from the nitritation tank were provided constantly. Transient changes in the DO concentration resulting in anoxic conditions combined with high concentrations of nitrite and ammonium serving as electron acceptor had been previously reported to favor this formation pathway [15]. Additionally, incomplete heterotrophic denitrification is also believed of being a potential source for the production of N_2O in the buffer tank due to carbon limiting conditions [15] or electron competition among the reduction steps of denitrification [30]. To the best of our knowledge, the N_2O concentrations have never been monitored before in an intermediate buffer tank of a two-stage system; nonetheless, the predominant conditions could be compared to those during the settling phase of a nitritation reactor. During the settling phase, an accumulation of dissolved N_2O was observed [29,31], but at a concentration several magnitudes lower as observed in this study. A significantly longer reaction phase for the metabolic N_2O production pathway in the buffer tank with an HRT of approximately one day and an infinite SRT was assumed to be responsible for the higher accumulation of N_2O.

As the SBR was intermittently fed with the effluent of the buffer tank, a transfer of dissolved N_2O into the second stage was observed, leading to average N_2O concentrations of 0.4 ± 0.4 mg/L (translates to $0.01\% \pm 0.01\%$ relative to $N_{removed}$ as the theoretical N_2O emission potential). This correlation is depicted in detail for one cycle of SBR in Figure 6. With the influent (Q_{feed}) coming from the buffer tank, the N_2O concentration increased to its maximum in the SBR, while the N_2O concentrations decreased slightly during the first aeration cycle of every batch interval. This is most probably due to the stripping rate of N_2O into the atmosphere exceeding the N_2O formation rate. During the stirred, non-aerated phases, a steep decline of N_2O concentrations was detectable, which could be attributed to denitrifying bacteria reducing N_2O to molecular nitrogen. In comparison to the buffer tank, a higher decay of biomass due to a higher TSS concentration is supposed to have provided an additional, readily degradable carbon source enabling denitrification. In the second aeration per interval, the N_2O concentrations increased again in contrast to the first aeration. As AnAOBs are not known to produce N_2O [21], this N_2O formation is expected to result from the activity of AOBs, which were abundant in the second stage, as indicated by the previous observations of a partial nitritation. This increase in N_2O during the second aeration interval of the SBR batch is assumed to be caused by the production rate of N_2O due to both nitrifier denitrification activity as well as inhibition of the nitrous oxide reductase of denitrifiers with the transition from anoxic to oxic conditions, being higher than the stripping rate. As this second stage of the deammonification process served not only as a pure anammox reaction but also as nitritation and was additionally fed with dissolved N_2O from the buffer tank, these results can hardly be compared to other studies due to the differing conditions.

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Nevertheless, the observed consumption of N_2O during unaerated phases attributed to heterotrophic denitrification is supported by previous investigations [32].

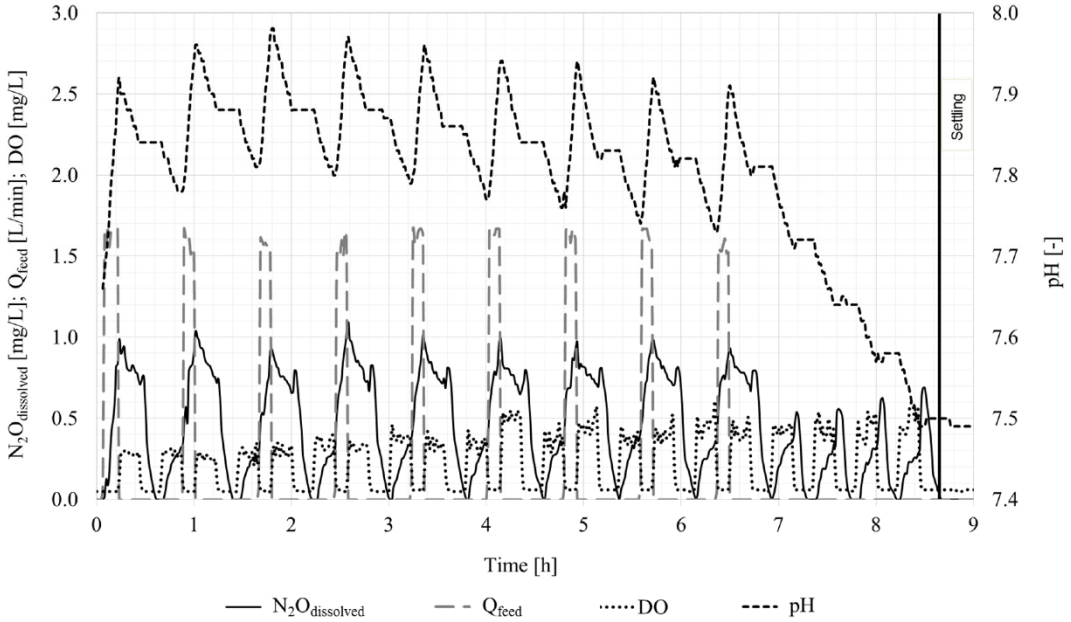

Figure 6. Development of dissolved N_2O, dissolved oxygen, and pH concentrations in SBR for one cycle (18 July 2015).

Even though a direct comparison of the SBR operated as the second stage of the deammonification and the MBBR operated as single-stage deammonification regarding the dissolved N_2O concentrations is difficult, certain similarities due to the supplementary aeration causing additional single-stage deammonification in the SBR are obvious. Considering the fact that the SBR was fed with high concentrations of dissolved N_2O from the buffer tank, it is not surprising that the maximal dissolved N_2O concentrations exceeded those of the MBBR, also causing a slightly higher average concentration and standard deviation. In addition, dissolved N_2O concentrations exhibited a comparable range, which is why neither the MBBR with biofilm carriers nor the SBR with suspended sludge seemed to have a clear advantage regarding a lower N_2O formation.

3.2.2. Mitigation Strategies

Considering the highly deleterious impact of N_2O on the environment, establishing efficient strategies to decrease biological N_2O formation and potential N_2O emissions into the atmosphere are crucial.

The buffer tank had a high N_2O formation potential, thus conditions should be adapted in such a way that N_2O formation is limited, restricting its diffusion into the atmosphere and transfer into subsequent reactors. Hence, one potential countermeasure is the use of a decreased HRT in the buffer tank to shorten the reaction time for both the proposed nitrifier denitrification and the incomplete heterotrophic denitrification pathway. Additionally, frequent removal of the biomass in the buffer tank is recommended to keep the abundance as low as possible. This could be implemented easily by an inlet and outlet installed close to the surface of the reactor enabling a gentle influent and effluent and therefore, a simultaneous sedimentation and withdrawal of the biomass at the bottom of the tank. Keeping the abundance of biomass as low as possible would be advantageous in various ways: the microbiologically produced dissolved N_2O could be decreased in this stage, leading to a lower diffusion into the atmosphere and a mitigated transfer of dissolved N_2O into the subsequent stage where it could potentially be emitted, e.g., due to aeration. Thus, this approach would definitely reduce

the carbon footprint of the whole deammonification plant. With this removed biomass being further processed in the digester under anaerobic conditions, dissolved N_2O would be reduced to ammonium, thus N_2O emission from this system is rather unlikely. However, even if N_2O were to be emitted, it would be captured in the gastight digester and incinerated together with the produced biogas.

Generally, an incineration of N_2O does not only render it to non-hazardous N_2, thus being no longer harmful to the environment, but it also increases the energy yield by 37% in comparison to combustion with oxygen [33]. Such an approach could be transferred to any deammonification plant or—in a broader context—to any reactor of biological wastewater treatment plant emitting N_2O. Deammonification reactors are often already covered to minimize temperature losses and therefore could be easily modified as gastight reactors. This amendment would enable capturing the total off-gas and its use as a supplement for ambient air when incinerating the biogas. Even though this is an end-of-pipe solution, it would eliminate safely any uncontrolled N_2O emission from deammonification processes.

For unaerated reactors, the N_2O emission is only driven by diffusion at the liquid–gas interface due to the gradient between the dissolved and gaseous concentrations, which was the case for the buffer tank. However, this advantage does not apply for aerated reactors, such as the nitritation step and the SBR, because the aeration increases the flux of N_2O into the atmosphere due to stripping effects. If anoxic phases proved to enable the reduction of dissolved N_2O concentrations, which was the case for the SBR, it is recommended to not interrupt these stages by aeration if possible to reduce these stripping effects. Thus, a rearrangement of the aeration strategy of SBR could mitigate N_2O emissions. After the intermittent feeding, which increased the dissolved N_2O concentration, an initial anoxic mixing phase instead of an initial aeration phase after feeding is highly recommended to enable the reduction of N_2O during anoxic phases and thereby decrease the possibility of stripping during subsequent aeration.

4. Conclusions

- A high nitritation performance in the first stage providing an effluent ratio of as close as 1 mol of ammonium to 1.32 mol of nitrite is crucial for a fully anoxic ammonium oxidation in the second stage and high overall degradation rates. If this ratio cannot be achieved, a prolonged HRT, elevation of the temperature above 30 °C, and increased aeration intensity are suggested to improve the nitritation under the countermeasures necessary to suppress NOB growth.
- Although the nitritation effluent did not meet the required ammonium-to-nitrite ratio during the study periods, both the SBR and MBBR in the second stage were able to achieve nitrogen turnover rates and degradation rates above 500 g N/(m^3·day) and 80%, respectively. This was only possible because both second stages acted partially as single-stage deammonification processes due to supplementary aeration, which is highly uncommon for the second stage of the deammonification process.
- Comparing MBBR and SBR performances, the latter achieved higher nitrogen removal and degradation, attributed to the higher inflow NO_2-N/NH_4-N ratio and longer HRT in the SBR, respectively. In contrast, the calculated single-stage ammonium conversion rate of the MBBR was found to be higher, enabling moderate compensation of the smaller nitrite inflow concentrations.
- The intermediate buffer tank exhibited the highest dissolved N_2O concentrations of all reactors. Prevalent anoxic conditions, high nitrite and ammonium concentrations, an HRT of approximately one day, and abundant biomass introduced from the nitritation reactor into the non-mixed and non-aerated buffer tank with an infinite SRT has been identified to provide favourable conditions for N_2O production.
- Nitritation with continuous aeration and the SBR in the second stage had lower dissolved N_2O concentrations in comparison to the buffer tank. A transfer of dissolved N_2O from the buffer tank into the SBR could be observed. The nitritation step and the SBR contributed to the emission of

N_2O due to the aeration; however, the specific amount could not be quantified due to missing off-gas measurements.

- N_2O mitigation strategies were identified, including the use of a decreased HRT in the buffer tank to shorten the reaction time for N_2O production and frequent removal of settled biomass. In the second stage, stripping effects could be limited by a rearrangement of the aeration phases with anoxic periods after feeding the SBR to allow for a reduction of N_2O or implementation of a complete anammox process without aeration.
- To avoid uncontrolled N_2O emissions from deammonification processes, the total off-gas could be collected by use of gastight reactors and then be applied as oxidant during the combustion of biogas, representing an end-of-pipe solution. Even if the proportion of N_2O to the total amount of the off-gas would be low, this approach would not only ensure an environmentally friendly solution as the N_2O is converted to inert N_2 when incinerated, but would also make use of the 37% higher energy yield of the oxidant N_2O in comparison to oxygen.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4441/8/12/578/s1, Figure S1: Two-stage deammonification pilot plant in Kempten (Allgäu), Figure S2: Pictures of aeration elements, Figure S3: Performance of nitritation in combination with downstream SBR in 2014, Figure S4: Performance of nitritation in combination with downstream MBBR in 2015, Figure S5: Dissolved nitrous oxide concentration in the nitritation, Figure S6: Dissolved nitrous oxide concentration in the buffer tank, Figure S7: Dissolved nitrous oxide, dissolved oxygen, ammonium, and nitrate concentrations in the SBR, Figure S8: Dissolved nitrous oxide, dissolved oxygen, ammonium, and nitrate concentrations in the MBBR operated as single-stage deammonification process, Table S1: Respiratory AOB and NOB activity tests of SBR.

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Author Contributions: Carmen Leix and the WWTP Kempten (Allgäu) conceived and designed the experiments. The team of the WWTP Kempten ran the pilot plant including data acquisition and chemical analysis. Carmen Leix and Rebecca Hartl were responsible for the dissolved N_2O measurements and analyzed all data. Carmen Leix wrote the paper. Jörg E. Drewes and Konrad Koch supervised this study and reviewed the manuscript. The final version was approved by all authors.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, and in the decision to publish the results.

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6 Paper III: Strategies for Enhanced Deammonification Performance and Reduced Nitrous Oxide Emissions [10]

The deammonification performance and associated N_2O emissions are dependent on operational conditions. While previous studies have investigated factors for high performances and low emissions separately, this study investigated them jointly. We hypothesized that *process conditions for maximized nitrogen removal rates and minimized N_2O emissions can be determined for the one-stage deammonification process* (hypothesis III.1). Furthermore, we postulated that *a negative correlation exists between the nitrogen removal rate and the emission of nitrous oxide for the one-stage deammonification process, thus, a simultaneous enhancement of the deammonification performance and a minimization of N_2O emissions is possible* (hypothesis III.2). Beyond that, the contribution of the side-stream deammonification process to the removal of trace organic compounds (TOrCs) was investigated. We hypothesized that *the one-stage deammonification process as side-stream treatment of ammonium-rich wastewater is able to reduce TOrCs* (hypothesis III.3).

To test these hypotheses, we performed a design of experiment (DoE) to develop two models for the prediction and optimization of both the nitrogen removal rate and the N_2O emissions. The choice of influencing factors followed two rules: 1) easily adjustable by WWTP operators and 2) sensitive enough to affect both response variables. Based on these principles, the cycle's initial pH value, the feeding strategy, and the aeration strategy were selected and varied on three levels, leading to a total of 13 different DoE runs. The pH value was varied at pH 7.2, pH 7.5, and pH 7.8, while the feeding and aeration strategy were changed from single feeding to (highly) intermittent feeding and continuous to (highly) intermittent aeration, respectively. Additionally, TOrCs removal was investigated for three selected DoE runs using liquid chromatography with tandem mass spectrometry (LC-MS/MS) after sample concentration by solid-phase extraction (SPE).

Both, the modeled and the experimental data suggested a weak positive correlation between the nitrogen removal rate and the N_2O emissions. Thus, a single optimizing operational set-point for the purpose of simultaneously maximizing the nitrogen removal rate and minimizing the N_2O emissions did not exist under the conditions tested. Therefore, hypothesis III.2 had to be rejected. However, various operational settings meeting a desired compromise between a more or a less efficient and environmentally friendly process could be derived using the models. Moreover, the two developed models enabled to define optimized operational settings for an economic and ecological deammonification process separately leading to the acceptance of hypothesis III.1. Thus, moderate settings of pH 7.46, intermittent feeding, and intermittent aeration were predicted to maximize the nitrogen removal rate, whereas N_2O emissions were predicted to be minimized at pH 7.80, single feeding, and a continuous aeration. Concluding,

[10] Leix *et al.* (2017); Supplementary information is attached in the Appendix.

these models allowed to target a desired combination of the plant's performance and N_2O emission for the development of effective process control strategies. Thus, they can be used as an useful tool for WWTP operators to establish a more efficient and/or environmentally friendly deammonification process.

From a set of 17 TOrCs[11], those with the highest concentrations in the influent (i.e., benzotriazole, carbamazepine, diclofenac, gabapentin, and metoprolol) were selected for the analysis of TOrCs removal by the one-stage side-stream deammonification process. Removal efficiencies of the three selected DoE runs with a fixed pH of 7.5, but different feeding and aeration strategies were all in a comparable range. Carbamazepine, diclofenac, and gabapentin exhibited negligible or low removal, whereas benzotriazole and metoprolol were moderately removed (49±9 % and 35±10 %, respectively). As the concentration of some biodegradable TOrCs could be reduced, hypothesis III.3 could be accepted, at least for some TOrC. TOrC removal efficiencies were compared and discussed in context of other biological nutrient removal processes, namely another side-stream partial nitritation-anammox process, conventional nitrification/denitrification, as well as main-stream partial nitritation-anammox at low temperatures (~ 15°C). With one exception regarding diclofenac removal, results suggested a similar removal of the remaining investigated TOrCs.

[11] As the examination of TOrCS removal was a side project of the dissertation, results are presented and discussed in the supplementary information of Leix *et al.* (2017), which is attached in the Appendix.

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Strategies for enhanced deammonification performance and reduced nitrous oxide emissions

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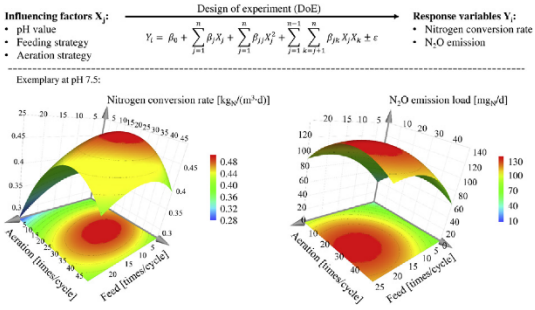
HIGHLIGHTS

- A nitrogen removal & N_2O emission model was developed using a design of experiment.
- Three influencing factors (pH value, feeding and aeration strategy) were studied.
- Nitrogen removal and N_2O emissions exhibited a weak positive correlation.
- Moderate settings (pH 7.46, intermittent aeration) maximized the nitrogen removal.
- Continuous aeration and elevated pH values were advantageous for N_2O mitigation.

GRAPHICAL ABSTRACT



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ABSTRACT

Deammonification's performance and associated nitrous oxide emissions (N_2O) depend on operational conditions. While studies have investigated factors for high performances and low emissions separately, this study investigated optimizing deammonification performance while simultaneously reducing N_2O emissions. Using a design of experiment (DoE) method, two models were developed for the prediction of the nitrogen removal rate and N_2O emissions during single-stage deammonification considering three operational factors (i.e., pH value, feeding and aeration strategy). The emission factor varied between 0.7 ± 0.5% and 4.1 ± 1.2% at different DoE-conditions. The nitrogen removal rate was predicted to be maximized at settings of pH 7.46, intermittent feeding and aeration. Conversely, emissions were predicted to be minimized at the design edges at pH 7.80, single feeding, and continuous aeration. Results suggested a weak positive correlation between the nitrogen removal rate and N_2O emissions, thus, a single optimizing operational set-point for maximized performance and minimized emissions did not exist.

Abbreviations: AOB, aerobic ammonium oxidizing bacteria; anammox, anoxic ammonium oxidation; AnAOB, anoxic ammonium oxidizing bacteria; ANOVA, Analysis of Variance; β, coefficient; BNR, biological nitrogen removal; C_0, center point; COD, chemical oxygen demand; DO, dissolved oxygen concentration; DoE, design of experiment; ε, error of the model; FA, free ammonia; n, amount of influencing factors; MLR, multiple linear regression; NDIR, nondispersive infrared; FNA, free nitrous acid; N_2O, nitrous oxide; NOB, nitrite oxidizing bacteria; Q^2, goodness of the prediction; R^2, coefficient of determination; RSM, response surface methodology; SBR, sequencing batch reactor; SRT, sludge retention time; TOrC, trace organic compounds; TSS, total suspended solids; VSS, volatile suspended solids; WWTP, waste water treatment plant; X_j, influencing factor j; Y_i, response variable i.

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1. Introduction

With increasingly strict legal requirements for effluent concentrations and initiatives for a reduced energy consumption of wastewater treatment systems, the deammonification process comprising a partial nitritation and an anoxic ammonium oxidation (anammox) step by aerobic and anoxic ammonium oxidizing bacteria (AOBs and AnAOBs) holds promise to meet both criteria. Firstly, it is a completely autotrophic nitrogen removal process and the organic carbon compounds required for denitrification during conventional biological nitrogen removal (BNR) processes can instead be used to enhance methane production during digestion (Kartal et al., 2010; Ma et al., 2016). Additionally, the energy consumption can be reduced by more than 50% in comparison to conventional BNR because ammonium is only oxidized partially to nitrite and no further oxidation to nitrate is necessary (Fux et al., 2002). The deammonification process has been successfully applied as side-stream treatment in wastewater treatment plants (WWTPs), whose inflow usually is characterized by high ammonium concentrations and high temperatures (Lackner et al., 2014). Recently, deammonification attracts more interests to be applied as a mainstream treatment for nitrogen removal to replace the conventional BNR process (Cao et al., 2017; Gilbert et al., 2015; Laureni et al., 2016; Ma et al., 2011; Winkler et al., 2012).

For a high deammonification performance, favorable conditions for both sub-processes of partial nitritation-anammox should be provided, such as a low dissolved oxygen concentration (DO) for the suppression of nitrite oxidizing bacteria (NOBs) (Ciudad et al., 2006) and nitrite, which acts as both substrate, but also as an inhibitor for AnAOBs (Lotti et al., 2012). Favoring conditions can be either maintained by altering the process conditions time-dependently (during single-stage deammonification) or spatially (during two-stage deammonification/single-stage deammonification as plug flow reactor or by utilizing prevailing spatial substrate gradients in biofilms, granules, or flocs). However, these factors amongst others were also reported to influence the production of dissolved nitrous oxide (N_2O) potentially being emitted into the atmosphere due to diffusion and stripping induced by aeration (Kampschreur et al., 2009b). Different N_2O production pathways have been reported, including nitrifier denitrification (or also termed 'aerobic nitrous denitritation' as proposed by Weißbach et al. (2017)) and hydroxylamine oxidation by AOBs as well as incomplete heterotrophic denitrification due to inhibition of N_2O reductase (Ding et al., 2016; Law et al., 2012; Rathnayake et al., 2015). In contrast, the metabolic pathway of AnAOBs is not known to be capable of N_2O production (Kartal et al., 2007). N_2O is both an ozone-depleting substance and a strong greenhouse gas with a global warming potential 298 times higher than that of carbon dioxide based on a 100-year time horizon (Intergovernmental Panel on Climate Change, 2013). Therefore, effective strategies for the mitigation of N_2O emissions are desirable to minimize adverse effects on the environment, even though a complete avoidance of N_2O formation will be hardly feasible, as N_2O is not only biologically, but also abiotically produced (Schreiber et al., 2012).

Ideally, high nitrogen removal rates would simultaneously come along with low N_2O emissions to create an incentive for WWTP operators to voluntarily implement N_2O mitigation strategies. However, previous studies regarding autotrophic nitrogen removal processes suggested a positive correlation. Simulation studies mimicking autotrophic nitrogen removal conditions altering DO concentration, granule size, temperature, and the ammonium load revealed that maximal N_2O emissions caused by AOB denitrification correlated with optimum conditions for AnAOBs (Van Hulle et al., 2012). Castro-Barros et al. (2015) also observed an increased N_2O formation and emission with increased ammonium conversion rates in a single-stage granular sludge reactor promoting the partial nitritation-anammox process by changes of aeration intensities. Other factors influencing deammonification performance and its N_2O emissions are conceivable, which is why more studies are needed to elucidate their effect in such systems.

For this reason, the aim of this study was two-pronged: a) to investigate the correlation between the nitrogen removal rate and N_2O emissions for the single-stage deammonification process in side-stream under varying operational conditions and b) to determine the optimal operational settings to achieve a maximized nitrogen removal rate at minimized N_2O emissions. A design of experiment (DoE) approach was adopted to evaluate the direct effects and interactions of the influencing factors simultaneously. This approach also enabled a less intensive, less costly, and more reliable experimental design via a reduced number of predesigned tests, compared with traditional univariate approaches (Ferreira et al., 2007).

The influencing factors chosen in the DoE design followed two rules: 1) easily to be adjusted by WWTP operators and 2) sensitive enough to affect both nitrogen removal rate and N_2O production. Based on these two rules, the cycle's initial pH value, the feeding strategy, and the aeration strategy were closely investigated. The pH value does not only influence the activity of the microorganisms (Jetten et al., 1999; Van Hulle et al., 2007), which perform necessarily as a symbiosis in a single-stage deammonification process, but also the concentration of free ammonia (FA, NH_3) and free nitrous acid (FNA, HNO_2) having an inhibitory effect on microorganisms (Anthonisen et al., 1976; Hellinga et al., 1999; Kim et al., 2012). In addition to influencing the performance of the deammonification process, pH was also reported to affect N_2O production (Kampschreur et al., 2009b; Law et al., 2012; Rathnayake et al., 2015). The feeding strategy, which can be varied from single to continuous feeding, has a direct influence on the ammonium concentration or load, on the pH value, as well as on the FA concentration. It can, therefore, affect both the performance and the N_2O formation due to varying substrate availabilities and changing ammonium concentrations (Domingo-Félez et al., 2014), possible ammonia shock loads (Burgess et al., 2002), and changing microbial activities. The aeration strategy, comprised of the aeration intensity as well as the aeration interval, influences both the DO concentration (including transient changes in anoxic and oxic phases) and stripping effects. Thus, it affects not only the nitrite production and consumption, but also the FNA concentration and the conditions for N_2O production influenced by the nitrite and DO concentration (Kampschreur et al., 2009b, 2008b; Peng et al., 2015). Furthermore, N_2O emissions are enhanced by active aeration (Law et al., 2012). While studies reported in the literature generally agree on an increase of N_2O production upon the presence of nitrite (Foley et al., 2010; Kampschreur et al., 2009b; Kampschreur et al., 2008a,b; Peng et al., 2015; Wunderlin et al., 2012), contradictory observations have been reported regarding the influence of DO on N_2O production, including both negative and positive correlations (Castro-Barros et al., 2015; Kampschreur et al., 2009b; Peng et al., 2014, 2015; Pijuan et al., 2014). Some of these studies were based on one-dimensional investigations or changed only one influencing factor at a time. In contrast, the DoE method has the advantage that a set of influencing factors can be investigated jointly by changing them all at a time creating a more comprehensive and precise picture of operational conditions these systems can experience.

The combined investigation of pH, feeding strategy, and aeration strategy (including also their interactions) influencing both the nitrogen conversion rate and N_2O emission has, to the best of our knowledge, never been reported before. The developed models enable the prediction of both response variables and thus allow to target a desired combination of the plant's performance and N_2O

emission as effective process control strategies. Thus, the models can be used as a helpful tool for WWTP operators to establish a more efficient and/or environmentally friendly deammonification process.

2. Materials and methods

2.1. Experimental setup

All experiments of the DoE were conducted in two identical gas-tight sequencing batch reactors (SBR) with a working volume of 10 L. The reactors were temperature-controlled via a circulation thermostat at 32 °C. A gas meter (Dr.-Ing. Ritter Apparatebau GmbH & Co. KG, Bochum, Germany) connected to the headspace of the reactor was used for the manual control of aeration intensities adjusting a needle valve ahead of initiating the experiments. The gas meter enabled the measurement of the total off-gas volume, which was comprised of the aeration gas and gas production by the process itself, to precisely balance the N_2O emissions of each cycle. The off-gas was collected in a gas bag for subsequent N_2O analysis. Additionally, the reactors were equipped with a stirrer, a peristaltic pump, and *in situ* probes for process monitoring. The reactors were time- and signal-controlled by a supervisory control and data acquisition (SCADA) system. A detailed description of the experimental setup can be found in Leix et al. (2016). Additionally, a flow schematic is provided in the Supplementary information (SI; Fig. SI-1).

2.2. Design of experiment (DoE)

A Box-Behnken design with three influencing factors (X_j) on three levels (1−, 0,+1), namely the cycle's initial pH value (X_1) at the beginning of a cycle, the feeding strategy (X_2), and the aeration strategy (X_3), has been developed to investigate which factors – including also their interactions - had a significant influence on the two response variables (Y_i), such as the nitrogen removal rate (Y_1) and N_2O emission (Y_2). The Box-Behnken design including one center point (C_0) was comprised of 13 DoE, each with a different combination of influencing factors, as shown in Table 1. To avoid a falsification of effect estimations with respect to the influencing factors due to a trend or other undetected changes of results, the order of DoE runs was randomized. Every DoE run was repeatedly studied. The statistical significance of main effects, two-way, and squared interactions of influencing factors was determined with an Analysis of Variance (ANOVA) at a 95% confidence level ($p < 0.05$). Based on these results, two mathematical models were fitted using multiple linear regression (MLR) described by the following quadratic polynomial equation in a n-dimensional design space:

$$Y_i = \beta_0 + \sum_{j=1}^{n} \beta_j X_j + \sum_{j=1}^{n} \beta_{jj} X_j^2 + \sum_{j=1}^{n-1} \sum_{k=j+1}^{n} \beta_{jk} X_j X_k \pm \varepsilon \quad (1)$$

with Y_i being the respective response variable, β_0, β_j, β_{jj}, and β_{jk} the coefficients of the intercept, the linear, squared, and two-way effect, respectively, n the amount of influencing factors (here n = 3), $X_{j/k}$ the respective influencing factor, and ε the error of the model. To improve the quality of the model (Q^2: measure for the goodness of the prediction), the statistically significant influencing factors were included into the model exclusively. Response surface methodology (RSM) was used to determine statistically optimum conditions for both the nitrogen conversion rate (maximized) and the N_2O emission (minimized) independently as well as their combination. Data were analyzed with the software MODDE Pro 11.01 (MKS Data Analytics Solutions, Malmö, Sweden).

2.3. Influencing factors and reactor operation

For the development of applicable mathematical models, the three levels of influencing factors needed to cover realistic conditions for the deammonification process. Following the summarized operational strategies of full-scale partial nitritation-anammox processes as suggested by Lackner et al. (2014), the pH values at the cycle beginning were adjusted to pH 7.2, pH 7.5, and pH 7.8, the feeding strategy from single feeding to intermittent to highly intermittent feeding (i.e., 1, 13, 25 feeding intervals per cycle), and the aeration strategy from continuous to intermittent to highly intermittent aeration (i.e., 1, 25, 49 aeration intervals per cycle) according to the Box-Behnken design (Tables 1, SI-1). The desired initial pH value was adjusted using sodium hydroxide (NaOH) or hydrochloric acid (HCl). Process water was dosed by a time controlled pump either directly at the beginning of the cycle for single feeding or evenly distributed over the entire cycle duration for (highly) intermittent feeding. A constant aeration volume of 120 L for every DoE run was provided, while aeration intensities and aeration intervals were adjusted, again evenly distributed over

Table 1
Box-Behnken design matrix with experimentally observed results regarding the response variables (Y_i) for the nitrogen removal rate and the N_2O emission as well as the respective emission factors (three influencing factors (X_j) were varied on three levels including the cycle's initial pH value: (−1) = pH 7.2, (0) = pH 7.5, (+1) = pH 7.8; Feeding strategy: (−1) = 1 time/cycle, (0) = 13 times/cycle, (+1) = 25 times/cycle; Aeration strategy: (−1) = 1 time/cycle, (0) = 25 times/cycle, (+1) = 49 times/cycle).

Box-Behnken design	Influencing factor (X_j)			Response variable (Y_i)				
Run order	pH value (X_1) [−]	Feeding strategy (X_2) [times/cycle]	Aeration strategy (X_3) [times/cycle]	Nitrogen removal rate (Y_1) [$kg_N/(m^3 \cdot d)$]	n [−]	N_2O emission (Y_2) [mg_N/d]	n [−]	Emission factor [%]
1	+1	0	+1	0.34 ± 0.04	2	107.9 ± 24.1	2	3.3 ± 1.1
2	+1	0	−1	0.24 ± 0.04	4	33.4 ± 2.1	3	1.4 ± 0.2
3	0	+1	−1	0.28 ± 0.02	5	93.9 ± 21.4	6	3.5 ± 0.7
4	−1	−1	0	0.42 ± 0.04	6	66.2 ± 26.1	7	1.5 ± 0.7
5*	0	−1	−1	0.36 ± 0.01	3	50.4 ± 12.6	5	1.6 ± 0.4
6	0	0	0	0.48 ± 0.01	3	125.7 ± 4.4	3	2.6 ± 0.2
7	+1	−1	0	0.34 ± 0.01	3	27.3 ± 15.7	4	0.7 ± 0.5
8	−1	0	+1	0.38 ± 0.02	3	92.8 ± 13.0	3	2.5 ± 0.5
9*	0	−1	+1	0.38 ± 0.01	3	44.6 ± 17.7	3	0.9 ± 0.0
10	−1	0	−1	0.27 ± 0.04	3	64.9 ± 43.2	4	2.7 ± 1.8
11*	0	+1	+1	0.38 ± 0.03	3	108.3 ± 57.8	4	2.5 ± 1.5
12	−1	+1	0	0.37 ± 0.07	3	139.8 ± 59.7	4	4.1 ± 1.2
13	+1	+1	0	0.34 ± 0.03	3	62.5 ± 8.5	3	1.9 ± 0.2
Total				0.35 ± 0.07	44	77.0 ± 42.8	51	2.3 ± 1.3

* Biological removal of trace organic compounds (TOrC) was analyzed for these runs as a side project with results being presented in the SI.

the whole cycle for (highly) intermittent aeration (also see Table SI-2).

Apart from changing the influencing factors for every DoE run, all other conditions were kept constant as far as possible. The cycle duration (including feeding and the reaction phase without settling) and the exchange volume were fixed to six hours and 10%, respectively. During feeding and reaction phases, the reactors were constantly stirred to guarantee homogeneity. An effluent concentration of 60 mg NH_4-N/L was targeted to provide comparable starting conditions after settling and decant phase for every DoE run. Gas bags for the collection of one cycle's off-gas volume were evacuated, flushed with N_2 and evacuated again, before they were connected to the gas meter. Before sealing the reactor gas-tightly and starting a new cycle, the reactor headspace was flushed with ambient air to assure that no N_2O was trapped in the headspace falsifying the results.

2.4. Inoculation

To guarantee comparable starting conditions for every new DoE run with a microbial community not being influenced by protraction of the previous DoE run, every new combination of operational parameters was freshly inoculated with 10 L of suspended sludge originated from a pilot-scale deammonification reactor with a 130 L working volume. This reactor was exclusively operated to provide inoculum of constant quality. As the nitrogen removal rate as well as the nitrogen degradation were relatively stable over the entire experimental period for the model development as well as for the validation of results, the relatively constant composition of the microbial community was assumed during the course of this study (Fig. SI-2).

2.5. Process water

Process water of the WWTP Garching/Munich was used as feed water. To avoid naturally occurring compositional fluctuations of the process water over time, one charge of process water required for both the model calibration and validation of the DoE was stored frozen in canisters at −20 °C. For every new DoE run, process water was gently thawed. The process water during the model calibration was characterized as follows: 1270 mg NH_4-N/L, 1280 mg/L COD, and pH 8.3. The Environment Protection Authority (2000) recommended a maximal storage time of 28 days until analysis. Despite the study duration being longer than this recommended storage time, freezing the process water for keeping its composition as stable as possible to mimic realistic wastewater conditions was still believed to be the best compromise. Nevertheless, the most severe change was observed for the COD concentration until the validation experiments could be conducted. During this time, the process water was characterized by the following water quality: 1245 mg NH_4-N/L, 800 mg/L COD, and pH 8.2.

2.6. Analytical methods

Ammonium, nitrite, nitrate, and COD concentrations were determined using photochemical testing kits (LCK303, LCK341 and LCK342, LCK340, LCK514; Hach Lange GmbH, Düsseldorf, Germany). To prevent a falsification of analyzed nitrate concentrations by disruptive nitrite ions, nitrite was eliminated by adding sulfamic acid (Wu et al., 1997). Total suspended solids (TSS) and volatile suspended solids (VSS) were analyzed according to Standard Methods (APHA, 2012). The gaseous N_2O concentration was analyzed via a gas analyzer using a nondispersive infrared sensor (NDIR) (X-STREAM XEGP, Emerson Process Management GmbH & Co. OHG, St. Louis, USA).

3. Results and discussion

3.1. Nitrogen removal rates and N_2O emissions for model calibration

The 13 runs of experiments following the Box-Behnken design with the different settings of the influencing factors were conducted repeatedly and in randomized order as presented in Table 1, leading to a total of 55 analyzed cycles. Even though both response variables were investigated jointly, some experiments were chosen to be evaluated more often regarding their specific nitrogen removal rate or N_2O emission to reduce fluctuations, thus, the amount of analyzed repetitions differed in some instances. For the development of the two mathematical models, the single results of both response variables were taken into account. Results are shown in Table 1 and also include the N_2O emission factor related to the nitrogen removed. The emission factors observed in this study varied between 0.7 ± 0.5% and 4.1 ± 1.2%. This is well within the range described by Kampschreur et al. (2009b) who reported 0–95% of the nitrogen load being emitted as N_2O in laboratory-scale studies and 0–15% in full-scale studies, respectively. These observations demonstrate that N_2O emission can vary largely between different studies which is why further investigations into N_2O emissions influenced by the process operation are crucial.

3.2. Models and optimal set-points

3.2.1. Model development and maximization of the nitrogen removal rate

The model for the prediction of the nitrogen removal rate was developed by taking into account all significant factors revealed by the ANOVA. Apart from the interaction term of 'pH·Aeration' and 'pH·Feed', all other main effects, two-way, and squared interactions were found to be significant. The absolute value of the effect indicates the degree of the factor's influence on the response variable. It can be seen that the squared effect of the aeration strategy had the highest effect on the nitrogen removal rate, followed by the squared effect of the cycle's initial pH value and so forth (Table 2). In contrast, the feeding strategy (including the main effect and the two-way interaction) had the lowest effect.

The model for the response variable of the nitrogen removal rate (Y_1) with the unit $kg_N/(m^3 \cdot d)$ was estimated according to Table 2 as follows:

$$Y_1 = -50.8659 + 13.7265 \cdot X_1 + 0.0034 \cdot X_2 + 0.0091 \cdot X_3 - 0.9200 \cdot X_1^2 - 0.0003 \cdot X_2^2 - 0.0002 \cdot X_3^2 + 0.0001 \cdot X_2X_3 \pm 0.0324 \quad (2)$$

with X_1 being the influencing factor of the pH value, X_2 representing the feeding strategy, and X_3 the aeration strategy. Further evaluation of the mathematical model for the prediction of the nitrogen removal rate is presented in Fig. SI-4.

Applying this model enabled the generation of 2D-response contour plots visualizing the impact of the three influencing factors on the nitrogen removal rate (Fig. 1). The corresponding 3D-response contour plots can be found in the SI (Fig. SI-5).

The response contour plots revealed that moderate operational settings for all influencing factors investigated (pH value, feeding strategy, and aeration strategy) influenced positively the performance of the deammonifying process. Conversely, settings were neither on the low (−1), nor on the high level (+1) of the Box-Behnken design advantageous for the nitrogen removal rate. Thus, the edges of the influencing factors have been successfully chosen.

The optimum set-point for a maximized nitrogen removal rate within the examined frame of operational settings was calculated

Table 2
Statistical analysis for the response variables nitrogen removal rate (Y_1) and N_2O emission (Y_2) (Aer: Aeration).

	Nitrogen conversion rate (Y_1)			N_2O emission (Y_2)		
	p-Value	Effect	β	p-Value	Effect	β
C_0	$7.9 \cdot 10^{-25}$	–	−50.8659	$2.4 \cdot 10^{-08}$	–	−17363.60
X_1: pH	$1.5 \cdot 10^{-03}$	−0.04	13.7265	$3.3 \cdot 10^{-03}$	−37.32	4700.12
X_2: Feed	$1.1 \cdot 10^{-02}$	−0.03	0.0034	$1.1 \cdot 10^{-05}$	54.08	6.27
X_3: Aer	$4.3 \cdot 10^{-07}$	0.08	0.0091	$7.3 \cdot 10^{-02}$	22.03	2.83
X_1X_1: pH·pH	$5.8 \cdot 10^{-08}$	−0.17	−0.9200	$1.9 \cdot 10^{-02}$	−57.15	−317.48
X_2X_2: Feed·Feed	$5.2 \cdot 10^{-03}$	−0.07	−0.0003	$6.3 \cdot 10^{-02}$	−44.45	−0.15
X_3X_3: Aer·Aer	$2.2 \cdot 10^{-09}$	-0.19	−0.0002	$2.5 \cdot 10^{-02}$	−54.54	−0.05
X_2X_3: Feed·Aer	$3.0 \cdot 10^{-02}$	0.04	0.0001	–	–	–
ε	–	–	±0.0324	–	–	±32.12
		$R^2 = 0.82$	$Q^2 = 0.75$		$R^2 = 0.50$	$Q^2 = 0.34$

by using a simplex algorithm. An extrapolation of results beyond this frame would contain an inestimable uncertainty which is why statements in this direction are not being discussed, also because they would cover rather uncommon operational set-points. A maximized nitrogen removal rate of 0.49 ± 0.03 $kg_N/(m^3 \cdot d)$ was calculated to be achieved at a cycle's initial pH value of 7.46 combined with 30 aeration and 11 feeding intervals per cycle. In a survey by Lackner et al. (2014), volumetric nitrogen loads of eight deammonifying systems comparable to the system tested here have been summarized with a variation from as low as 0.04 $kg_N/(m^3 \cdot d)$ up to 0.65 $kg_N/(m^3 \cdot d)$. Assuming a maximal nitrogen removal of 89% according to the stoichiometry (Strous et al., 1998), minimal and maximal nitrogen removal rates of 0.03 $kg_N/(m^3 \cdot d)$ and 0.58 $kg_N/(m^3 \cdot d)$ could be achieved, respectively. Thus, the maximal predicted nitrogen removal rate of 0.49 ± 0.03 $kg_N/(m^3 \cdot d)$ can be ranked into the upper level of nitrogen removal rates in comparison to other comparable deammonification systems.

Intermittent aeration with evenly distributed aerated (~5.5 min) and unaerated phases (~6.5 min) at a moderate aeration intensity (~44 L/h) seemed to be the most suitable condition for both the partial nitritation and the anammox process. Such an aeration strategy generally creates moderate DO concentrations in comparison to continuous and highly intermittent aeration, which would lead to a permanent low DO concentration or peaks with high DO concentrations, respectively. Mean, minimal, and maximal DO concentrations for the three different aeration strategies are summarized in Fig. SI-3. Comparing different aeration strategies, it has been reported that continuous aeration will lead to a higher performance of the deammonification process (Joss et al., 2009). This could not be confirmed by the model under the precondition of a constant aeration volume over a set cycle duration to render different aeration strategies comparable, but might be applicable to conditions where the aeration intensity is increased. Consequently, the intermittent aeration is not only more cost-effective compared to continuous aeration due to aeration savings observed here, but creates also unaerated lag-phases to suppress the growth of NOBs (Gilbert et al., 2014; Kornaros et al., 2010).

Additionally, operation should be aimed for a pH value of approximately 7.5 to promote a high performing deammonification process as a symbiosis of partial nitritation and anammox. If deviations from this ideal set-point are not avoidable, pH values should be controlled to be rather below than above this target set-point as the nitrogen removal reduction is less pronounced at a lower pH-range (Fig. 1). This observation is in line with favorable pH values observed before for AOBs and AnAOBs (pH 6.5–pH 8.0 for AOBs (Van Hulle et al., 2007) and pH 6.7–pH 8.3 for AnAOBs (Jetten et al., 1999)) as well as with the inhibitory effect of FA, as the FA concentration increases with an increasing pH value (inhibitory effect on AOBs starts at threshold values of 10 mg NH_3-N/L with complete inhibition at 150 mg NH_3-N/L (Anthonisen et al., 1976)). In comparison, no inhibition of AOBs by FNA could be assumed because nitrite did not accumulate during the single-stage deammonification process (inhibitory effect on AOBs at threshold values above 0.2 mg HNO_2-N/L (Hellinga et al., 1999)).

As for the feeding strategy, again a lower loss of the plant's performance can be expected when operated rather below the optimal set-point represented by a moderate intermittent feeding than above. This impact became less conspicuous the further the aeration strategy approached continuous aeration conditions. Nevertheless, a change of the feeding strategy from a single to a highly intermittent feeding was not predicted to severely affect the nitrogen removal rate (Fig. 1).

3.2.2. Model development and mitigation of the N_2O emissions

A model for the prediction of N_2O emissions was developed correspondingly to the model for the estimation of the nitrogen removal rate. The ANOVA revealed the linear and squared influencing factors to be statistically significant, apart from the linear influencing factor 'aeration' and the squared influencing factor 'feed·feed'. However, their removal would have negatively influenced the quality of the model (Q^2), which is why these terms were retained in the statistical model with the squared pH value having the highest effect on the N_2O emissions, followed by the squared aeration strategy, and so forth (Table 2).

The model for the response variable of the N_2O emission (Y_2) with the unit mg_N/d was estimated according to Table 2 as follows:

$$Y_2 = -17363.60 + 4700.12 \cdot X_1 + 6.27 \cdot X_2 + 2.83 \cdot X_3 - 317.48 \cdot X_1^2 - 0.15 \cdot X_2^2 - 0.05 \cdot X_3^2 \pm 32.12 \quad (3)$$

Further evaluations of the mathematical model for the prediction of the N_2O emission are provided in the SI (Fig. SI-6).

This model was applied for the generation of the 2D-response contour plots visualizing the impact of the three influencing factors on the N_2O emission (Fig. 2). The corresponding 3D-response contour plots can be found in the SI (Fig. SI-7).

Based on the model, the following correlations between the three influencing factors and the N_2O emission could be derived, again only being valid within the limits of the investigation area. A continuous aeration was predicted to be more favorable than (highly) intermittent aeration for the purpose of reduced N_2O emissions. With respect to the aeration regime, a previous study by Joss et al. (2009) observed the same correlation, while another study found no difference (Yang et al., 2013). Generally, N_2O formation was believed to be promoted by low DO concentration levels (Kampschreur et al., 2008b, 2009b). Oxygen limiting conditions can occur during continuous rather than (highly) intermittent aeration, thus continuous aeration would be counterproductive in

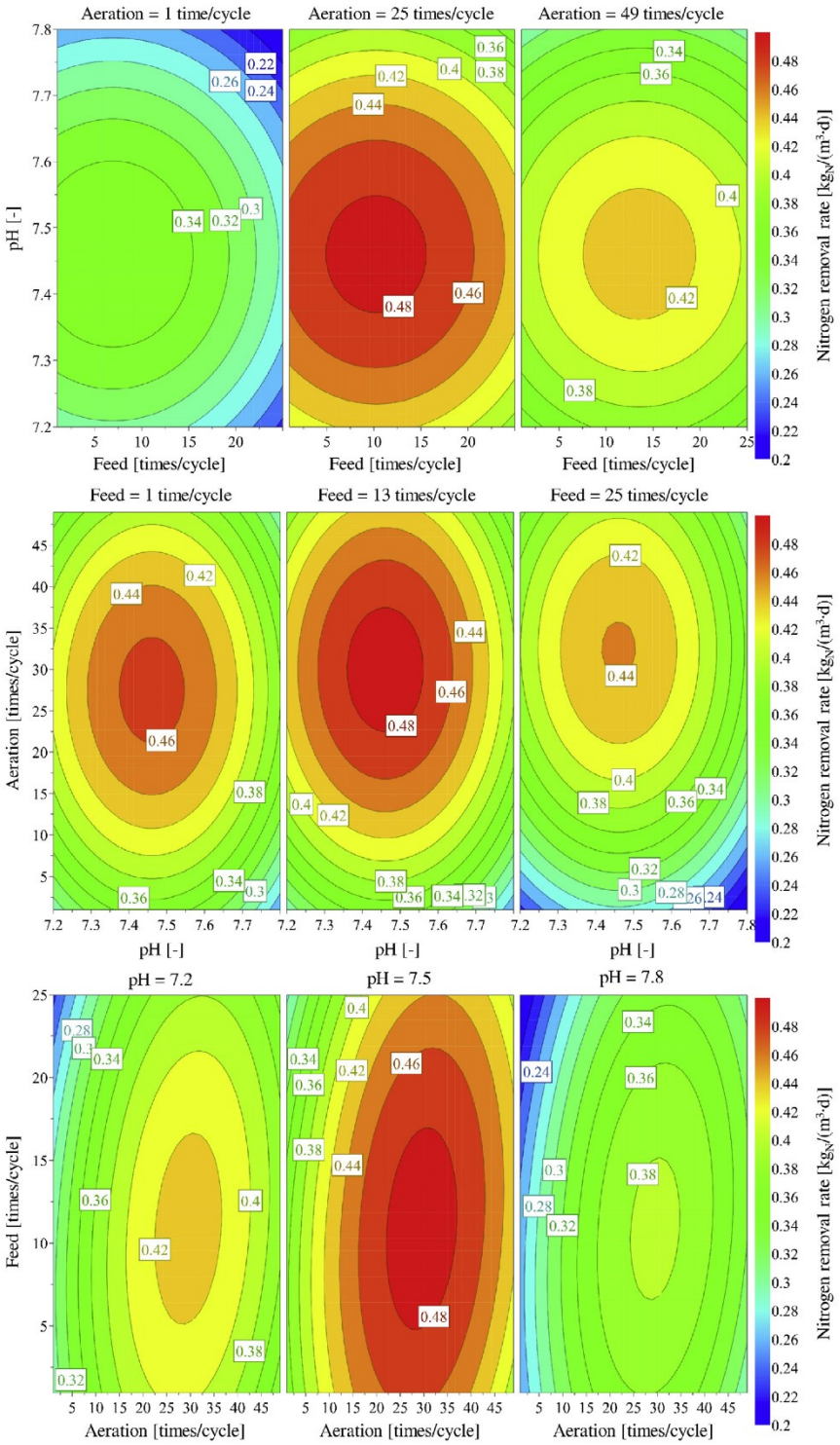

Fig. 1. 2D-response contour plots for the influencing factors pH value, feeding strategy, and aeration strategy on the response variable of nitrogen removal rate (Y_1).

terms of low N_2O emissions relating to this observation. However, also the contrary case of low DO concentrations resulting in decreased N_2O emissions has been reported (Kampschreur et al., 2009a; Peng et al., 2014), which is in agreement with the findings of this study and could be explained by the combined consideration of DO and nitrite concentrations. Nitrite itself was observed

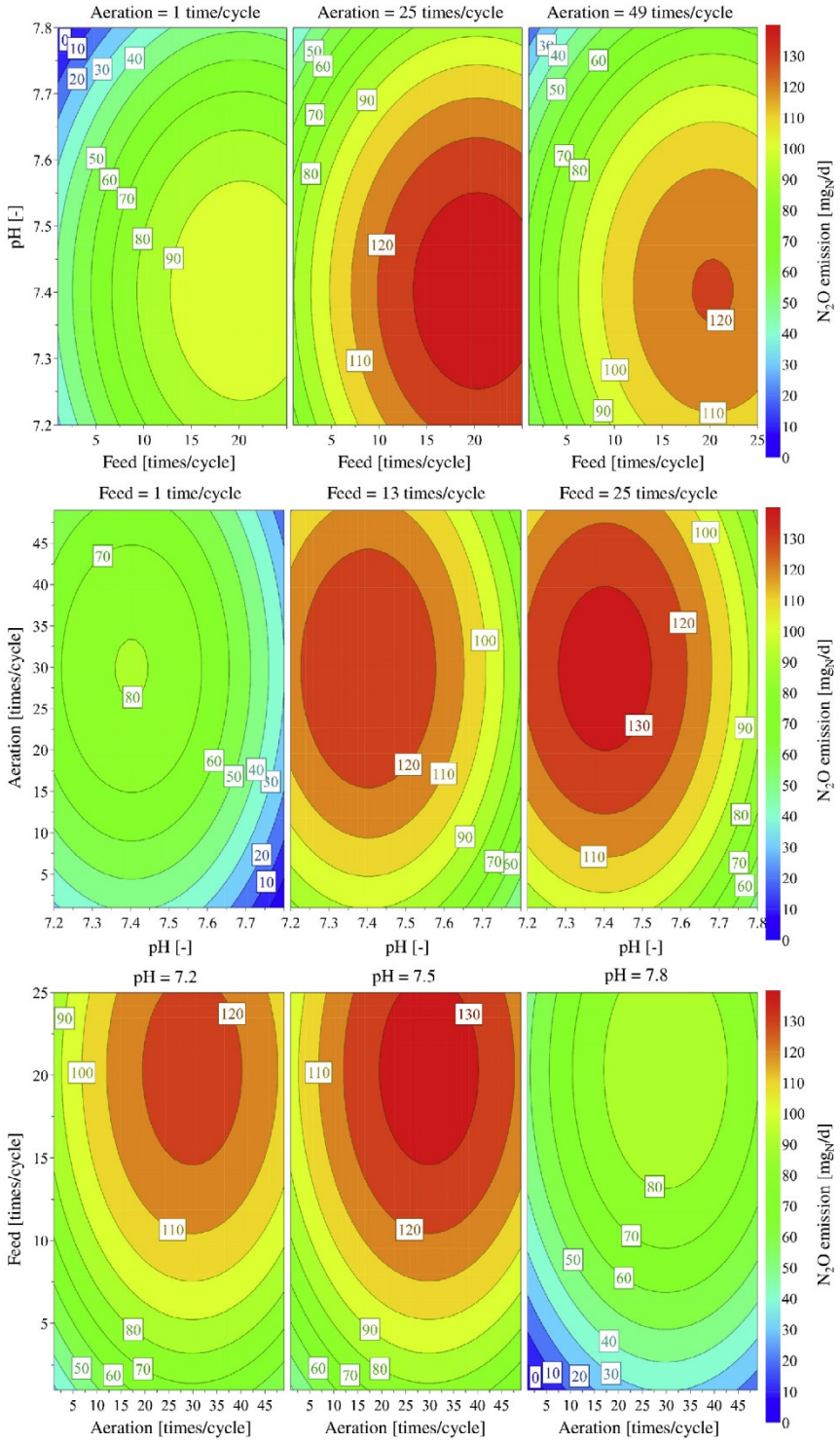

Fig. 2. 2D-response contour plots for the influencing factors pH value, feeding strategy, and aeration strategy on the response variable of N_2O emission (Y_2).

to generally trigger N_2O formation, but Peng et al. (2014, 2015) claimed that low nitrite concentrations potentially are responsible for a reduced stimulative effect on the AOB denitrification pathway, even though this N_2O production pathway is encouraged at lower DO levels. These explanations apply for the investigated system here as well, as continuous aeration causing a low DO

concentration enables an immediate consumption of produced nitrite by the anammox process in deeper layers of microbial aggregates and thus, prohibits an accumulation of nitrite. Additionally, a lower aeration intensity not only decreases the stripping effect, but could have also been hold responsible for a lower N_2O formation rate as observed by Castro-Barros et al. (2015). Furthermore, continuous aeration also prohibits sudden changes in the DO concentration (especially the recovery from anoxic conditions), which were demonstrated to be favorable for the N_2O production (Castro-Barros et al., 2015; Yu et al., 2010). For all these reasons, continuous aeration seemed to be advantageous over (highly) intermittent aeration for mitigating N_2O emissions.

Furthermore, less frequent feeding causing a higher gradient of ammonium concentration was observed to result in lower N_2O emissions. Decreasing ammonium concentrations have been demonstrated before to decrease N_2O peaks as well as N_2O production rates (Domingo-Félez et al., 2014). Therefore, also potential N_2O emissions could be expected to be lower, which would favor the approach of a (highly) intermittent feeding strategy. However, others could not find such an indication (Kampschreur et al., 2009a). Nevertheless, ammonia shock loads were observed to influence the N_2O production rate, yet having reached its maximum at ammonia loads exceeding 1.6 mg NH_3-N/g TSS (Burgess et al., 2002). Considering all possible conditions during the DoE, nearly all settings fulfilled this load threshold (ammonia concentrations were calculated according to Anthonisen et al. (1976)). Thus, N_2O formation was most likely only induced once by single feeding instead of multiple times by (highly) intermittent feeding. Therefore, the amount of produced dissolved N_2O, which is potentially emitted, could be reduced. Furthermore, an infrequent feeding strategy for mitigated N_2O emissions is most likely also related to a further increase of the pH value at the beginning of the cycle if feeding takes place rarely.

With respect to the pH value as the most influencing factor, the lowest N_2O emissions were observed with the highest pH value of pH 7.8 whereby a change from 'level 0' to 'level +1' caused a considerable higher decrease of N_2O emissions than a change to 'level −1' (Fig. 2). These observations are in good agreement with Rathnayake et al. (2015) who found the N_2O emissions to peak at pH 7.5 and to decrease with lower and higher values. However, this study only investigated the partial nitritation system, whereas N_2O emissions of the entire deammonification system were investigated in this study providing more general conclusions. Inhibited AOBs and with it decreased activity of AOB denitrification and reduced production of intermediates being available for N_2O formation due to elevated FA concentrations at higher pH values causing also a decreased nitrogen removal rate could be one explanation for the observations. FNA has been demonstrated to inhibit strongly the N_2O reduction during denitrification (Zhou et al., 2008). FNA built-up was avoided by both an elevated pH value and a continuous aeration strategy enabling for an immediate consumption of produced nitrite. Thus, these conditions are favorable for a simultaneous reduction of dissolved N_2O leading to a decrease of potentially emitted N_2O and are in line with the predictions of the model.

To reduce the N_2O emissions to a minimum, an optimum setpoint was calculated to be at the edges of the operational settings investigated, namely at pH 7.8, a single feeding strategy, and a continuous aeration. Whether a further reduction of the N_2O emissions beyond the edges of the influencing factors investigated would be possible, should be evaluated in future studies, whereby a further increase of the pH value is the only possibility since the feeding and aeration strategy are already at the lowest edge. However, the performance of the deammonification process would most likely further decrease with an additional increase of the pH value (see Fig. 1), which would already drop by 46% to a nitrogen removal rate of 0.24 ± 0.03 $kg_N/(m^3 \cdot d)$ in comparison to the mean value at the proposed optimized operational settings for N_2O reduction.

3.2.3. Practical operation strategies to achieve high nitrogen removal rates and reduced N_2O emissions

Ideally, a maximization of the nitrogen removal rate would come along with a minimization of the N_2O emissions for an economic and an environmentally friendly operation of the deammonification process. However, the results of the DoE tests revealed that such a negative correlation did not exist between those two response variables for single-stage deammonification (Fig. 3). More specifically, only a weak positive correlation was identifiable, which is supported by a low R^2, that is yet in a comparable range for the measured values by the DoE with $R^2 = 0.086$ and the predicted values by the models with $R^2 = 0.107$. These findings

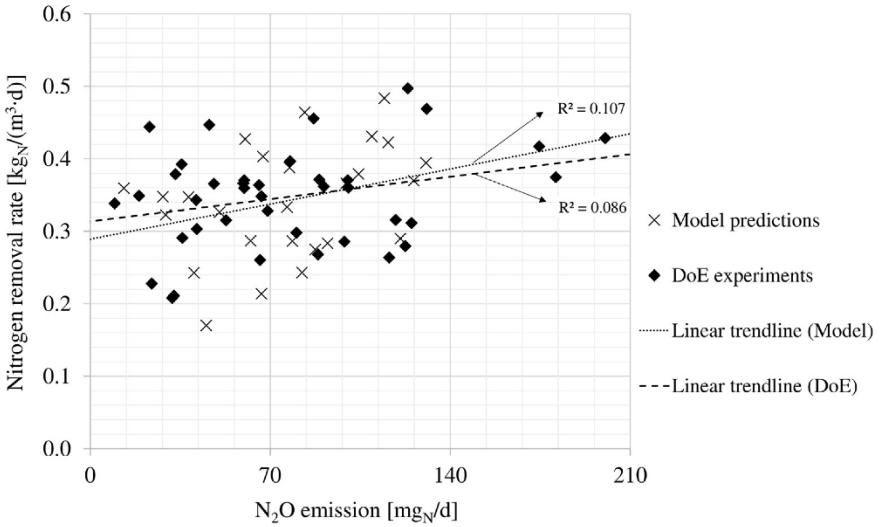

Fig. 3. Correlation between the nitrogen removal rate (Y_1) and the N_2O emission (Y_2) for both the model predictions and the DoE experiments.

Table 3
Validation of both models for the estimation of the nitrogen removal rate and the N_2O emission by comparing predicted vs. experimental results.

Nitrogen removal rate [$kg_N/(m^3 \cdot d)$]			N_2O emission [mg_N/d]		
Prediction	Experiment	Validated* (Y/N)	Prediction	Experiment	Validated* (Y/N)
Low N_2O emission (pH 7.80, fourfold feeding, continuous aeration)					
0.25 ± 0.03	0.24	Y		6.9	Y
	0.25	Y	7.5 ± 32.1	4.6	Y
	0.27	Y		6.7	Y
Moderate nitrogen removal rate and N_2O emission (pH 7.32, single feeding, highly intermittent aeration)					
0.37 ± 0.03	0.37	Y		162.1	N
	0.38	Y	61.1 ± 32.1	139.1	N
	0.36	Y		148.1	N
High nitrogen removal rate (pH 7.46, eleven-fold feeding, intermittent aeration (30 times/cycle))					
0.49 ± 0.03	0.41	N		222.2	N
	0.38	N	123.7 ± 32.1	155.8	Y
	0.42	N		258.9	N

* Test, if experiments for validation met the prediction criterion.

are supported by a comparison of predicted values for all possible combinations of settings (see Table SI-3) proving that a single optimized operational setting did not exist. Consequently, a compromise between the two aims of reducing the N_2O emissions and improving the nitrogen removal rate is needed for an environmentally friendly or efficient operation of deammonification, e.g. at pH 7.7, a single feeding, and a moderate intermittent aeration (here represented by 22 times/cycle). With these settings, the nitrogen removal rate is predicted to be 0.41 ± 0.03 $kg_N/(m^3 \cdot d)$, thus exceeding the mean by 17%. In contrast, the N_2O emissions were estimated to be decreased by 55% in comparison to the mean to 49.6 ± 32 mg_N/d. For an even more environmentally friendly operation, settings at pH 7.8, with single feeding, and fewer aeration intervals of 11 times/cycle were calculated to lead to a more than fivefold decrease of the N_2O emissions if a nitrogen removal reduction of approximately 10% is acceptable. If the focus is on a high nitrogen removal rate, operational settings for instance at pH 7.55, single feeding, and a higher aeration interval of 28 times/cycle would allow for an increase of the mean performance by over 31% while N_2O emissions would still be below the mean value.

3.3. Validation and reliability of the mathematical models

Both models for the prediction of the nitrogen removal rate and the N_2O emission were validated repeatedly and simultaneously at three different set-points for the purpose of low N_2O emissions, of a moderate nitrogen removal rate at moderate N_2O emissions, and of a high nitrogen removal rate. Details regarding the operational settings with their specific model predictions and experimental results for both response variables are listed in Table 3.

Results demonstrated a good agreement between the predicted and experimental values for both response variables at settings for low N_2O emissions. The experimentally evaluated values had a low deviation from the predicted mean, thus were all securely within the prediction range and therefore able to validate both models at this set-point. In contrast, experiments for the validation of the models at settings leading to a high nitrogen removal rate did not meet the prediction criterion with one exception for the N_2O emissions. At settings for a moderate nitrogen removal rate and N_2O emissions, the validation experiments could confirm the predictions for the nitrogen removal rate while results of the N_2O emissions exceeded the prediction range. Thus, two out of three cases could be validated for the nitrogen removal rate model, while only one was confirmed by the N_2O emission model.

From a statistical point of view, this fact can be explained by the quality of the models. The prediction accuracy of both models was limited to some extent, which is reflected by Fig. 4 depicting the correlation between observed and predicted values. The model for the nitrogen removal rate prediction had a coefficient of determination of $R^2 = 0.82$, whereas R^2 of the N_2O emission model was well below with $R^2 = 0.50$. Even though a wide range of emitted N_2O concentrations have been reported before for different WWTPs (Foley et al., 2010; Kampschreur et al., 2009b), N_2O emissions were expected to be comparable within the repetitions of each run of the DoE as the settings of influencing factors were the same and all other conditions were kept stable as far as possible. However, the N_2O emission exhibited rather high fluctuations within the repetitions (Table 1 and Fig. 4), which influenced directly the quality of the model. Thus, the N_2O emission model exhibited a low Q^2 value and therefore can be more characterized as a trend model. Consequently, the model for nitrogen removal rate was expected to have a higher prediction accuracy with 75% of the experimental data being expected to meet the predictions within one standard deviation ($Q^2_{N-removal\ rate} = 0.75$), and therefore being more reliable than the model of N_2O emission with only 34% ($Q^2_{N2O\ emission} = 0.34$), which could be reproduced by the validation experiments (Table 3).

From a practical point of view, different error sources are conceivable, which were attempted conscientiously to be minimized, however, could not be completely excluded. These might have negatively influenced the results of the DoE and thus, the quality and the validation probability of the models. The accuracy of photochemical testing kits and the feeding pumps as well as the precision of the pH value adjustment and of the initial ammonium concentration at the beginning of each cycle are generally limited. Additionally, rapidly changing process conditions have been reported to increase N_2O emissions (Kampschreur et al., 2009b). Even though a fast change in the pH value has not been described in that context, the adjustment of the pH value at the beginning of the cycle might have stressed the microorganisms and therefore, increased the N_2O production potentially dependent on the induced pH gradient.

Although conditions apart from the pH value, the aeration strategy, and the feeding strategy were tried to being kept constant, it cannot be excluded that also other unidentified factors could have influenced the response variables, such as a possible alternation of the sludge retention time (SRT). Due to a mediocre settling behavior of the sludge during the DoE experiments, no surplus sludge removal was necessary to keep the TSS concentration at a stable level of 2.0 ± 0.2 g/L, whereas the TSS concentration increased up to 3.6 g/L until the validation experiments, consequently prolonging the SRT. Preliminary validation results demonstrated that N_2O emissions were well below the predicted N_2O emission (results not shown), which is supported by the negative correlation

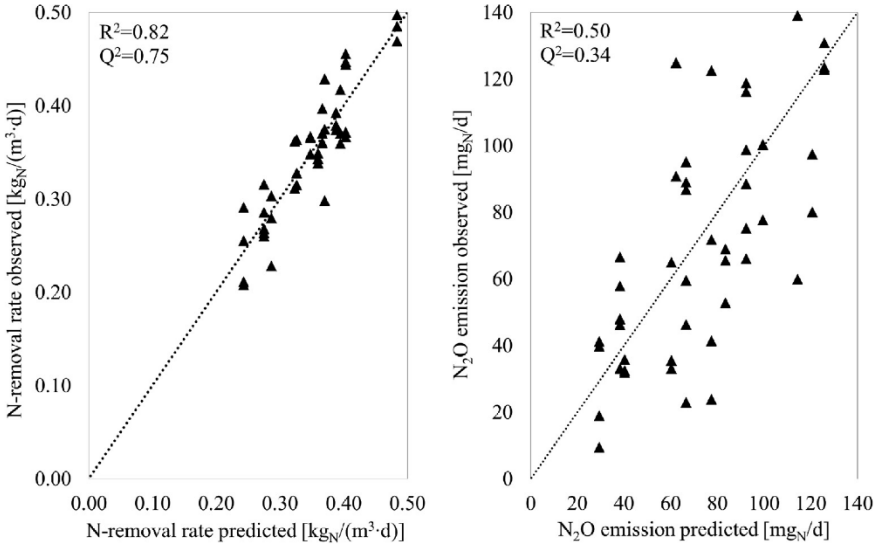

Fig. 4. Observed vs. predicted values for the model of the nitrogen removal rate (left) and the N_2O emission (right).

of N_2O emissions and SRT described in a previous study (Noda et al., 2003). Nitrogen removal rates were also below the model predictions. This can be explained by a higher endogenous respiration at higher TSS concentrations, while the aeration volume over one cycle was kept constant as one fundamental condition of this experimental setup. Thus, the available dissolved oxygen for the purpose of the single-stage deammonification process was likely reduced causing the nitrogen removal rate to drop. Consequently, the TSS concentration was adjusted for the validation experiments to match those during the DoE experiments and therefore enable again for comparable conditions, which could improve the nitrogen removal rate, however also shortened the SRT. In contrast to the preliminary validation experiments, the N_2O emissions exceeded the predictions in two out of three validation cases (Table 3). A possible explanation for the deviating results could potentially also be found in the changed composition of the process water between the calibration experiments and the validation experiments. Even though the process water had been stored at −20 °C to minimize differences in quality over the entire experimental period, an alteration was observed which was especially apparent for the COD concentration. Thereby, the COD concentrations during the validation experiments were in average decreased by 38% in comparison to those during the model development and calibration. This circumstance is supposed not to have negatively influenced the validation at settings for low N_2O emissions with continuous aeration, because potential denitrification for the reduction of N_2O is known to be inhibited by oxygen (Oh and Silverstein, 1999). However, decreased COD concentrations causing a hypothesized decreased denitrification activity could be an explanation, why the N_2O emissions exceeded the predicted values for the other two cases during which anoxic phases were supplied. For these reasons, it is more likely that not all predicted values were reproducible. Consequently, changing COD concentrations of the process water as well as the SRT would be interesting additional influencing factors to be investigated jointly in further studies.

Generally, the models were not expected to predict the exact values for every deammonification plant as the influencing factors apart of the pH value are hardly directly transferable. However, to prove that the models were rather qualitatively than quantitatively reliable, the most significant effects for both models were tested in additional trend validation experiments. Thus, the aeration strategy has been changed on all three levels for the investigation of the nitrogen removal rate, while the pH value and the feeding strategy were kept stable (pH 7.5; feed = 13 times/cycle). For the trend validation of the N_2O emissions, the effect of the pH value was examined with the feeding and aeration strategy being unchanged (feed = 25 times/cycle; aeration = 30 times/cycle). In Fig. 5, the changes of the nitrogen removal rate and N_2O emissions related to the lowest values at changing conditions are depicted.

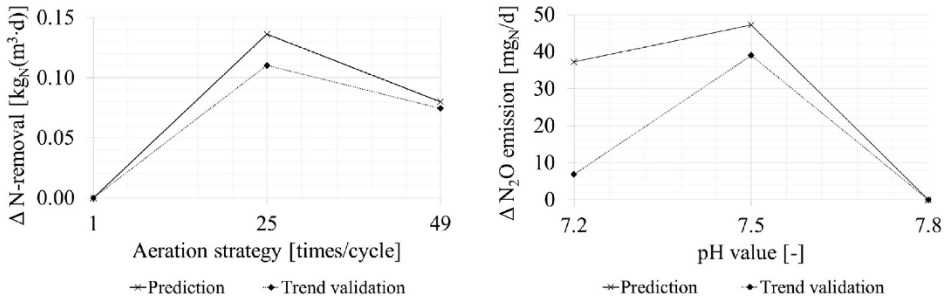

Fig. 5. Trend validation for the model of the nitrogen removal rate (left) and the N_2O emissions (right).

Results demonstrated a good agreement between the development of the trend validation values and the predicted values of both response variables and therefore, could confirm the qualitative applicability of both models. Only the experiment for the trend validation of the N_2O emission at pH 7.2 diverged clearly, however, it still could mimic the predicted trend of N_2O emissions at changing pH values. Therefore, it could be proved that the developed mathematic models are suitable to give qualitatively recommendations for improving the nitrogen removal rate and reducing the N_2O emissions.

4. Conclusions

Two models were developed via a systematic DoE approach for the prediction of the nitrogen removal rate (Y_1) and N_2O emissions (Y_2) of a single-stage deammonification process influenced by three adjustable operation factors (pH value, aeration and feeding strategy).

The highest degree of influence on Y_1 and Y_2 had the squared effect of the aeration regime and the pH value, respectively. $Y_{1,max}$ was predicated at a pH value of 7.46, intermittent feeding and aeration, while $Y_{2,min}$ was predicted at a pH value of 7.80, single feeding, and continuous aeration. Furthermore, results suggested a weak positive correlation between Y_1 and Y_2.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.03.182.

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7 Discussion, Conclusions, and Future Research

The deammonification process discovered in the mid-1990 is a relatively new technology of biological nitrogen removal (Mulder *et al.* 1995). Due to its economic operation (Jetten *et al.* 2001) and the added benefit to further increase the total nitrogen removal capacity of a WWTP (Fux *et al.* 2002), it is not surprising that there is a high interest and growing number of full scale installations worldwide (Lackner *et al.* 2014). Even though the end product of the deammonification process would ideally only be inert nitrogen gas, N_2O can be formed as an undesired byproduct by biotic and abiotic reactions (cf. Chapter 2.3.2). Thus, the growing number of full scale installations also imposes the risk of increasing, unintentional N_2O emissions, which cause harmful long-term effects on the environment (cf. Chapter 2.3.1). So far, WWTP operators are not forced to restrict their N_2O emissions by legislative regulations, which is why the willingness to implement N_2O mitigation strategies is likely to increase if the nitrogen removal rate could be improved simultaneously.

Therefore, this dissertation focused on two key research objectives: strategies for i) an enhanced deammonification performance and ii) reduced N_2O emissions for side-stream deammonification processes. For every objective, hypotheses were formulated (cf. Chapter 3 and Figure 5) and tested by a set of experiments with results being presented in **Paper I – Paper III** (cf. Chapter 4 – 6). In the following, results derived during this doctoral thesis are discussed in the light of strategies for enhanced deammonification performance and for reduced N_2O emissions separately (Chapter 7.1 and Chapter 7.2) as well as jointly (Chapter 7.3). Additionally, practical suggestions and knowledge gaps for future research are elucidated in Chapter 7.4.

7.1 Strategies for Enhanced Deammonification Performance

The primary aim of wastewater treatment with the deammonification process is to achieve a nitrogen removal rate as high as possible. The summary of system configurations in Chapter 2.2.3 revealed that various options for the deammonification application exist due to different reactor designs, microbial aggregations, and process operation. To derive recommendations towards an enhanced deammonification performance for as many system configurations as possible, both one-stage deammonification systems (**Paper I** and **Paper III**) and two-stage deammonification systems (**Paper II**) employing suspended sludge (**Paper II** and **Paper III**) and biofilm (**Paper I** and **Paper II**) have been investigated.

Optimal conditions favoring the different groups of microorganisms individually are crucial to achieve the goal of high nitrogen removal rates (cf. Chapter 2.2.2). However, they clearly differ from each other for the two most important guilds of AOBs and AnAOBs involved in the PN/A process, especially with respect to DO concentrations and growth kinetics. While dissolved oxygen is essential for the ammonium oxidation of AOBs (Schmidt *et al.* 2003), AnAOBs require anoxic conditions (Joss *et al.* 2011) and have an estimated 9- to up to 38-fold

longer doubling time (cf. Chapter 2.2.2). Clearly, two-stage deammonification systems and MBBRs employing biofilm carriers can more easily provide constantly favorable conditions for partial nitritation and the subsequent anammox process than one-stage systems with suspended sludge. This is related to the possibility to decouple conditions spatially, either in two independent reactors or over the gradient developing over the biofilm depth, respectively (cf. Figure 3).

Nevertheless, the maximum achievable nitrogen removal efficiency of a two-stage deammonification system is, inter alia, restricted by the initial performance of the partial nitritation step, as the amount of produced nitrite serves as substrate for AnAOBs in the second stage and therefore, limits the final elimination of remaining ammonium. This difficulty was addressed in **Paper II**, where the performance of partial nitritation was insufficient for the nitrogen removal rate required by the WWTP operators. The effluent ratio of ammonium to nitrite was far from reaching 1:1.32, which is necessarily required for a complete ammonium removal by the anammox reaction in the second stage according to the underlying stoichiometry (Strous *et al.* 1998; Schmidt *et al.* 2003; Kuenen 2008; Jetten *et al.* 2009). One reason was associated with successive calcification of the aeration membranes. For a well performing partial nitritation as a prerequisite for a high deammonifying performance, clogging of the aeration modules must be prevented as an essential condition to constantly ensure a generation of fine air bubbles and therefore, an enhanced oxygen transfer into the water. This can be achieved by e.g. occasional short aeration pulses with high aeration intensities to induce high turbulences, or careful acidification if clogging is due to calcification, as in this specific case. Further well-known strategies for an enhanced partial nitritation are prolonging the HRT and increasing the oxygen availability by increasing the aeration duration and/or the aeration intensity. However, these strategies also impose the risk of favoring NOB growth which necessarily needs to be suppressed as NOBs compete with AnAOBs for the substrate nitrite. For this reason, the DO concentrations besides the nitrate production need to be carefully monitored. With increasing TSS concentrations, endogenous respiration concurrently increases, which in turn decreases the oxygen availability for ammonium oxidation. This was identified to be the reason for a reduced nitrogen removal rate in **Paper III** at a constant aeration flow rate. Therefore, the aeration must be frequently adjusted to the microbial activity as well as the TSS concentration. Nevertheless, excess oxygen strictly needs to be avoided to successfully suppress NOBs. To counteract the modest performance of the partial nitritation in the first stage, the WWTP operator decided to additionally aerate the second stage, which is highly uncommon in two-stage deammonification systems (**Paper II**). This enabled an additional one-stage deammonification side-process in the second stages besides the initially purposed exclusive anammox reaction. Even though this measure could guarantee that the demanded removal efficiencies were met, it is not recommended for two-stage deammonification systems. This approach consequently sacrifices the benefit of distinctly and independently developing microbial communities under individually adjustable ideal conditions in both reactors of the PN/A process, which is one of the biggest advantages of such systems.

However, this enabled to compare reactor performances of suspended sludge in SBR- and biofilm in MBBR-mode hypothetically being operated as one-stage deammonification systems (**Paper II**). Results demonstrated that nitrogen removal rates of the MBBR operation exceeded those of the SBR operation[12]. Key results of **Paper III** revealed that intermittent aeration and feeding as well as a pH value of approximately pH 7.5 were advantageous for an enhanced one-stage deammonification performance. As both SBR and MBBR were intermittently aerated and fed, these influences could not been hold responsible to be the decisive reasons for their difference. With respect to the pH value, however, the MBBR was operated closer to the favorable pH value than the SBR, which might have been one crucial factor for higher removal rates.

Beyond that, the inoculum for investigations in **Paper I** originated from the same MBBR reactor investigated in **Paper II**. Results demonstrated that small quantities of residual suspended biomass were present besides the biofilm carriers. IFAS systems – by design a hybrid of attached and suspended growth microorganisms – are operated at significantly higher TSS concentrations (**Paper I**). Thus, the investigated MBBR can be classified as an IFAS system at a much smaller biomass concentration. In IFAS systems, the positive effect of the considerable higher concentration of suspended biomass on the nitrogen removal rate has been reported in a previous study (Veuillet *et al.* 2014). In comparison, we could demonstrate that even these small amounts of biomass suspension severely influenced the deammonifying performance of a MBBR which could be another indication why the calculated one-stage deammonification performance of the MBBR was in advantage over the SBR in **Paper II**.

The findings in **Paper I** also underline the additional potential to further enhance the performance of MBBR systems by intentionally accumulating microorganisms in suspension. FISH imaging demonstrated that suspended sludge was almost exclusively composed of AOBs. This is not surprising, as AOBs can improve their oxygen accessibility by increasing their surface area when being present as flocs than being clustered in the dense packed biofilm structure. In this way, nitrite supply for AnAOBs, securely protected from dissolved oxygen in the biofilm depth, can be increased and therefore, enhances the total nitrogen removal rate. Due to the biofilm growth, microorganisms (and more particular, AnAOBs) are not only less exposed to elevated DO concentrations, but also easily protected from washout in comparison to systems with suspended growth.

These different aspects point out that two-stage deammonification systems and MBBRs have some advantages over one-stage deammonification systems with suspended sludge regarding the simplicity to supply constantly favorable microbial conditions for an enhanced performance. The latter configuration is nevertheless the most commonly implemented full scale system (Lackner *et al.* 2014). This might be related to lower construction costs, space requirements, and

[12] In comparison, the total SBR's average nitrogen removal rate was higher than the MBBR's due to higher nitrite influent concentrations.

workload associated with one compared to two reactors. Furthermore, the availability of inoculum from other one-stage deammonification plants, which has been successfully applied for a fast start-up, might be another reason why more and more one-stage plants are installed. In contrast, biofilm growth is a time-consuming process (the built-up of only a thin biofilm layer required three to four months in the study of **Paper II**) if biofilm carriers are not introduced pre-colonized. To develop strategies for enhanced deammonification performance with an outcome as great as possible, also one-stage deammonification system with suspended sludge were investigated (**Paper III**). An important requirement of this study – apart of the influencing factors having an effect on the performance – was the practical applicability of the developed strategies for WWTP operators. For this reason, the aeration and feeding strategy as well as the pH value at the beginning of the cycle, which are easily adjustable by WWTP operators, were chosen as influencing factors and methodically varied based on a DoE method. This approach had the advantage that a large combination of typical process operations – thus a mixture of continuous/intermittent/highly intermittent aeration with single/intermittent/highly intermittent feeding and pH 7.2/7.5/7.8 – could be investigated. However, this method also induces a complex interaction of further factors that are indirectly affected by the changing conditions, such as the DO, nitrite, FA and FNA concentrations, which needed to be considered as well. Based on the developed model, optimal operational conditions leading to a nitrogen removal rate of 0.49±0.03 $kg_N/(m^3 \cdot d)$ were predicted at moderate settings of intermittent aeration and feeding as well as a pH value of pH 7.46. As the microorganisms necessarily need to perform as a symbiosis in an one-stage system, these operational settings seemed to be the best compromise for all participating guilds. It was revealed that the aeration strategy was the most important influencing factor of the three factors tested. Intermittent aeration could ensure optimal, alternating conditions for AOBs and AnAOBs and also has the benefit of providing unaerated lag-phases for NOB suppression (Kornaros *et al.* 2010; Gilbert *et al.* 2014). In contrast, the feeding strategy played a subordinate role, however, influences the FA concentrations together with the pH value, which acts as both a substrate as well as an inhibitor (cf. Chapter 2.2.2). As the composition of the wastewater is generally not constant, but subjected to dynamic changes, and the aeration strategy influenced by the duration and aeration intensity is hardly directly transferable from laboratory to full scale applications, results of this study can be characterized rather qualitatively than quantitatively.

7.2 Strategies for Reduced N_2O Emissions

N_2O formation is subjected to various complex mechanisms, including biotic, but also abiotic production pathways (cf. Chapter 2.3.2). This collection of formation pathways might be yet incomplete and in the future supplemented by further discovered production pathways, as hypothesized by (Schreiber *et al.* 2012). As AnAOBs are not suspected to contribute to N_2O formation (Kartal *et al.* 2007a), biological N_2O formation by the deammonification process can be therefore related to nitrifier and denitrifier activity. Based on the current state of knowledge,

the high disadvantage of BNR processes[13] seems to be the fact that N_2O formation is not going to be completely avoidable. Thus, under any operational conditions, N_2O formation inducing potential N_2O emission will take place. This statement can be supported by several observations, e.g. that at any DO concentration, N_2O was produced (Zheng *et al.* 1994), yet, dissolved oxygen is essential for the oxidation of ammonium (cf. Chapter 2.2.1). Furthermore, N_2O formation was reported to increase upon increasing ammonium oxidation (Law *et al.* 2012a; Domingo-Félez *et al.* 2014). This also suggests that N_2O avoidance would only be achievable without any biological activity which would push the original purpose of wastewater treatment ad absurdum. Even if biological N_2O formation could hypothetically be completely suppressed in any manner, chemical reactions would still contribute to N_2O production. Nevertheless, different mitigation strategies can at least aim for reduced N_2O formation and subsequent emission and therefore diminish the negative effects on the environment.

N_2O formation is triggered by various influencing factors (cf. Chapter 2.3.3), which again are partly interconnected, such as the DO concentration and SRT influencing the nitrite concentration, nitrite concentration and pH value influencing FNA concentration, etc. This closely linked interplay emphasizes that N_2O mitigation strategies are complex and might not be able to tackle all problems at once. Especially chemical reactions causing N_2O formation seem to be hardly controllable, as they originate from various intermediate products of the ammonium oxidation (e.g., NH_2OH, NO_2^-/HNO_2). Furthermore, abiotic N_2O formation can be catalyzed by metal ions, thus, it is also dependent on the wastewater composition, which can likewise affect biotic N_2O formation by toxic or inhibitory compounds (cf. Chapter 2.3.2). Again, different N_2O mitigation strategies are needed for different reactor designs. In addition, biological systems are highly dynamic and continuously subjected to changes, e.g. due to an alternation of the composition of the wastewater, which does not only influence N_2O formation as discussed above, but also the microbial community and activity, etc. Therefore, constant adjustments of the operational settings are required to keep the deammonification process in balance and at a high performance which can influence N_2O formation on their part. However, N_2O mitigation for these small scale changes are hardly feasible, but must be targeted on a more general scale.

Elevated nitrite concentrations should generally be avoided as they trigger N_2O formation by both nitrifier and denitrifier activity (cf. Chapter 2.3.3). This could be supported by **Paper II**, where the buffer tank (interconnecting the first and the second stage) was identified to cause highest dissolved N_2O concentrations of all investigated reactors. Elevated nitrite and ammonium concentrations, anoxic conditions, and a constant supply of microorganisms represented ideal conditions for N_2O formation. These conditions were comparable to those during an unaerated phase or the settlement phase of a nitritation reactor, which have been reported before to cause as well an accumulation of dissolved N_2O, yet at a lower level of concentrations (Gabarró *et al.* 2014; Ju *et al.* 2015). Clearly, elevated nitrite concentrations are not intended to be avoided in a

[13] Even though this dissertation focused on deammonification systems, this statement can be generalized to BNR processes, as N_2O formation by nitrifier and denitrifier activity is part of all BNR processes.

two-stage deammonification system. Quite the contrary, high ammonium conversion rates to nitrite are desired for a well performing entire system. However, anoxic conditions at the presence of nitrite should be avoided to reduce N_2O formation (Wunderlin *et al.* 2012; Peng *et al.* 2015), which indicates a continuous aeration to be advantageous over an intermittent aeration in the first nitritation stage. These continuously elevated DO concentrations can help to reduce N_2O formation via nitrifier denitrification, however, hydroxylamine oxidation and abiotic reactions will take place nonetheless (cf. Chapter 2.3.2). This could also be supported by results of **Paper II**, where the DO concentration reached 3 mg/L, but dissolved N_2O was still detected. Ideally, a buffer tank or a settling phase should be omitted to avoid anoxic phases (which would lead to a sludge retention time as long as the hydraulic retention time for the latter case and could simultaneously be favorable for NOB-washout; cf. Chapter 2.2.2). However, if this is not achievable, settling should preferably take place in a buffer tank because dissolved N_2O would subsequently be transferred into the unaerated second stage. Here, N_2O emission will only be diffusion-driven as in comparison to being actively stripped by aeration in the first stage. Furthermore, to avoid nitrite peaks and rapidly changing conditions triggering N_2O formation (cf. Chapter 2.3.3), rather continuous feeding into the second stage instead of a pulse loading is believed to mitigate N_2O formation.

(Zheng *et al.* 1994) proposed to avoid incomplete nitrification (thus nitrite accumulation) in order to decrease N_2O formation by increasing both the DO concentrations and the SRT. This is an applicable N_2O mitigation strategy for nitrification, but the first stage of a two-stage deammonification process aims for nitrite accumulation and short SRT (at elevated temperatures) to favor the growth of AOBs over NOBs (cf. Chapter 2.2.2). Nevertheless, prolonging the SRT was demonstrated to reduce N_2O formation during denitrification (Hanaki *et al.* 1992; Noda *et al.* 2003). An increased SRT was also suspected to be the reason for N_2O emissions deviating from the predicted values in **Paper III**. Furthermore, higher TSS concentrations inducing a higher decay of biomass, which can serve as a readily degradable carbon source for denitrification, was hypothesized to be accountable for observed N_2O reduction in **Paper II**. Thus, extending the SRT in a one-stage deammonification system and in the second stage of a two-stage deammonification system seems to be an applicable N_2O mitigation strategy.

The aeration strategy comprised of the aeration duration and volumetric aeration flow rate influences not only the DO concentration, but indirectly also the amount of produced nitrite, nitrate, and dissolved N_2O. Furthermore, it provokes stripping and thus N_2O emissions. Therefore, the aeration strategy has a manifold influence on the N_2O formation and the emissions. One of the key results of **Paper III** was that continuous aeration in a one-stage deammonification process was favorable over (highly) intermittent aeration with respect to mitigated N_2O emissions[14]. Clearly, the DO concentration of a deammonification process

[14] Aeration strategies were directly comparable, as the total aeration volume over every cycle was the same.

performed in one reactor at continuous aeration needs to be as low as the anammox process can simultaneously be executed[15]. This was an interesting finding, as the effect of the DO concentration on the N_2O formation is conflicting in literature. Observations point into opposite directions, with both increasing and decreasing DO concentrations triggering N_2O formation for nitrifying, denitrifying, and deammonifying systems (cf. Chapter 2.3.3). It still needs to be elucidated, what exactly is the reason for these diverging observations. However, it is most likely related to an interaction of many factors in the complex formation, but also reduction of N_2O. One reason for the advantageous continuous aeration towards a reduced N_2O formation is the fact that nitrite can immediately be consumed. Thus, a nitrite accumulation is prevented. Therefore, nitrite for N_2O formation is available to a much lesser extent than under (highly) intermittent aeration. Furthermore, continuous aeration prohibits a transition from anoxic to oxic conditions and vice versa, which was observed to increase N_2O formation (Ahn *et al.* 2010; Yu *et al.* 2010; Law *et al.* 2012a; Castro-Barros *et al.* 2015). Due to the lower aeration intensity at continuous in comparison to (highly) intermittent aeration, also the stripping effects were less. As the performance was predicted to decrease under these optimized conditions for minimized N_2O emissions, another explanation could also be given by reduced ammonium oxidation and with that a reduced availability of intermediate products for N_2O formation (cf. Chapter 2.3.3).

Apart from the aeration strategy, **Paper III** also demonstrated that the pH value was the most important of the three tested factors with respect of influencing the N_2O emissions. At pH 7.8, N_2O emissions were at its lowest level. Decreasing the pH value to pH 7.5 caused by far a more severe increase of N_2O emissions as compared to a change from pH 7.2 to pH 7.5. Elevated FA concentrations at elevated pH values decreasing the activity of AOBs and with it the nitrifier denitrification besides a reduced formation of intermediates available for N_2O formation, but no inhibition of denitrification by FNA concentrations were discussed to be the reasons. Furthermore, the model predicted that the less frequently feeding takes place, the lower the N_2O emissions can be expected to be. This partly conflicts with previous studies, where ammonium shock loads and higher ammonium concentrations were related to increased N_2O formation (cf. Chapter 2.3.3). According to that, continuous feeding would be suggested to be favorable over intermittent feeding for the purpose of reduced N_2O formation and potential subsequent N_2O emission. With N_2O formation possibly reaching a maximum at an ammonia shock load of 1.6 mg_N/g TSS (Burgess *et al.* 2002), single feeding causing only once N_2O formation might as well be advantageous over intermittent aeration, as suggested by **Paper III**. Beyond that, the feeding strategy also directly influences the pH value as well as the FA concentration.

Often, studies are based on one-dimensional investigations. The big advantage of the DoE method applied in **Paper III** was that several influencing factors could be investigated in parallel, which enabled to evaluate not only direct effects, but also interactions of the influencing factors. Furthermore, the entire deammonification system was investigated in comparison to other

[15] The better the AnAOBs are protected from dissolved oxygen, the higher the DO concentration can be, e.g. in huge flocs, granules, or even biofilm systems (protection increases in this order).

studies, where only partial steps were examined. Thus, this approach enabled to provide more general conclusions. Nevertheless, results also demonstrated that N_2O emissions were subjected to a high variability within the repetitions of the DoE runs, even though the process was operated at conditions as constant as possible. This again highlights, how sensitive N_2O formation, and therefore, how challenging N_2O mitigation is.

Even if N_2O mitigation strategies are applied, a complete avoidance of N_2O formation being potentially emitted is believed to be impossible (as discussed above). However, any uncontrolled N_2O emissions could be safely eliminated if they were captured and incinerated, as proposed in **Paper II**. Even though this is an end-of-pipe solution, environmentally unfriendly N_2O could be converted into non-hazardous N_2. Additionally, the energy-yield by an incineration of CH_4 with N_2O instead of oxygen can be increased by 37 % (Scherson *et al.* 2013). This approach would not only be a safe and environmentally friendly solution, but also offers the opportunity to further decrease the energy demand of BNR processes by intentionally producing N_2O (Weißbach *et al.* 2017b). As deammonification plants are often already covered to reduce heat losses, an upgrade to a gastight reactor should be easily feasible. Furthermore, additional costs for piping and gas pre-conditioning would arise. However, these costs might be subsidized in the future, if the brisance of deammonifying processes emitting N_2O will gain more attention.

7.3 Strategies for Simultaneously Enhanced Deammonification Performance and Reduced N_2O Emissions

In comparison to other studies, **Paper III** not only investigated the entire deammonification process instead of partial reactions under a methodically variation of three instead of one influencing factor. It also combined the joint identification of the nitrogen removal rates and the N_2O emissions. This enabled an advantageous holistic approach for optimization strategies.

Against the desirable and ideal case of a simultaneous reduction of N_2O emissions at increased nitrogen removal rates, the two response variables pointed towards a weak positive correlation, yet with a high similarity of experimental and modeled data ($R^2_{experiments}$=0.086; R^2_{models}=0.107). This was already indicated by contradictory results for separately improved nitrogen removal rates and reduced N_2O emissions (pH=7.46 vs. pH=7.8; intermittent vs. continuous aeration; intermittent vs. single feeding). Thus, a single set-point for the desired simultaneous economic and ecologic operation under the tested conditions could not be identified. This conclusion can be supported by previous observations, where an increased ammonium oxidation activity was hypothesized to promote N_2O formation by nitrification (Law *et al.* 2011); cf. Chapter 2.3.3).

Nevertheless, the models could be used to target a desired compromise between an efficient and environmentally friendly deammonification process. Exemplarily, three different options were presented in **Paper III** and compared to their percentage deviation from the mean derived from the DoE experiments. Settings for highly reduced N_2O emissions at minor losses of the

nitrogen removal rate (Scenario I), for considerable reduced N_2O emissions at a little improvement of the nitrogen removal rate (Scenario II), and for a higher performance, yet still at slightly reduced N_2O emissions (Scenario III) were proposed and evaluated for two different weightings regarding nitrogen removal rates and N_2O emissions (Figure 6). If the nitrogen removal rate would be as important as N_2O emissions (weighting 1/1), Scenario I would be the best compromise; in contrast, Scenario III would represent the choice of settings, if the WWTP operator would put the preference on the nitrogen removal rate being thrice as important as N_2O emissions (weighting 3/1). Thus, the decision on how to regulate the deammonification process will inter alia be dominated by future legislative regulations. Beyond that, many more scenarios for a desired compromise can be developed by using the two models for the prediction of the deammonification performance and the N_2O emissions presented in **Paper III**.

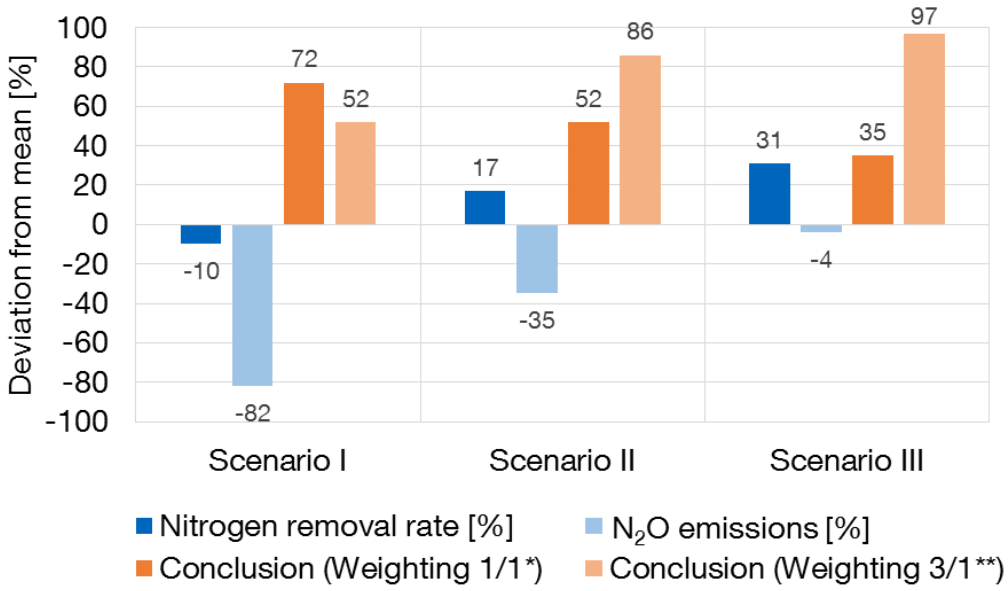

Figure 6. Three different scenarios for a compromise between an economic and ecologic operation of the deammonification process (the conclusions are calculated as the sum of the nitrogen removal rate value and the absolute value of the N_2O emissions; *same importance for the nitrogen removal rate and N_2O emissions; **nitrogen removal rate being thrice as important as N_2O emissions)

7.4 Practical Suggestions, Knowledge Gaps, and Future Research

A certain extent of uncertainty regarding the reproducibility of the results remains, if experiments are not repeated a number of times, e.g. in triplicate. Ideally, experiments are run in parallel, which is, however, not always feasible due to limited resources (e.g. number of available reactors). An alternative approach is to carry out the experiments repeatedly in series, which was applied in **Paper I**. This had the added benefit that the positive relation between the VSS concentration and the nitrogen removal rate could be demonstrated due to varying VSS concentrations in the three independent inoculations. In contrast, for the DoE method in **Paper III**, experiments were only repeated within one inoculation without a repetition in triplicate. This different approach was chosen, as a compromise was necessary between an experimental

duration as short as possible to reduce the risk of a changing microbial community[16] in the pilot scale reactor exclusively operated to supply inoculation material of constant quality for the 13 different DoE runs, but as long as necessary for the adaptation of the microorganisms to the new settings of each DoE run and the subsequent repetitions for data acquisition[17]. Nevertheless, a microbial adaptation over the long term causing decreasing N_2O emissions might be possible, as indicated by (Kampschreur *et al.* 2009b). Therefore, future studies could use DoE settings that led to the highest N_2O emissions and investigate, if an operation over several months would cause the N_2O emissions to drop.

Furthermore, extending the DoE method with further influencing factors at more levels would enable an even more detailed insight into the deammonification process with its performance and N_2O emissions. Interesting additional factors for a joint investigation in future studies could be changes in the COD/N ratios and the SRT. These two factors were identified to have undesirably influenced the results of **Paper III**. However, increasing the amount of influencing factors also increases the amount of DoE runs and consequently the experimental duration which should be kept as short as possible for the precondition of a microbial community as constant as possible. Increasing the levels would comparably increase the amount of experiments, too. Even if the latter approach would enable a more detailed breakdown of the influencing factors themselves, the outcome of increasing the amount of the factors at a minimal number of levels (n_{min}=3) in comparison to increasing the amount of levels will be more advantageous in generating a broader picture of the process and therefore is preferably recommended. Additionally, testing the entire DoE method again with another deammonifying microbial community would be an interesting approach to evaluate, if results are comparable and thus, transferable to any other one-stage deammonification system.

Throughout all experiments of **Paper I**, **Paper II**, and **Paper III**, real reject water was used. This had the advantage that realistic conditions could be provided. Furthermore, no resources needed to be consumed for the preparation of synthetic wastewater which can cause high expenses when the process is operated over years. When the process is run at pilot scale, the preparation of the considerable high amounts of synthetic wastewater is even hardly feasible. However, synthetic wastewater has the advantage that a stable composition of the wastewater can be provided throughout the experiments and the effect of added substances, e.g. on the N_2O formation, can be investigated in detail. In contrast, real wastewater is subjected to fluctuations over the year or even in the short term[18]. Thus, a decision between the two different types of wastewaters with their advantages and disadvantages needs to be taken depending on the purpose of the experiments. For the model calibration and the validation in **Paper III**, we decided to store one charge of real wastewater frozen to provide a constant composition of the wastewater for the deammonifying process without those mentioned fluctuations. After several

[16] This was a pilot scale reactor of 130 L working volume.
[17] This was a laboratory scale reactor of 10 L working volume.
[18] Extinguishing agent used for firefighting was for instance once identified to have been responsible for a short break down of the performance and considerable foaming in the pilot scale reactor.

months, changes of the ammonium concentration were inconsiderable, but the COD concentration had decreased noticeably by 38 %[19]. According to the (Environment Protection Authority 2000), samples can be stored frozen for 28 days until analysis. Thus, for experiments exceeding a duration of four weeks, the usage of synthetic wastewater rather than thawed real wastewater is recommended to prevent the impact of compositional fluctuations of the wastewater on the results.

As discussed in Chapter 2.3.3, Chapter 7.2, and **Paper III**, observations regarding the DO concentration on the N_2O formation are ambivalent in literature, with both increasing and decreasing DO concentrations triggering N_2O formation. It is not clear yet, what exactly causes these differential results, but might be a breakthrough for further N_2O mitigation strategies. This highlights the need to close this knowledge gap. The abundance of different microbial groups within a deammonifying community might give an indication of the amount of N_2O formation. Thus, one approach could be to couple observations regarding the N_2O formation with an analysis of the microbial community. Based on these results, a shift towards a microbial community that produces less or consumes more N_2O could be initiated by adjusting the operational conditions.

The DO concentration is closely connected to the aeration which in turn influences the stripping effects and therefore, enhances N_2O emissions into the atmosphere. This offers several options to be investigated in future studies for mitigated N_2O emissions. It could for instance be examined, to which extent N_2O emissions could be decreased if the proportion of pure oxygen in the air would be increased, thus exceeding the atmospheric concentration. This would require an aeration flow rate to a lesser extent and therefore, could decrease the stripping effects. As this approach entails additional costs for the supply of pure oxygen, it is likely to be only feasible in full scale applications, if N_2O emissions are going to be restricted in the future. As N_2O formation does not necessarily equal N_2O emission, the dissolved N_2O concentration might be used as a control signal to regulate the aeration, e.g. to hinder an activation of aeration at a set dissolved N_2O threshold concentration to avoid enhanced N_2O emission. This, however, requires an effective removal of dissolved N_2O which is under the exclusive responsibility of denitrifying microorganisms. As the availability of carbon sources is limited in process water, the effect of adding alternative carbon sources, such as primary effluent, at peaking dissolved N_2O concentrations under anoxic conditions, could be an interesting investigation. Nevertheless, this might induce an undesired shift of the microbial community in the long run with denitrifiers dominating AnAOBs. This strictly needs to be prevented in terms of a stable deammonification performance that should be preferred over mitigated N_2O emissions. Therefore, the influence of the additional carbon source on the microbial community would be needed to be closely monitored and investigated when testing such an approach. Furthermore, the influence of an improved reactor design, e.g. deeper reactor depths for better solubility of both N_2O and oxygen

[19] The reason how the COD has been decreased during a storage of -18 °C remains unexploited.

as well as more gentle mixing, on reduced N_2O emissions would be another interesting approach for future investigations.

Long start-up phases of MBBRs due to a time-consuming built-up of biofilm might be one reason, why suspended sludge systems are preferred over biofilm growth systems. However, observations presented in **Paper II** pointed towards a possibility to accelerate biofilm formation by an artificial calcification of carriers prior to inoculation. This could be supported by a previous study, where (Goode and Allen 2011) found a positive correlation between the calcium concentrations and the biofilm thickness and density. Thus, a detailed study regarding the effect of calcium on biofilm growth and accumulation could provide interesting findings.

Due to the highly variable and dynamic N_2O emissions of BNR processes, neither the evaluation of N_2O emissions based on a single point sample, nor on a default emission factor as proposed by the IPCC will provide a reliable and representative inventory (cf. Chapter 2.3.1). Furthermore, these uncertainties and a lacking standardized measurement procedure for full-scale installations render a direct comparison of N_2O emissions between different WWTP impossible. During our measurement campaign at a full scale deammonification plant (Thaler *et al.* 2017), it was revealed that less than 10 % of the expected aeration flow was emitted at one point in the reactor by use of a sampling hood (results not published). Thus, if results were extrapolated based on this single measurement point, the N_2O emission would severely deviate from those measured as emissions from the entirely covered reactor. Therefore, a standardized measurement protocol should be developed which covers different system configurations and operations and clearly defines the amount and spatial distribution of sampling spots, the logging interval, the measurement duration (ideally over the entire year to also include seasonal fluctuations), the measurement principle, etc. Discussions at the 'Nitrous oxide emissions from biological wastewater treatment - Expert Meeting and Workshop' in Bochum in 2016 also revealed that such a procedure is lacking so far, but necessary for comparability.

Finally, the recently presented process of oxygenic denitrification offers an opportunity to avoid an formation of N_2O by denitrification through a direct conversion of NO into N_2 (Zhu *et al.* 2016). Interestingly, the required *nod* genes for this process were remarkably abundant in different kinds of wastewater treatment systems, which also included the inoculum of **Paper I**, **Paper III,** and the second stage MBBR of **Paper II**. Therefore, supplying conditions that favor oxygenic denitrification inheres a potential to decrease N_2O formation. However, these conditions still need to be determined.

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Appendix

A.1 Calculation of free ammonia and free nitrous acid

Free ammonia (FA; NH_3) and free nitrous acid (FNA; HNO_2) concentrations can be calculated as follows (Anthonisen *et al.* 1976):

$$NH_3 - N = \frac{NH_4 - N \times 10^{pH}}{e^{(6344/T)} + 10^{pH}}$$

$$HNO_2 - N = \frac{NO_2 - N}{e^{(-2300/T)} \times 10^{pH}}$$

with:

NH_3-N:	Free ammonia nitrogen [mg_N/L]
HNO_2-N:	Free nitrous acid nitrogen [mg_N/L]
NH_4-N:	Ammonium nitrogen [mg_N/L]
NO_2-N:	Nitrite nitrogen [mg_N/L]
pH:	pH value [-]
T:	Temperature [K]

A.2 Supplementary information

The respective supplementary information (SI) as part of the presented **Papers I - III** are attached in the following section.

A.2.1 SI of Paper I

The role of residual quantities of suspended sludge on nitrogen removal efficiency in a deammonifying moving bed biofilm reactor

- Supplementary Information -

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I. FISH Analysis

FISH protocol

Samples of 400 µl were fixed by adding 1200 µl of 4 % PFA-PBS-solution pH 7.0 – pH 7.4 (Table I-1) and incubated at 4 °C for 24 h. Removal of 4 % PFA-PBS-solution, subsequent washing step with 1x PBS pH 7.4 (Table I-2) and its removal was achieved with interim steps of centrifugation at 10,000 g for 5 min (Centrifuge 5417 R, Eppendorf AG, Hamburg, Germany). Samples were resuspended in 400 µl of 1x PBS and 400 µl of ice-cold ethanol and stored at -20 °C. Defrosted sample volumes of 9 µl were immobilized in wells of gelatine-coated slides (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany), air dried and dehydrated for 3 min in an ethanol-series of 50 %, 80 % and 100 %. 8 µl of hybridization buffer as a mixture according to a 30 % formamide concentration of 600 µl formamide, 1,000 µl Milli-Q, 360 µl of 5 M NaCl, 40 µl of 1 M Tris/HCl pH 8.0, and 2 µl of 10 % SDS pH 7.2 were added to wells and mixed with 1 µl of fluorescent rRNA-targeted oligonucleotide probe for staining. Probes applied for detection of AOBs and anammox bacteria are listed in Table 1. Next, samples were incubated in hybridization oven (H. Saur Laborbedarf, Reutlingen, Germany) for 90 min at 46 °C in a moisture chamber (50 mL Greiner tubes). Slides were transferred to preheated washing solution consisting of a mixture of 1,000 µl of 1 M Tris/HCl pH 8.0, 50 µl of 10 % SDS pH 7.2, 1020 µl of 5 M NaCl, and 500 µl of 0.5 M EDTA pH 8.0, incubated for 20 min at 48 °C in a water bath (Grant Instruments, Cambridge, United Kingdom), then dipped into cold Milli-Q-water and air-dried. Before image acquisition, slides were covered with non-hardening antifadent mountant solution AF 1 (Citifluor Ltd, London, United Kingdom).

Table I-1. Chemical composition of 4 % PFA-PBS solution.

	4 % PFA-PBS-solution, pH 7.0-7.4
Paraformaldehyde	4.0 g
3x PBS	33 mL
Milli-Q	65 mL

Table I-2. Chemical composition of 1x PBS and 3x PBS.

	1x PBS, pH 7.4	**3x PBS, pH 7.4**
NaCl	8.00 g	24.00 g
KCl	0.20 g	0.60 g
Na_2HPO_4	1.44 g	4.32 g
KH_2PO_4	0.20 g	0.60 g
Milli-Q	1,000 mL	1,000 mL

FISH images

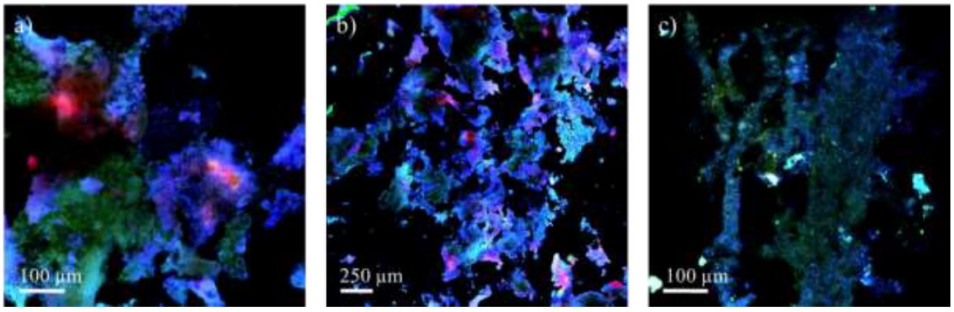

Figure I-1. *Bacteria*, AOBs, and *Brocadia fulgida* as representative for anammox bacteria simultaneously detected by FISH. a) and b): Biofilm; c): Suspended sludge. *Bacteria* are detected by probe EUB 338 (labelled with FLUOS; blue), AOBs by probe NSO 1225 (labelled with CY 3; green), and *Brocadia fulgida* by probe Bfu 613 (labelled with CY 5; red). Due to the simultaneous binding of EUB 338 (blue) with NSO 1225 (green) as well as with Bfu 613 (red), AOBs appear green to turquoise, while anammox bacteria can be distinguished by different shades of pink to purple in the FISH analysis.

II. Experimental results

Table II-1. Operation conditions for repetitions I, II and III with respect to biofilm carriers and suspended sludge (BC+SB), suspended sludge (SB), and biofilm carriers (BC).

	Q_{air} [L/h]	Oxic phase per interval [s]	Anoxic phase per interval [s]	DO set point [mg/L]	pH control [-]	Activation of DO control [-]	Aeration ratio* [%]	No. of cycles [-]
(I) BC+SB	50	240	840	0.1-0.3	> 7.5	-	0.78	6
(I) SB		240				-	0.78	6
(I) BC		240				X	0.16	5
(II) BC+SB		360				-	0.70	9
(II) SB		360				-	0.70	6
(II) BC		360				X	0.09	4
(III) BC+SB		360				-	0.70	4
(III) SB		360				-	0.69	6
(III) BC		360				X	0.25	4

*Aeration ratio: t_{ON}/t_{ON+OFF}

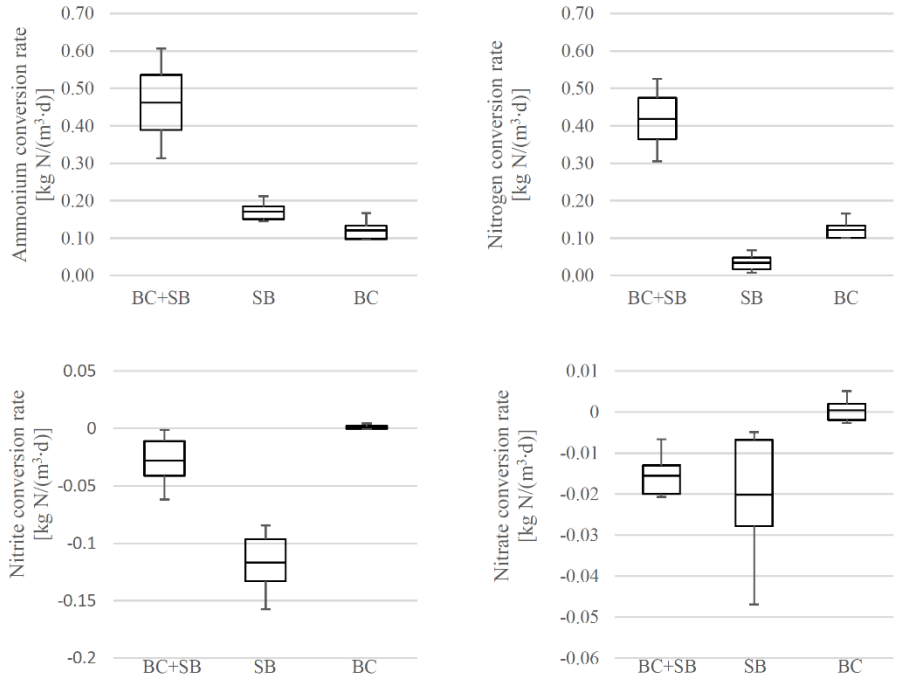

Figure II-1. Conversion rates of ammonium, nitrite, nitrate and total nitrogen for experiments of biofilm carriers and suspended sludge (BC+SB), suspended sludge (SB), and biofilm carriers (BC) (error bars display average maximum and minimum values for the repetitions, outer lines the 1st and 3rd quantile, and middle line the mean).

A.2.2 SI of Paper II

Water **2016**, *8*, 578; doi:10.3390/w8120578

Supplementary Materials: Performance and N_2O Formation of the Deammonification Process by Suspended Sludge and Biofilm Systems—A Pilot-Scale Study

Carmen Leix, Rebecca Hartl, Christian Zeh, Franz Beer, Jörg E. Drewes and Konrad Koch

Figure S1. Two-stage deammonification pilot plant in Kempten (Allgäu): (**a**) Overview (1: Sedimentation; 2: Nitritation; 3: Buffer tank; 4a: Moving bed biofilm reactor (MBBR) with biofilm carriers; 4b: Sequencing batch reactor (SBR) with suspended sludge); (**b**) SBR with suspended sludge; (**c**) MBBR with biofilm carriers.

Figure S2. Pictures of aeration elements (**left**: calcified membrane tube; **right**: recurrently acidified membrane plate).

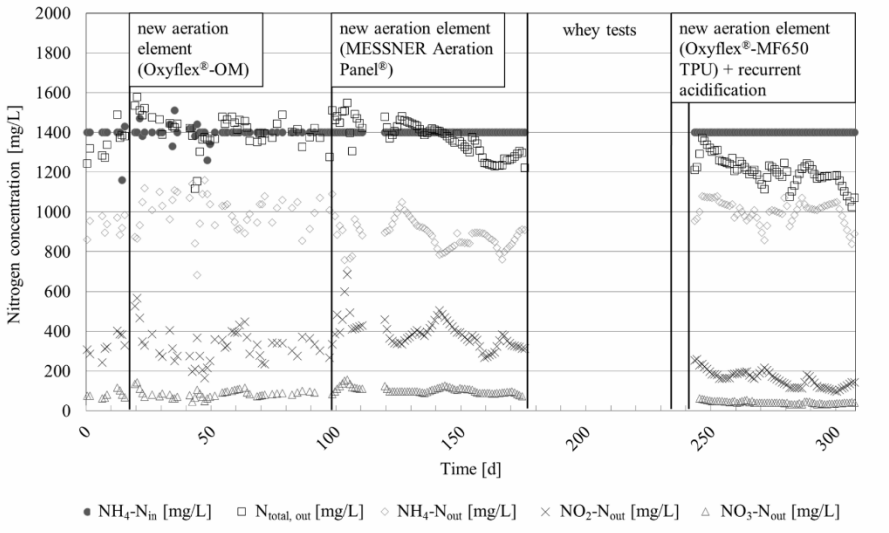

Figure S3. Performance of nitritation in combination with downstream SBR in 2014 (NH_4-N_{in} was assumed to be 1400 mg/L for the rest of the year after stable development during observation in February and March).

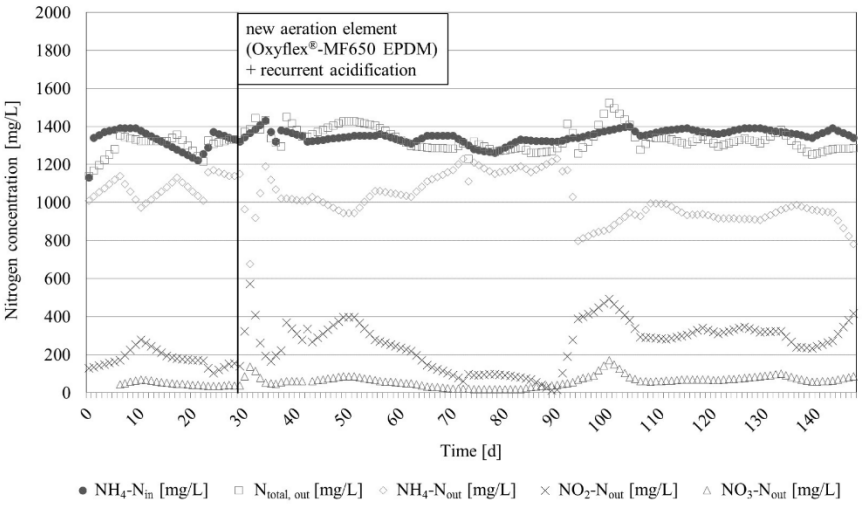

Figure S4. Performance of nitritation in combination with downstream MBBR in 2015.

Table S1. Respiratory AOB and NOB activity tests of SBR *.

	Respiratory Activity [g O_2/(g VSS·Day)]	Conversion Rates [g N/(kg VSS·Day)]
AOB	1.13	330
NOB	0.08	72

Notes: * The respiratory activity of ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) was investigated ex situ over the oxygen consumption and correlated to the volatile suspended solids concentration (VSS). For AOB activity only, sodium azide (NaN_3) was added with a final concentration of 1 mg N/L for the inhibition of NOBs. For NOB activity only, allylthiourea (ATH) was added to a final concentration of 5 mg ATH/L for the inhibition of AOBs. For all tests, supply of sufficient substrate (ammonium and additionally nitrite for the NOB activity test) needed to be ensured. Sludge was aerated until oxygen saturation and oxygen consumption was monitored. According to the stoichiometry, 3.43 g O_2/(g NH_4-N) are necessary for the ammonium oxidation, whereas 1.14 g O_2/(g NO_2-N) are needed for the nitrite oxidation. Results of activity tests indicate a strong abundance of AOBs besides a successful suppression of NOBs in the second stage of the deammonification plant and therefore are evidence—apart from the calculated single-stage ammonium conversion rates—that the second stage partly acted as a single-stage deammonification process.

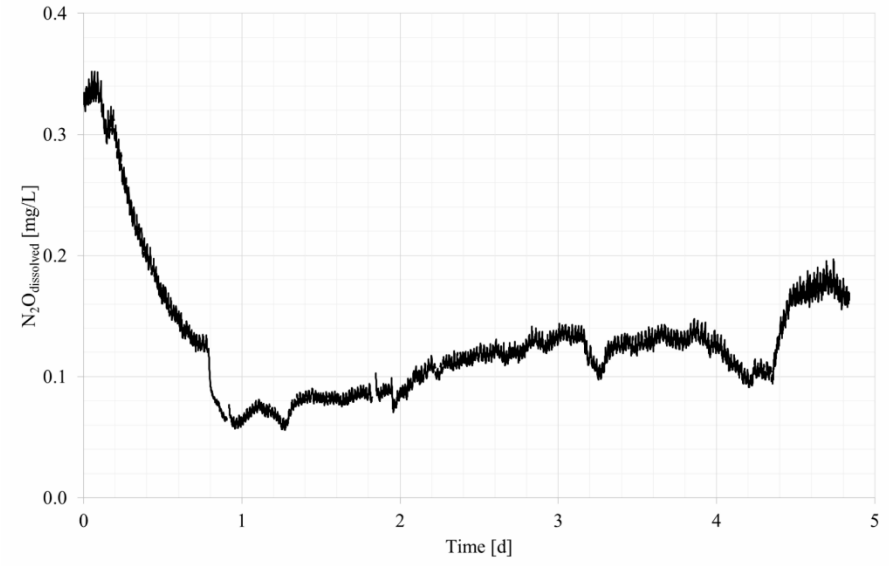

Figure S5. Dissolved nitrous oxide concentration in the nitritation.

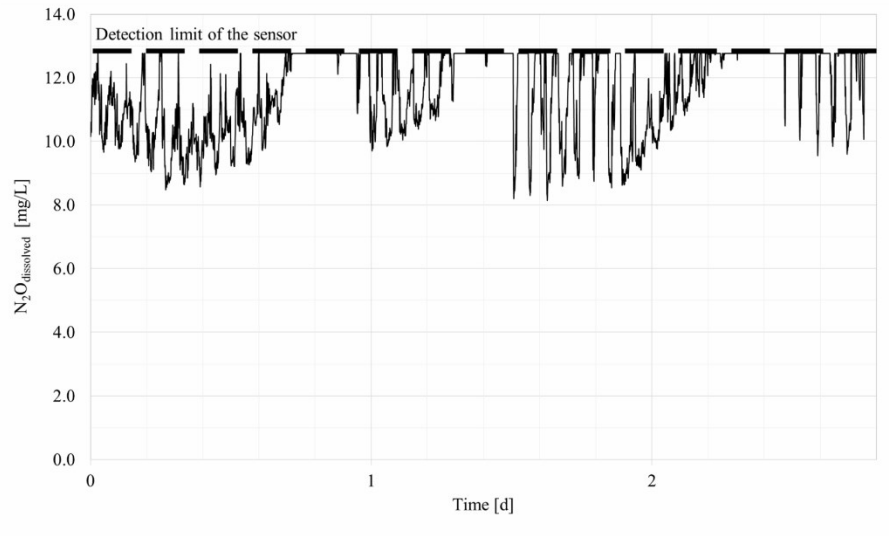

Figure S6. Dissolved nitrous oxide concentration in the buffer tank.

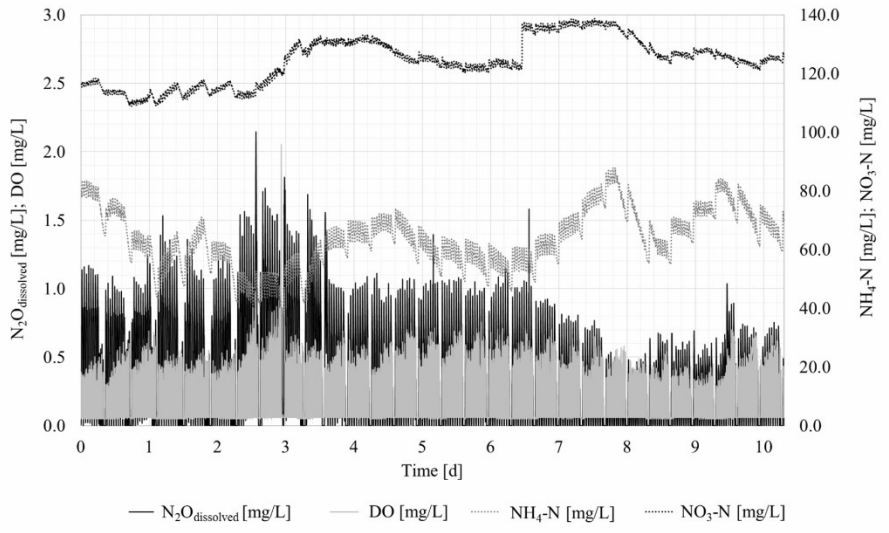

Figure S7. Dissolved nitrous oxide, dissolved oxygen, ammonium, and nitrate concentrations in the SBR.

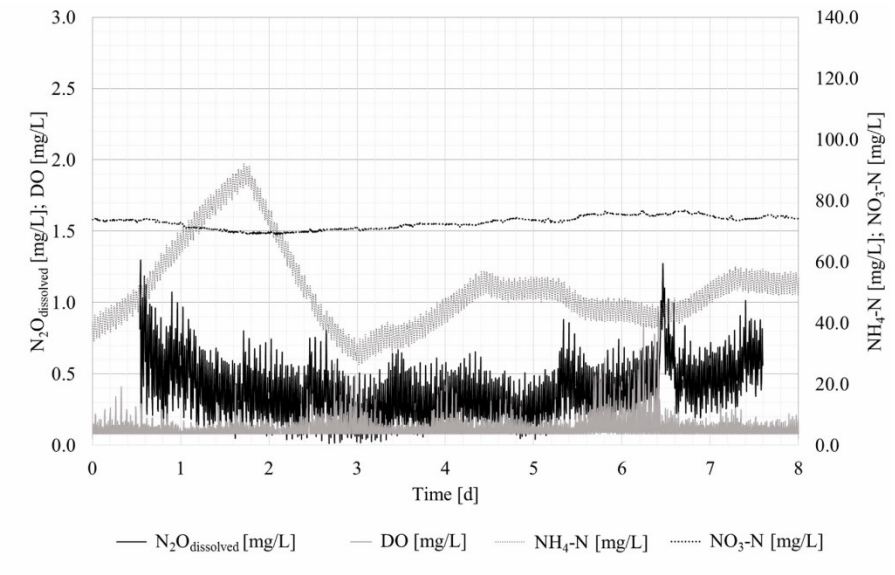

Figure S8. Dissolved nitrous oxide, dissolved oxygen, ammonium, and nitrate concentrations in the MBBR operated as a single-stage deammonification process.

A.2.3 SI of Paper III

Strategies for enhanced deammonification performance and reduced nitrous oxide emissions

- Supplementary Information -

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I. Experimental setup

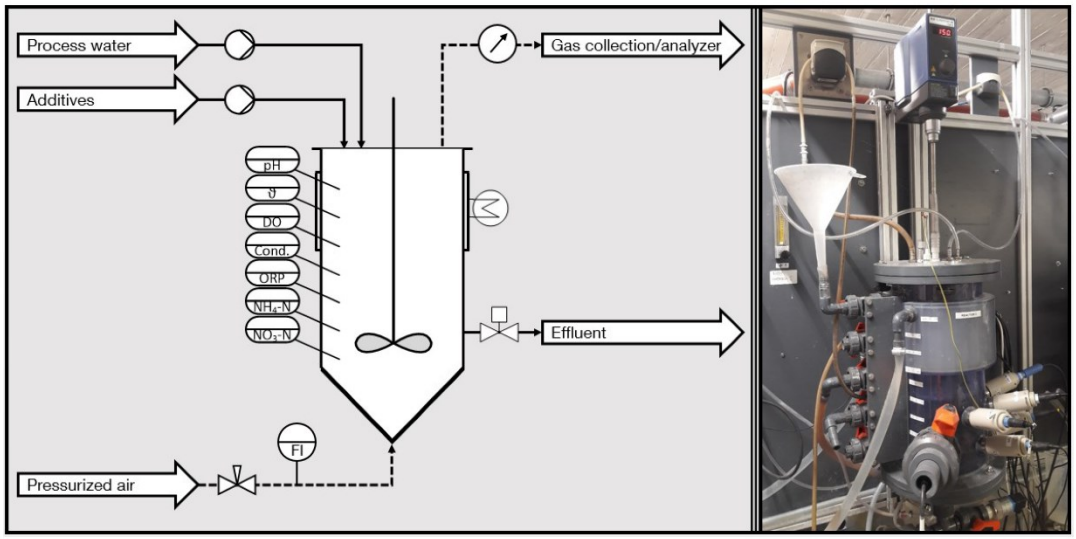

Figure SI- 1: Experimental setup as flow schematic (left; solid line: liquids; dashed line: gases; FI: flow indication; ϑ: temperature; DO: dissolved oxygen concentration; Cond.: conductivity; ORP: oxidation-reduction potential) and in reality (right)

II. Performance of the pilot-scale deammonification plant

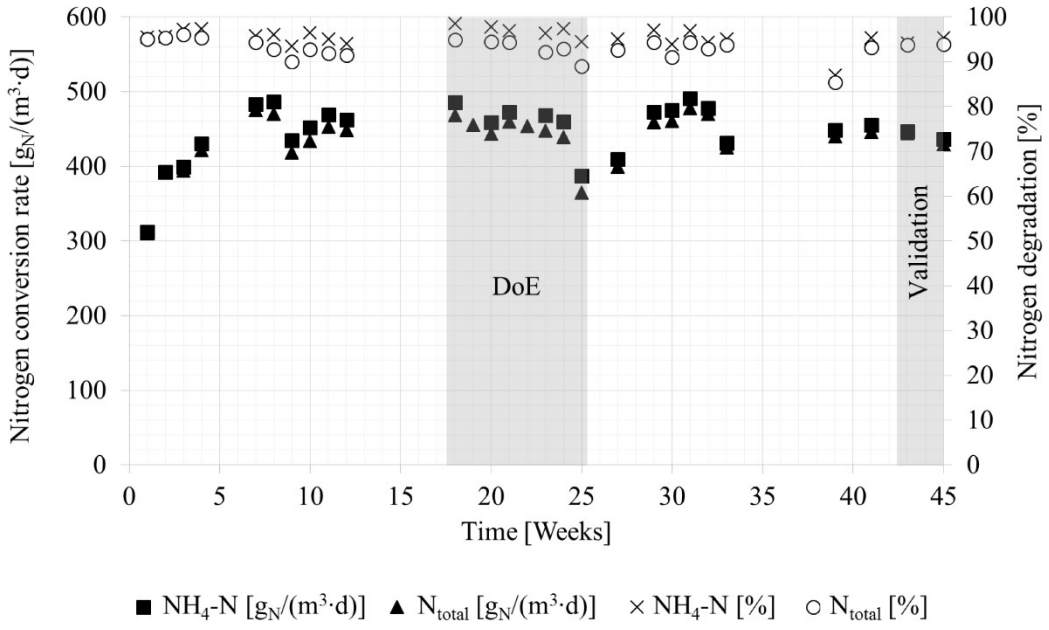

Figure SI- 2: Nitrogen conversion rate and nitrogen degradation of the pilot-scale deammonification plant over the course of operation providing a stable microbial community for the inoculation for every new run of DoE

III. Design of experiment

Table SI- 1: Overview of the three influencing factors on three levels

Influencing factors	Influencing variable	Unit	Level		
			-1	0	+1
X_1	pH value	[-]	7.2	7.5	7.8
X_2	Feeding strategy	[times/cycle]	1	13	25
X_3	Aeration strategy	[times/cycle]	1	25	49

Table SI- 2: Settings for the influencing factor of the aeration strategy

Level [-] **Aeration [times/cycle]**	**-1** **1**	**0** **25**	**+1** **49**
Aeration intensity [L/h]	20	40	60
Aeration strategy	continuous aeration	intermittent aeration	highly intermittent aeration
Aeration interval (t_{on}/t_{off})	1·(21600s/0s) ≙ 6h/6h	25·(432s/432s) ≙ 3h/6h	49·(147s/294s) ≙ 2h/6h
Total aeration proportion for cycle duration of 6 h	100%	50%	33.3 %

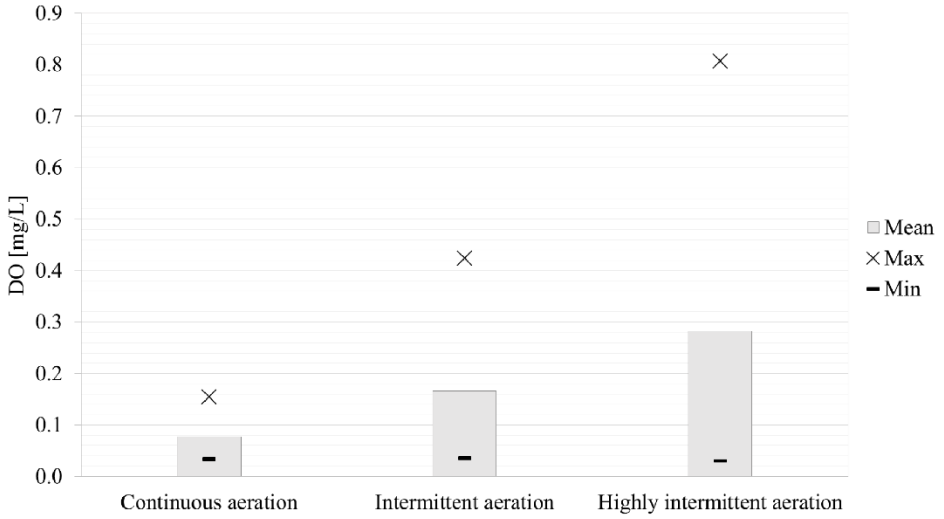

Figure SI- 3: Minimal, mean, and maximal dissolved oxygen (DO) concentrations for the three different aeration strategies

L

IV. Mathematical models

IV.1 Model for the prediction of the nitrogen removal rate

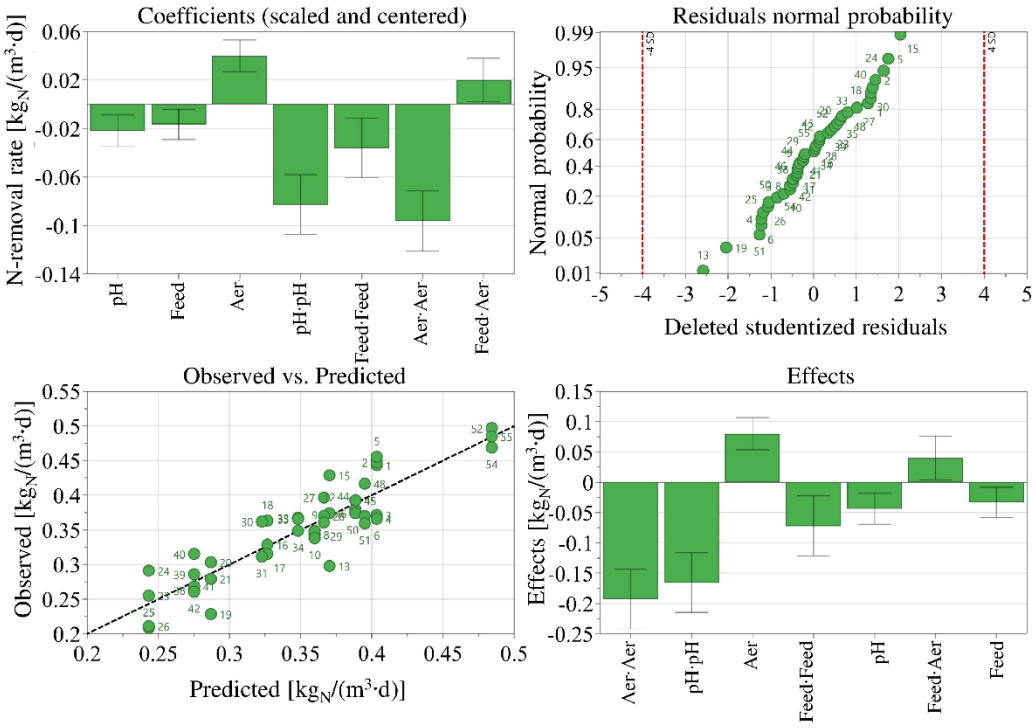

Figure SI- 4: Overview plot for the mathematical model of nitrogen removal (Aer: aeration strategy)

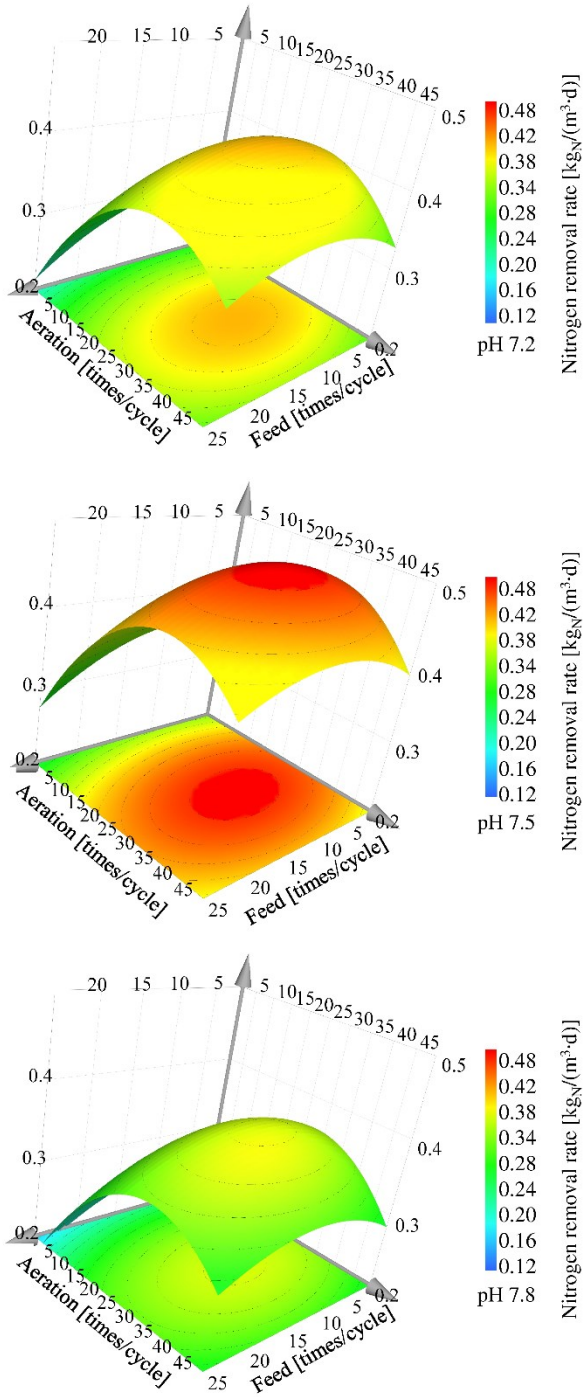

Figure SI- 5: 3D-response contour plots for the influencing factors pH value, feeding strategy, and aeration strategy on the response variable of nitrogen removal (Y_1) at pH 7.2, pH 7.5, and pH 7.8

IV.2 Model for the prediction of N_2O emission

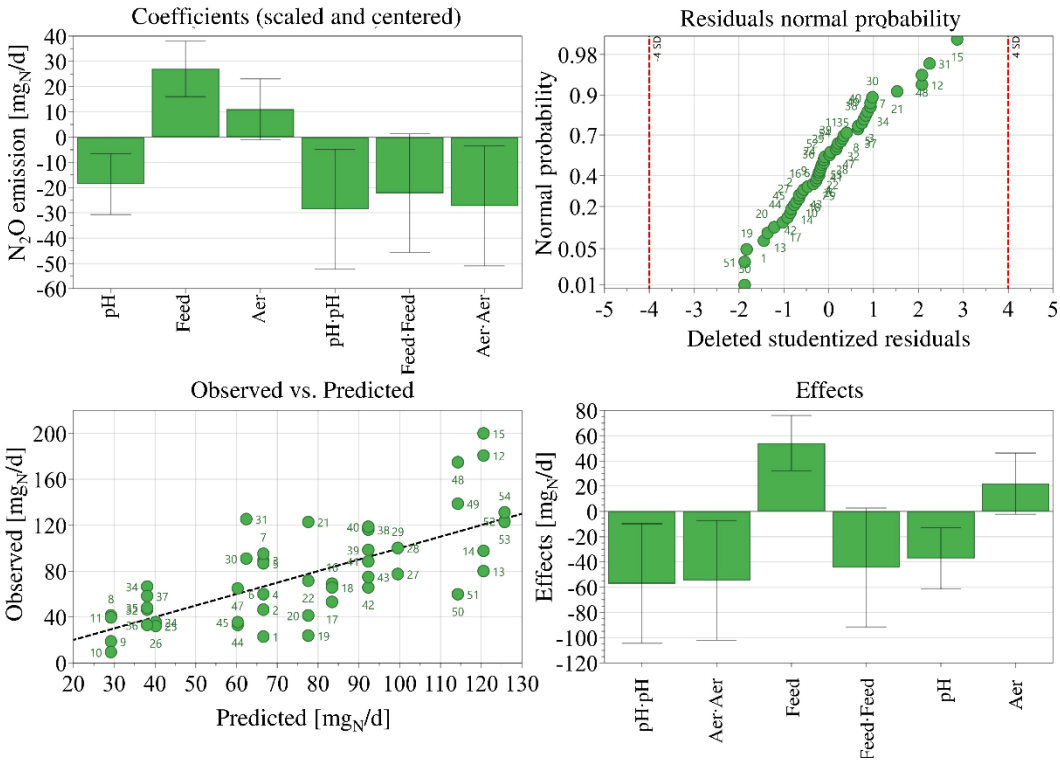

Figure SI- 6: Overview plot for the mathematical model of N_2O emission (Aer: aeration strategy)

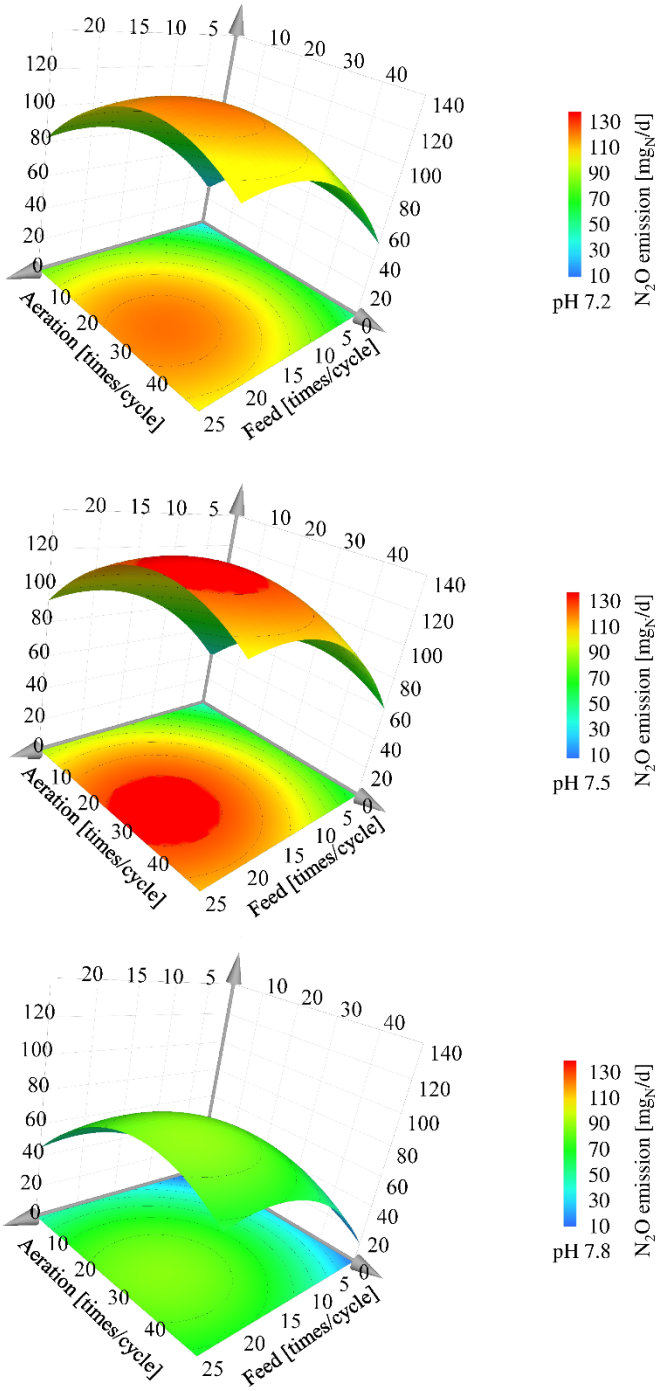

Figure SI- 7: 3D-response contour plots for the influencing factors pH value, feeding strategy, and aeration strategy on the response variable of N_2O emission (Y_2) at pH 7.2, pH 7.5, and pH 7.8

IV.3 Comparison of nitrogen removal and N_2O emission

Table SI- 3: Comparison of predicted nitrogen removal and N_2O emission for all possible combinations of settings (level -1, 0, +1 included) and their percentage share of the mean (N-removal$_{mean}$ = 0.35 $kg_N/(m^3 \cdot d)$; N_2O emission$_{mean}$ = 77.0 mg_N/d)

Operational settings			Prediction		Percentage share of the mean		
pH	Feed	Aeration	N-removal	N_2O emission	N removal	N_2O emission	Emission Factor
[-]	[times/cycle]	[times/cycle]	[$kg_N/(m^3 \cdot d)$]	[mg_N/d]	[%]	[%]	[%]
7.8	25	1	0.17	45.13	49%	59%	2.7
7.2	25	1	0.21	66.54	61%	86%	3.1
7.8	13	1	0.24	40.31	69%	52%	1.7
7.8	1	1	0.24	82.33	70%	107%	3.4
7.5	25	1	0.27	87.49	79%	114%	3.2
7.8	1	49	0.28	92.31	81%	120%	3.3
7.2	13	1	0.29	78.60	82%	102%	2.7
7.2	1	1	0.29	62.34	82%	81%	2.2
7.8	25	49	0.29	120.62	83%	157%	4.2
7.8	13	49	0.32	29.34	92%	38%	0.9
7.8	25	25	0.33	50.28	93%	65%	1.5
7.2	1	49	0.33	-8.95	94%	-12%	-0.3
7.2	25	49	0.33	76.51	95%	99%	2.3
7.5	13	1	0.35	38.22	99%	50%	1.1
7.5	1	1	0.35	28.25	100%	37%	0.8
7.8	1	25	0.36	13.08	103%	17%	0.4
7.2	13	49	0.37	99.55	105%	129%	2.7
7.2	25	25	0.37	125.77	106%	163%	3.4
7.8	13	25	0.38	104.36	109%	135%	2.8
7.5	1	49	0.39	77.52	111%	101%	2.0
7.5	25	49	0.39	130.59	113%	170%	3.3
7.2	1	25	0.40	67.16	115%	87%	1.7
7.2	13	25	0.42	115.80	121%	150%	2.7
7.5	13	49	0.43	60.25	122%	78%	1.4
7.5	25	25	0.43	109.52	123%	142%	2.5
7.5	1	25	0.46	83.42	133%	108%	1.8
7.5	13	25	0.48	114.34	138%	148%	2.4

V. Biological removal of trace organic compounds

A wide range of emerging trace organic compounds (TOrC) including pharmaceuticals, personal care products, and household chemicals have been detected in wastewater treatment plant influents as well as in the effluents. As these TOrCs can adversely affect human and ecosystem health (Alidina et al., 2014), removal during wastewater treatment is desired. However, information regarding a possible additional removal capacity of the side-stream deammonification process is still limited.

Therefore, the TOrC removal by the single-stage deammonification process was screened by analyzing a set of 17 TOrCs using liquid chromatography with tandem mass spectrometry (LC-MS/MS) after sample concentration by solid-phase extraction (SPE) following the protocol as described by Alidina et al. (2014). Three removal mechanisms exist, namely the mechanism of biotransformation, sorption to the biomass and/or volatilization. However, the specific removal pathways were not investigated as this was beyond the scope of this study.

Figure SI-8 displays the process water composition with respect to the 17 TOrCs being under investigation analyzed in the winter/spring months February and March.

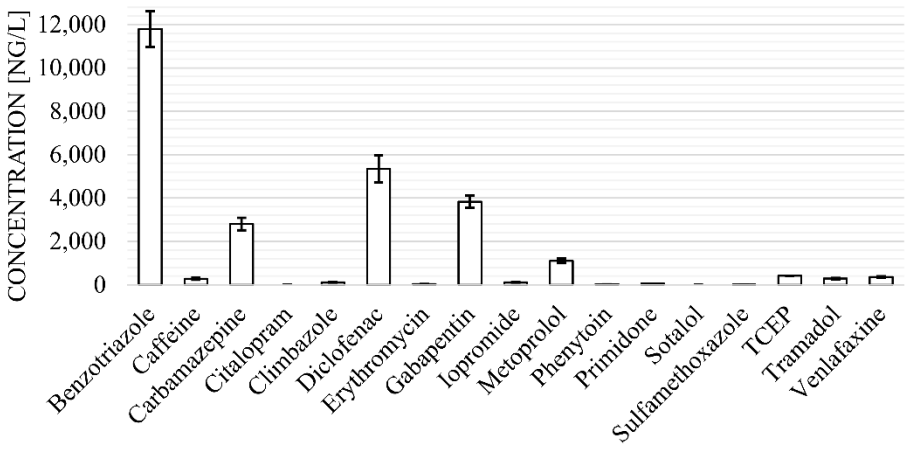

Figure SI-8: Trace organic compounds concentrations of process water (n=3); TCEP: Tris(2-chloroethyl)phosphate

TOrCs with the highest concentrations in the influent (benzotriazole, carbamazepine, diclofenac, gabapentin, and metoprolol) were selected and analyzed (single investigation) for the runs DoE 5, DoE 9, and DoE 11 to investigate the TOrCs removal by the partial nitritation-anammox process (Figure SI-9). For all runs, the pH value at the beginning of the cycle was set to pH 7.5, whereas the feeding strategy and aeration strategy varied from single to highly intermittent feeding as well as continuous to highly intermittent aeration (see Table 1 in the manuscript).

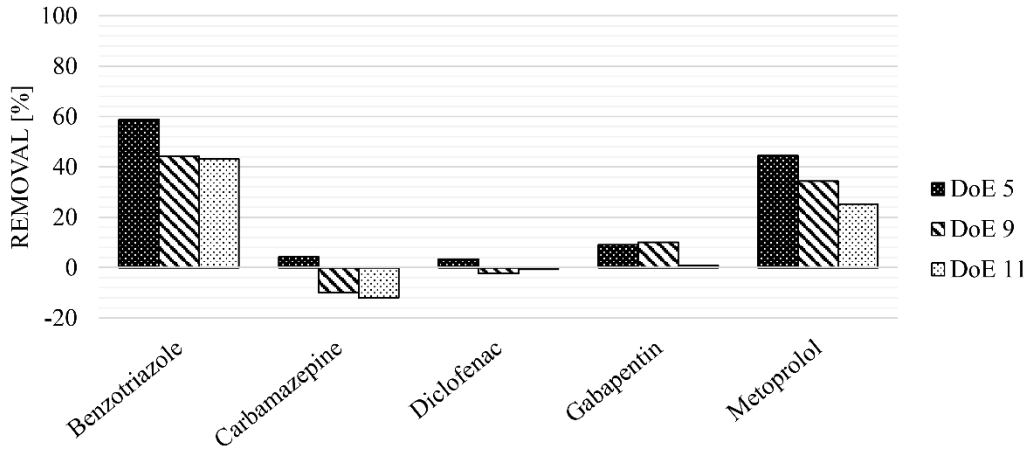

Figure SI-9: Removal of trace organic compounds of single-stage partial nitritation-anammox analyzed for the runs DoE 5, DoE 9, and DoE 11 (n=1)

Removal efficiencies of the three DoE runs with a differing feeding and aeration strategy were all in a comparable range. Even though DoE 5 seemed to remove best four out of the five elected TOrCs, no explicit conclusions regarding favorable conditions for TOrC removal can be drawn based on these single investigations. Carbamazepine, diclofenac and gabapentin exhibited negligible or low removal. This is in line for the removal rate of carbamazepine investigated also in side-stream partial nitritation-anammox by Alvarino et al. (2015) and nitrification/denitrification (Falas et al., 2016) as well as with investigations regarding main-stream partial nitritation-anammox at low temperatures (~ 15°C) for the TOrCs of carbamazepine and diclofenac (gabapentin was not investigated in these studies) (Laureni et al., 2016). However, Alvarino et al. (2015) and Falas et al. (2016) observed a diclofenac removal efficiency of 36±1% and ~ 25%, respectively, whereas no removal took place in our system for all three DoE runs.

In contrast, the side-stream deammonification process contributed moderately to the removal of benzotriazole and metoprolol. These removal efficiencies were comparable to those in a nitrifying and denitrifying process (Falas et al., 2016) and even exceeded the removal of benzotriazole by main-stream partial nitritation-anammox, where no removal could be detected (metoprolol was not investigated in this study) (Laureni et al., 2016).

Thus, the side-stream deammonification process removed four out of the five investigated TOrCs (apart of the diclofenac removal) nearly identically to the biological nutrient removal processes discussed here.

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Falas, P., Wick, A., Castronovo, S., Habermacher, J., Ternes, T.A., Joss, A., 2016. Tracing the limits of organic micropollutant removal in biological wastewater treatment. Water Res. 95, 240–249. 10.1016/j.watres.2016.03.009.

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