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FACULTY OF BIOSCIENCE ENGINEERING

DEPARTMENT OF GREEN CHEMISTRY AND TECHNOLOGY
BIOCO RESEARCH GROUP

PhD Dominika Grubba/Derwis – assessment by Eveline Volcke

Manuscript title

The impact of sulfur transformations on nitrogen removal processes in systems with granular sludge

Overall assessment

This PhD thesis deals with the integration of nitrogen, sulfur and carbon conversions in wastewater treatment systems with granular sludge.

The work starts with a very comprehensive literature review on the incorporation of the sulfur cycle in nitrogen removal systems (paper I). A most substantial amount of experimental work has been conducted, in the form of three series of long-term and experiments, each time in two parallel lab-scale SBR-type reactors, containing granular sludge and operated under non-aerated conditions:

- paper II (160 days): influent containing ammonium and nitrite (no nitrate), sulfate only in SBR2
- paper III (200 days) influent containing ammonium, nitrite (no nitrate), COD (periodically in both reactors) and sulfate only in SBR2
- paper IV (180 days): influent containing ammonium, nitrate (no nitrite), sulfate (periodically in both reactors), COD only in SBR2

The large amount of experimental results were presented in very well cared-for graphs and analysed through mathematical analysis, calculating the process rates. Extensive microbial community analysis has been performed as well. Given the large scale of data collection and the wealth of results in this complex research area, the interpretation of the results was not straightforward. I found the full interpretation of the figures sometimes hampered by data which are not readily available (e.g., influent conditions not on the same figure) and by the use of abbreviations. The mathematical analysis should have been further documented to allow reproducibility of the method by other researchers. I would also have encouraged further sharpening of the research hypotheses and their relation to the experimental plan and the key conclusions.

Overall, I judge the overall scientific level and originality of the work as very good and I look forward to discussing with the candidate to get further insights.

General questions

For the candidate's consideration - not necessarily to be addressed in the manuscript

Please elaborate on the experimental design. How did you decide on the influent concentrations/combinations of the different compounds to be tested, on the duration of the different experimental phases and the duration per study (per paper)?

All experiments were conducted for synthetic wastewater and under non-aerated conditions. How does this impact the applicability of the findings?

Do you consider SRAO/sulfammox to be a single reaction? Is it carried out by a dedicated species? A fixed stoichiometry was assumed for the SRAO reaction in the calculation of the process rates. What is the reference for that stoichiometry? Is the SRAO stoichiometry really fixed?

The summary mentions that 'Advanced stoichiometric models introduced in this work offer new opportunities for optimizing reactor configurations'. Please elaborate on this.

Chapter-wise comments

Summary

The summary describes the aim of the doctoral dissertation and the most important results.

Requested revision
Please indicate to which chapters (papers or PhD thesis section) each of the listed results correspond.

1. Introduction

The introduction states the importance of and challenges in integrating the N, S and C cycles in wastewater treatment, as well as the originality of the research. The **description remains quite general**, with little specific facts and figures as to why the coupling of N-S-C cycles is beneficial.

Requested revision
Please add a figure / table to allow the reader to understand which are the conversions under study, in particular what is SRAO (sulfate reduction ammonium oxidation) and SDAD (S-dependent autotrophic denitrification) – introduce the reactions in terms of substrates and products.

2. Objective and scope

The requested addition of a figure in Section 1 is also important for a good understanding of this section.

3. Methodology

This section gives a good overview of the laboratory setup, the calculations and the microbial analyses applied in this dissertation.

Please elaborate on the methodology to calculate the process rates
Section 2.4 of paper II indicates that the maximum slopes of concentration profiles were used to determine the specific process utilization rates, and that a stoichiometric analysis was carried out using the Solver utility in Excel from four given reactions. ***It is not clear to me how exactly the overall rates were decomposed and attributed to the specfic processes. I would like to see this described in detail.*** It would also be most helpful if the Excel files with the calculations were provided as supplementary information with the corresponding chapters.

- How **sensitive are the results to the assumed reaction stoichiometries** (e.g., assumed anammox stoichiometry, neglecting biomass growth)?
  Note: the value 1.32 in the anammox stoichiometry is not determined by Chen et al. (2018) as you state in Paper II, Section 3.2, but by Strous et al. (AMB, 1998) You may also want

to consider other anammox stoichiometries reported in literature, such as the ones from Lotti et al. (WR, 2014) or from Jia et al. (ESWRT, 2018).

- Were the **results presented in Paper II, Figure 2** obtained directly from the concentration profiles (slopes) or from stoichiometric analysis and decomposition of process rates (in Excel)?
- Paper II Figure 2a: would you expect the sum of the individual rates for Anammox and SRAO to equal the rate for 'Anammox +SRAO'? This seems not the case in the figure.

- Paper III starts from the same four reactions as paper II, but adds three heterotrophic reactions (as COD is added, in contrast to paper II). How does this influence the identifiability of the process rates?
- The supplementary material of paper III gives the separate catabolic and anabolic reactions – why? The anabolic reactions are neglected in the calculation, aren't they?
- Paper III Section 3.2. "The optimization process yielded highly accurate results with a single set of stoichiometric coefficients. … The measured and calculated values of rates and rations matched very accurately." How were the rates measured exactly – independently from the calculations?

Paper I: Critical review on incorporation of the sulfur cycle in sustainable nitrogen removal systems

Grubba et al., 2022, Journal of Cleaner Production.

This review contains an **exceptionally large amount of information, in a well-structured way**. It first reviews the operational conditions and performances of stand-alone S-dependent processes (Section 3 and Fig. 1), namely (3.1) S-dependent autotrophic denitrification, (3.2) heterotrophic sulfate reduction and (3.3) sulfammox. This is followed by a review of wastewater treatment systems integrating these processes (Section 4) organised according to (4.1 – Fig. 3) SANI-like processes, (4.2 – Fig. 4) systems with anammox and (4.3 – Fig. 5) those incorporating sulfammox. Section 5 then summarizes the operating conditions and performances of those systems, in the same order. Modelling is briefly touched upon in Section 6, while Section 7 discusses general process application opportunities and challenges.

The large amount of information is illustrated with **very clear, well-cared for figures**.
While the review article summarizes a huge amount of valuable information, **further steps could have been made regarding the synthesis** (references are often discussed one by one rather than compared), **focus and critical interpretation**. Nevertheless, I see the difficulty in balancing completeness with focus. I would therefore call it a **comprehensive review rather than a critical review**.

Specific comments and questions to be addressed in the manuscript

(page numbers refer to the paper, not to the PhD thesis)

- The introduction (section 1) may be not so easy to follow for somebody who does not yet have a background in the reactions concerned. It would be easier to follow if the reactions were introduced in terms of substrates and products.

- p4 'it was shown that autotrophic denitrification with S compounds emitted significantly less N_2O than heterotrophic denitrification.' Only 1 reference is given for this statement (Cui et al., 2019) – some others are given in the S.I. (Huang et al., 2019, Sun and Nemati, 2012, Yang et al., 2016 - line 22) even though it is not clear whether the latter

refer to the N_2O emissions or rather to other process characteristics. *Please specify and strengthen the references for this statement.*

Questions for the candidate's consideration

- Do you consider (autotrophic) sulfammox to be a standalone process? On which basis? Is Sulfammox really a separate species? (Fig1)

- Less N_2O emissions with autotrophic denitrification than heterotrophic denitrification? References? (see specific comment above). Seems not so well supported by the references – still in the thesis summary it is said that the literature review highlights that S compounds as e donors lead to less greenhouse gas emissions.
- See also p17 (introduction) 'Combination of PD and anammox has been shown to reduce energy demand and lower GHG emissions compared to conventional methods (Al-Hazmi et al., 2023, Bioresour Technol).' – any more proof in the latter reference?
- p8 "The SANI process reduced GHG emission by 36% compared to conventional N removal processes (Lu et al., 2011)". In which sense?

- p12 'Using specific N, S and C removal processes independently of each other is much easier to maintain than the processes combining these cycles. Is that really so? On which basis do you conclude this?

Paper II: Integration of the sulfate reduction and anammox processes for enhancing sustainable nitrogen removal in granular sludge reactors

Derwis et al., 2023, Bioresource Technology.

Overall assessment and questions for the candidate's consideration

- In this study, 'it was tested whether **sulfate reduction and anammox occur independently or are related to each other**' (cfr. abstract). It was found that 'a higher ammonium utilization rate and sulfate utilization rate were achieved' when increasing influent sulfate concentrations (also stated in abstract). However, these findings were not related to the ***tested hypothesis, on which I miss a clear answer***. Do sulfate reduction and anammox occur independently or are related to each other? *The goal and the conclusions of this study need to be made more clear.*
- In the highlights and in the introduction, it is mentioned that the performance of anammox and SRAO was compared **with and without nitrite addition**. However, this is not mentioned in the abstract, nor related to the tested hypothesis. *Please relate to the tested hypothesis.*
- Overall, I find the results from this study being described quite specifically (cfr. the abstract), while mixed with general discussion from the literature. For instance, it is indicated that increasing the influent sulfate concentrations lease to a higher ammonium utilization rate – which applications do you see for this finding? In particular, what if sulfate removal is not targeted? *Please elaborate on the more general results from this study.*

Specific question

Section 3.6 'The main process for sulfate removal was the independent reduction of sulfate by SRB using organics compounds.' I do not see the meaning of this sentence at this point. In this study, there were no organics present, were there?

Paper III: The role of the combined nitrogen-sulfur-carbon cycles for efficient performance of anammox-based systems

Derwis et al., 2024, Science of the Total Environment.

In this paper, the effect of sulfate and organic carbon on anaerobic ammonium oxidation was studied. As in paper II, long-term experiments were conducted on two parallel non-aerated SBRs, only one of which (SBR2) was fed with sulfate. Organic carbon was fed periodically in both reactors.

It was found that the ammonium utilisation rates and nitrite utilization rates were higher with **sulfate addition** (SBR2) (Section 3.1, p5). From this, it was hypothesized that 'sulfate as an additional electron acceptor fostered the proliferation of bacteria responsible for anaerobic ammonium oxidation. (Section 4.1, p10).' *You are referring to anammox bacteria, aren't you? How do you see this exactly?*

In both reactors, the ammonium uptake rate and nitrite uptake rate were higher during the phases with **COD addition** (Section 3.1, p5). *Why is that exactly?*

Sulfate production occurred during periods with COD addition, while sulfate reduction took place during periods with COD addition (Section 3.1, p7).

"The strong correlation between sulfate production and COD uitilization was implictly due to mixotrophic dentrification by SDAD (sulfur-based autotrophic denitrificaiton) and increased activity of autotrophic denitrifiers using sulfide/S0 and heterotrophs using COD" (Section 4.2 p11). *In which sense are these coupled?*

It is mentioned that the presented systems are suitable for treatment of wastewate rfrom mining, fermentation, landfil leachate and pulp and paper production (Section 4.3, p12). *Do these wastewaters contain nitrite/nitrate?*

It is shown in this study that it is important to have a certain sulfate concentration and to have the right C:N balance for anammox - it is said that applying alternating COD conditions is essential. But *with real wastewater, the influent composition cannot be manipulated, can it? So how to make the system perform in an optimal way?*

Paper IV: Enhancing nitrogen removal in the partial denitrification/anammox processes for sulfate - rich wastewater treatment: Insights into autotrophic and mixotrophic strategies

Derwis et al., 2024, Journal of Environmental Management

This paper contains a third series of long-term (180 days) experimental work in two parallel SBRs. In contrast to the studies of paper II and paper III, there was no nitrite present in the influent but the reactors were instead fed with nitrate, besides ammonium, and sulfate (periodically). Organic carbon (COD) was fed only in SBR2.

It was demonstrated that sulfate-dependent systems are capable of generating nitrite to facilitate ammonium oxidation.

Specific comments and questions

5. Conclusions (p10) "Optimal N/S and N/S/C ratios were determined to maximize TN and sulfate utilization in SBR1 and SBR2". *What are the optimal ratios and how were they determined exactly? Is it really the ratios as such which are optimal or do they depend on the concentrations of certain influent concentrations?*

2.4 Determination of specific process rates (p3). "All cacluated process rates underent rigorous statistical analysis and were subjected to N-S-C mass balances (Table S3)."
Please explain how to interpret the mass balances from Table S3.

Formatting/style

PhD title:
remove 'the' =>
"Impact of sulfur transformations on nitrogen removal processes in systems with granular sludge"
Alternative:
"Impact of sulfur transformations on nitrogen conversion processes in systems with granular sludge"

Table of contents

Please also include subsections of paper I
Also paper II, III, IV?

List of abbreviations

- Check for completeness. Not all abbreviations are included.
  E.g. for paper I: AMD, AAOB, PNASD....
- Is AnAOB (in list of abbreviations) the same as AAOB (in Chapter I)?

In general, I think the readability of the manuscript would be substantially improved by limiting the amount of abbreviations.

Typos

Summary p1 nitrites => nitrite (nitrite is a strong acid, there is only 1 type of 'nitrite')
List of abbreviations p16 WWTP = ~~municipal~~ wastewater treatment plant
p24 'a working volume of either 10L' => 'a working volume of 10L each'

p26: (on paper III) 'The dynamic changes in influent concentations of NH4+, NO2-, NO3-, ...'
There was no nitrate addition in the study of paper III, was there?
If not, please leave out 'NO_3^-' and state explicitly that there was no nitrate addition.

Signature

Gent, 22 June 2025

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