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DEVELOPMENT AND IMPLEMENTATION OF A MONITORING SYSTEM FOR ANAMMOX BIOMASS

Master thesis

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Diplomingenieur

submitted by:

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Abstract

This thesis was imbedded in a project focussing on the implementation of deammonification for middle scale wastewater treatment plants in Austria. Therefore, a monitoring scheme was developed to analyse the system's state, the biomass activity and fitness in a cost and time efficient way. To obtain a sufficient monitoring system, a literature review of existing monitoring approaches has been conducted and several methods have been tested for their applicability. The two most promising tools according applicability and expressive power – heme c concentration analysis and specific Anammox activity assessment (SAA) – have been established at the laboratory of the Insitute of Sanitary Engineering and Water Pollution Control. Heme c concentration measurement was based on fluorescence spectroscopy. The method establishment included several optimization steps to obtain an appropriate calibration. SAA was analysed by UV-Vis spectroscopic measurements of the N-compounds according to the stoichiometry of the Anammox process. To assess their accuracy and applicability, the two tools were evaluated during a multifactorial inhibition assay at the presence of several inhibitor combinations. For this purpose, a 2^3 set-up was chosen analysing the effect of nitrite, Fe(III)Cl_3 and a coagulant/polymer on the Anammox biomass. The experiment included four measuring cycles over a run time of twelve days. For the evaluation of the methods' accuracy, several linear models were fitted to the experimental data. It could be proven that the combined addition of a high (105 mg/L) NO_2-N and polymer concentration inhibits the biomass, while the addition of Fe(III)Cl_3 enhances its performance. Heme c concentration was correlated with nitrogen removal rate and Fe(III)Cl_3, SAA was able to show the adaption of the biomass to the inhibitors and fluctuated faster than heme c concentration.

Abstract in german

Diese Arbeit ist Teil des „DEKO"-Projektes, das die Implementierung von Deammonifikation auf mittleren Kläranlagen in Österreich anstrebt. Um dies zu erreichen wurde ein Monitoring-System entwickelt, das den Zustand des Systems und Aktivität und Fitness der Biomasse kosten- und zeiteffizient abbilden soll. Zu diesem Zweck wurden in der Literatur bereits angewandte Monitoring-Methoden analysiert und einige ausgewählte im Labor auf ihre Anwendbarkeit überprüft. Dies führte zur Etablierung der zwei Methoden, die bezüglich Einfachheit in der Anwendung bei gleichzeitiger Aussagekraft die vielversprechendsten Aussichten lieferten: Häm c Analyse und spezifische Anammox Aktivitätsmessung (SAA), im Labor des Instituts für Siedlungwasserbau, Industriewasserwirtschaft und Gewässerschutz. Die Messung der Häm c Konzentration basierte auf Fluoreszenzspektroskopie. Die Methode wurde für die Anammox-Biomasse durch mehrere Maßnahmen optimiert. SAA wurde über Bestimmung der Stickstoffkonzentrationen und die Stöchiometrie der Anammox Bakterien bestimmt. Um die Genauigkeit der Methoden zu überprüfen wurde ein 2^3 multifaktorielles Inhibierungsexperiment durchgeführt. Die drei potentiellen Hemmstoffe Nitrit, Fe(III)Cl_3 und ein Polymer wurden auf ihren Einfluss auf die Anammox Biomasse untersucht. Das Experiment hatte eine Laufzeit von zwölf Tagen und beinhaltete vier Zyklen um kumulierende Effekte abbilden zu können. Um die Performance der Methoden zu überprüfen wurden die Ergebnisse mit mehreren linearen Modellen ausgewertet. Es konnte gezeigt werden, dass die kombinierte Zugabe einer hohen (105 mg/L) NO_2-N Konzentration und eines Polymers zur Inhibierung der Biomasse führt, während Fe(III)Cl_3 positiv auf die Abbaurate wirkt. Die Häm c Konzentration korrelierte mit der Stickstoffabbaurate und der Fe(III)Cl_3 Zugabe, SAA konnte die Adaptierung der Biomasse an die Hemmfaktoren zeigen und fluktuierte stärker als die Häm c Konzentration.

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Abbreviations

WWTP	Wastewater treatment plant
Anammox	Anaerob ammonium oxidizing bacteria
SIG	Institute of Sanitary Engineering and Water Pollution Control
DEKO	Deammonification for small and middle scalewastewater treatment plants in Austria
AOB	ammonia-oxidzing bacteria
SHARON	Single reactor system for high activity ammonium removal over nitrite
PNA	Partial nitritation – Anammox process
CANON	Completely autotrophic nitrogen removal over nitrite
AA	Anammox activity
SAA	Specific Anammox activity
SBR	Sequencing batch reactor
DO	Dissolved oxygen
hh	Hydrazine hydrolase
hd	Hydrazine dehydrogenase
HAO	hydrazine/hydroxylamine oxidoreductase
qPCR	Quantitative polymerase chain reaction
FISH	Fluorescence in situ hybridization
SS	Suspended solids
VSS	Volatile suspended solids
HZS	Hydrazine synthesis
HZO	Hydrazine oxidase

1. Introduction

Due to ongoing settlement development and the high average age of wastewater disposal infrastructure in Austria, many wastewater treatment plants (WWTP) already reached their design capacity. To increase capacities, the construction of additional reactor volume is cost and space intensive. Process optimization is an alternative to increase the performance avoiding substantial investment. Modern wastewater treatment plants combine mechanical and biological treatment steps to remove course matter, organic carbon and nutrients from wastewater. Within the process chain, the nitrogen conversion and removal is a sensitive and energy intensive step.

Chargeback from sludge dewatering sums up to 20 % of total nitrogen inflow load. The reduction or separate treatment of this ammonium rich reject water can therefore reduce the necessary energy amount. The implementation of a deammonification driven nitrogen removal for ammonium rich wastewater resulting from sludge treatment can be a promising method (Wett et al., 2007). Using this process, the plant capacity in terms of nitrogen removal increases due to the removal of ammonia nitrogen of the recycle flow (reject water) from sludge dewatering.

Deammonification, which is characterized by partial nitritation combined with anaerobe ammonium oxidation, uses the capability of the 1999 discovered Anammox bacteria (Strous et al., 1999) to convert ammonium and nitrite directly to dinitrogen gas. Through the exclusion of nitratation and denitrification, the oxygen demand can be reduced. The large scale feasibility of the Anammox process has been proven through several implementations (Lackner et al., 2014). Especially the treatment plant in Strass i. Zillertal, Austria (Wett et al,. 2007), is well investigated.

Nevertheless, the Anammox process and possible inhibiting factors are yet not fully understood. This lack of knowledge leads to difficulties in the introduction of deammonification at middle scale WWTPs. As these plants do not possess personnel capacity to run a complex monitoring system or adapt the process engineering accordingly to the specific needs of the two involved bacterial species, ammonia oxidising bacteria (AOB) and Anammox bacteria.

A national research project (‘Deammonification for middle scale wastewater treatment plants in Austria’), in which this thesis is embedded, focuses on these problems hindering deammonification from being implemented at a broader scale at municipal WWTPs. Besides the optimization of process engineering, the development and implementation of an adapted, time- and cost-efficient monitoring presents a major field of interest in the project.

Research on possible monitoring methods has already been conducted (Podmirseg et al., 2015), but the proposed monitoring scheme has not yet been tested in long-term experiments or under the influence of possible inhibitors that may occur during standard plant operation. Further, the current operation scheme of deammonification is designed for large scale WWTPs with a well-equipped lab and trained staff to analyse the biomass. Therefore, further research on the applicability of an efficient monitoring at middle scale municipal WWTPs and its relevance for inhibiting conditions has to be conducted.

As the research on inhibiting factors is dominated by inhibitors which are found in industrial wastewaters (Jin et al., 2012; Zhang et al., 2012; Zhang et al., 2014) and substrate inhibition (Carvajal-Arroyo et al., 2013; Dapena-Mora et al., 2007) determined by short term inhibition assays, knowledge of the effect of commonly process additives on deammonification is sparse. Nevertheless, the application of deammonification in the treatment of reject water from sludge dewatering requires this research to enhance a stable process with optimal treatment performance.

2. Objectives

The aim of this thesis is the elaboration of a monitoring scheme for Anammox-biomass using standard equipment, which is already available at middle-scale wastewater treatment plants. Nevertheless, the monitoring methods shall give a complete and detailed overview of the status and performance of the system.

In order to develop an appropriate monitoring scheme, the following detailed objectives had to be achieved:

- Evaluation of existing monitoring methods
- Combination of promising existing methods
- Development of an adapted monitoring protocol

Finally, it was the goal to apply the developed approach as basis to determine the impact of different possible inhibitors occurring under practical conditions of municipal wastewater treatment plants.

In the first part of the thesis, the fundamentals of the anaerobe ammonium oxidizing process such as microbiology, chemistry and process technology are presented. Besides, the general concept of nitrogen removal through biological treatment in wastewater treatment plants will be discussed. Afterwards, the main concepts for implementing the Anammox-process in sanitary engineering will be introduced.

Chapter 4 describes the methods used such as different nitrogen-measurement tools, heme analysis and particle tracking.

In the results and discussion section, the obtained nitrogen, heme C and particle size data are presented and evaluated. Their advantages and disadvantages are discussed and a suggestion for the usability of the methods is made. At last, a summary of the thesis is given and the most important conclusions are drawn.

3. Fundamentals

At conventional wastewater treatment plants, reject water redistributed from sludge dewatering into the main stream has a high influence on the treatment capacity of the plant as it presents up to 20% of the inflow load and possesses a low C:N ratio (Schaubroeck et al., 2015, Figure 1). Especially for plants reaching their design maxima or already overloaded plants, the implementation of an optimized reject water treatment presents an option to increase their treatment capacity and prevent costs arising from the construction of additional reactor volume.

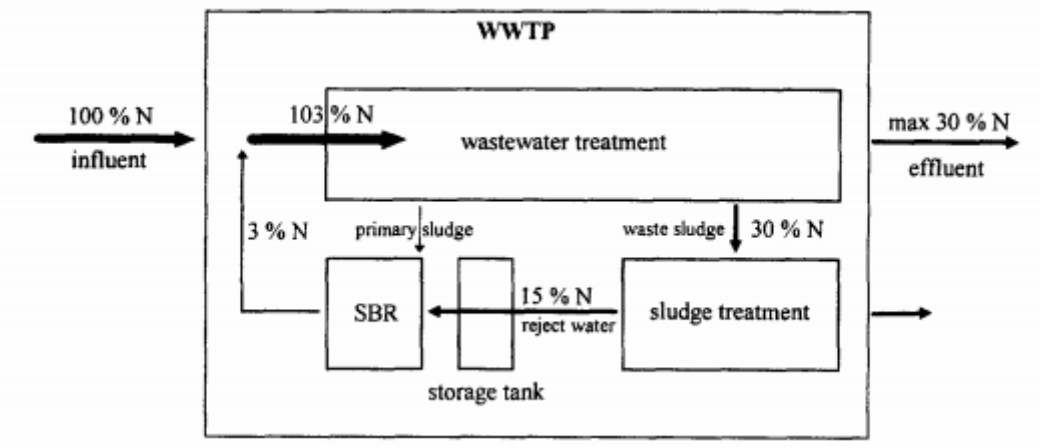

Figure 1: Simplified process schematic of the biological stage for a WWTP with side stream treatment of reject water from sludge treatment as installed at the WWTP AIZ Strass i. Zillertal. The SBR works as a PNA reactor (Wett et al., 1998).

This side stream treatment of process water from sludge dewatering has to be suitable for wastewater with high ammonium loads and a low C:N ratio (Egli et al., 2001). Several large-scale implementations (Wett et al., 2007, Lackner et al. 2014) have shown that deammonification is feasible to treat reject water efficiently and reduces the nitrogen backflow to the main stream from sludge treatment.

While one of the two microbial species used in the deammonification process – the aerobe ammonium oxidizing bacteria – are already well investigated, the second one – Anammox bacteria- were discovered just 20 years ago. Therefore, this chapter focuses at first on the Anammox process and the involved bacteria (3.1.1 and 3.1.2) to clarify the bacteria's kinetics, their living environment and hence the conditions for the application. Secondly, the implementation of the deammonification process (3.1.3) and possible inhibitors are discussed (3.2).

3.1 Principles of the Anammox process

While Hamm and Thompson already assumed in 1941 that anaerobic ammonium oxidation could be the missing link in the nitrogen cycle and Broda stated in 1977 that a lithothrop was missing, the proof of a planctomycete being the missing link was not made until 1999 by Strous et al. Beforehand, van de Graaf et al. (1995 and 1996) and Mulder et al. (1995) found proof of the Anammox process in a WWTP and a denitrification pilot plant in the Netherlands, but could not identify the responsible bacteria. Strous et al. (1999) were able to enrich the chemolithoauothropic bacteria which were characterized as planctomycete, Candidatus Brocadia anammoxiadans later on (Kuenen and Jetten, 2001).

As stated by Strous et al. (1999), "anaerobic ammonium oxidation (Anammox) is the biological conversion of ammonium and nitrite to dinitrogen gas". This shortening of the standard ammonium depletion process over nitrification and denitrification applied by most wastewater treatment plants using activated sludge systems, may provide many advantages. Anyhow, the process is still not fully understood.

This chapter gives an overview on the biochemical and microbiological basics of the Anammox process and the responsible planctomycetes.

3.1.1 Microbiological specification of Anammox bacteria

The “missing lithotroph” (Strous et al., 1999) being responsible for the Anammox process has been identified as planctomycete by Strous et al. (1999). This bacterial order has a compartmented structure with “membrane-bound cell compartments” (Strous et al., 1999) which allows their identification. Some basic properties of the Anammox bacteria are:

- Doubling time of 11-20 days
- Coccoid cells < 1 µm
- Obligate anaerobs

Until today five genera of Anammox with 15 species are known (Sonthiphand et al., 2014) occurring in marine or non-saline water and treatment plants. The five genera with the number of species are:

- Candidatus Brocadia: 3 (Kartal et al., 2008, Oshiki et al., 2011, Rothrock et al., 2011)
- Candidatus Kueninia: 1 (Schmid et al., 2000)
- Candidatus Scalindua: 9 (Kuypers et al., 2003, Woebken et al., 2008, Hong et al., 2011, Fuchsmann et al., 2012, Dang et al., 2013, van de Vossenberg et al., 2013)
- Candidatus Anammoxoglobus: 1 (Kartal et al., 2007)
- Candidatus Jettenia: 1 (Quan et al., 2008)

Most of these Anammox species have been discovered in engineered systems, but several species have been detected in natural ecosystems, which confirms that Anammox are more widespread than previously assumed and main contributors to the marine nitrogen cycle (Sonthiphand et al., 2014; Kartal et al., 2007).

As Anammox belong to the phylum *Planctomycetes* they have some special properties such as

- The absence of peptidoglycan
- Proteinaceous cell wall
- Compartmentalized cytoplasm
- Two membranes at the inner cell wall and no outer membrane

Yet, unlike most planctomycetes – which are aerobic chemoorganoheterotrophs – Anammox are anaerobic chemolithoautotrophs with a unique cell structure (van Niftrik et al., 2004): They possess a third membrane surrounding the so called Anammoxosome, which is characterized as “bacterial ‘organelle’ or vacuole without ribosomes” (van Niftrik et al., 2008). Therefore the cytoplasm of Anammox cells is separated into three compartments which are “bounded by individual bilayer membranes” (van Niftrik et al., 2008). These factors lead to a proposed cell scheme, which is shown in Figure 2 and Figure 3. As it has been surveyed that the enzymes responsible for Anammox catabolism are mainly situated at the Anammoxosome (van Niftrik et al., 2004). This supposedly plays a key role in the Anammox process.

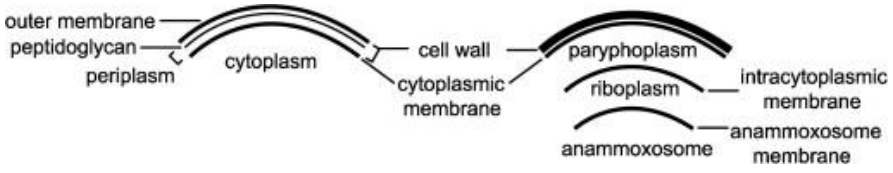

Figure 2: Schematic drawing comparing a Gram-negative bacteria cell-plan (left) and an Anammox bacterium (van Niftrik et al., 2008).

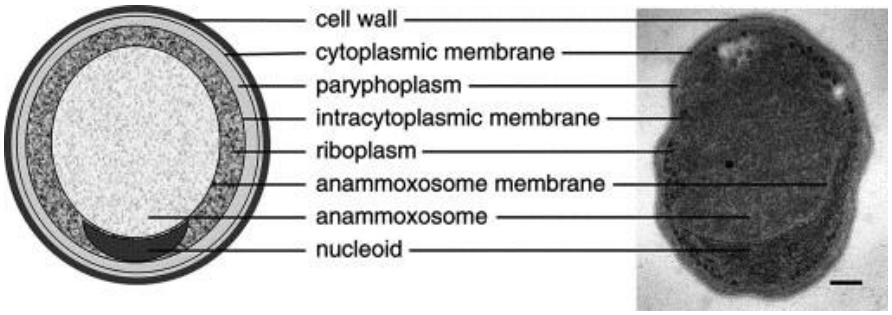

Figure 3: Cellular compartmentalization in Anammox bacteria. Left: schematic drawing, right: thin section of cryosubstituted Candidatus "Brocadia Anammoxidans" displayed via transmission electron microscopy. Bar 100 nm. (van Niftrik et al., 2004).

Figure 4 shows the curved and highly folded configuration of the bilayer Anammoxosome membrane and the storage of iron in the Anammoxosome by electron-dense particles (van Niftrik et al., 2008). This finding is supported by research conducted by Strous et al. (2006) who found two iron storage genes in the genome of Candidatus K. stuttgartiensis. The reason for the iron storage could lay in the facultative iron respiration conducted by Anammox or in the enhanced iron need for the production of heme-containing enzymes which accumulate to >20% [w/w] of the total cell protein (van Niftrik et al., 2008). In Candidatus Kuenenia stuttgartiensis 61 c-type cytochrome proteins have been observed in the Anammoxosome (Strous et al., 2006). As these proteins, which are involved in the electron transport chain, and the hydrazine/hydroxylamine oxidoreductase (HAO) have both been located in the Anammoxosome, the Anammox reaction assumably happens in the Anammoxosome (Jetten et al., 2009).

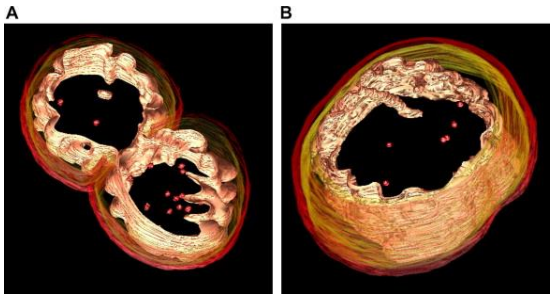

Figure 4: Snapshots of "Candidatus K. stuttgartiensis" electron tomography models showing the curved Anammoxosome membrane and iron particles inside the Anammoxosome. (A) Dividing Anammox cell. (B) Single cell with deep protrusions of the Anammoxosome membrane into the Anammoxosome. Models show (from out- to inside) cell wall (in transparent red), intracytoplasmic membrane (in transparent yellow), Anammoxosome membrane (in pink), and Anammoxosome particles (in red) (Niftrik et al., 2008).

Another atypical structure in the Anammox cell configuration are the lipids of the Anammox membrane: They are a combination of ether- and ester-linked membrane lipids, which are typical for either Archaea or Bacteria and Eukarya, but normally do not occur together in one organism. Additionally, the ethers and fatty acids include ladderanes. These ladderane membrane lipids possess a unique structure which has so far been found nowhere in nature except Anammox bacteria (van Niftrik et al., 2008).

The modelled (Sinninghe Damsté et al., 2002) tightly packed ladderane lipid membranes could be developed in response to the very slowly proceeding Anammox metabolism. Low enzymatic turnover rates enables diffusion of protons and intermediates through membranes (Strous et al., 2006) and the ladderane membrane could prohibit or at least decrease proton leakage and the loss of intermediates produced during the Anammox metabolism as the ladderanes' density makes them impermeable for apolar compounds (van Niftrik et al,. 2008).

3.1.2 Biochemical reflections on the Anammox process

Anammox bacteria shorten the process of oxidizing ammonium through directly using ammonium and nitrite, following the equation postulated by Strous et al. (1999).

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- - +0.13\ H^+$$

$$\rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O$$

This equation can be separated in two processes: hydrazine hydrolisation through hydrazine hydrolase and hydrazine oxidation through the enzyme hydrazine oxidoreductase which forms a metabolic system - including the obligatory nitritation before the Anammox process- as described in Table 1.

Table 1: Enzymes of the nitritation and nitratation/Anammox cycle and the reactions they catalyze. Reactions are shown as redox half reactions where the enzyme itself acts as the primary electron acceptor or donor (adapted after Jetten et. al., 2009).

Process/enzyme	Reaction			$E^{0'}$ (V/e^-)	Location
Ammonia monooxygenase	$NH_4^+ + O_2 + H^+ + 2e^-$	→	$NH_2OH + H_2O$	0.73	Transmembrane
Hydroxylamine oxidoreductase	$NH_2OH + H_2O$	→	$NO_2^- + 5H^+ + 4e^-$	-0.06	Periplasm
Nitrite oxidoreductase	$NO_2^- + H_2O$	→	$NO_3^- + 2H^+ + 2e^-$	-0.43	Membrane associated
Hydrazine hydrolase	$NH_4^+ + NO + 2H^+ + 3e^-$	→	$N_2H_4 + H_2O$	0.34	Anammoxosome
Hydrazine oxidoreductase	N_2H_4	→	$N_2 + 4H^+ + 4e^-$	-0.75	Anammoxosome

The identification of the involved enzymes - Table 1Figure 5 - and cell compartments leads to a proposed catabolic pathway as can be seen in Figure 5. The responsible enzymes include several cytochrome molecules. The importance of these molecules for the Anammox process is explained in more detail in chapter 3.3.2.

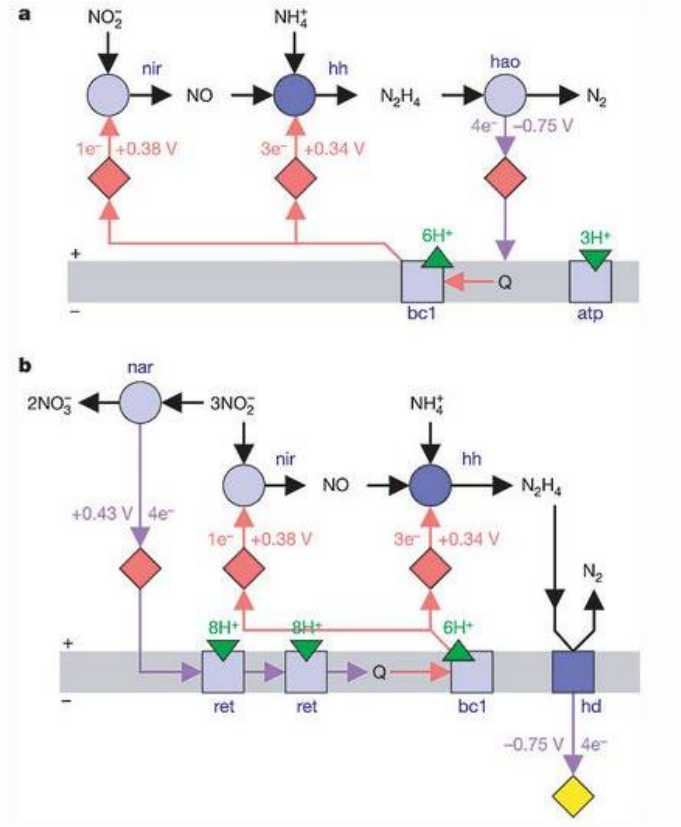

Figure 5: a) Anammox central catabolism with nitric oxide as intermediate, electron transport and energy conservation; b) combination of central catabolism with nitrate reductase to generate high potential electrons for the acetyl-CoA pathway. Red diamonds, cytochromes; yellow diamond, ferredoxin; red arrows, reductions; purple arrows, oxidations. (Strous et al., 2006).

The pathways of Figure 5 reveal two enzymes which are unique to Anammox bacteria: hydrazine hydrolase (hh) and hydrazine dehydrogenase (hd). As described in Table 1, hydrazine hydrolase produces hydrazine out of ammonium and nitrite while hydrazine dehydrogenase converts hydrazine into dinitrogen gas and transfers the revealed electrons into ferredoxin (Strous et al. 2006).

As Anammox bacteria obtain their energy for maintenance and growth from a chemolitothropic conversion of ammonium and nitrite (almost 1:1) to dinitrogen gas possessing and energy of $\Delta G°' = -275$ kJ mol^{-1} NH_4^+. and use exclusively bicarbonate as carbon source for the synthesis of their cell biomass (Jetten et al., 2009), they are autotrophs.

Although Anammox bacteria have a high affinity to their substrates and are capable of depleting them until very low concentrations (K_s < 5 µM) are reached, they posses a slow metabolism forming "15 – 80 µmol of N_2 per g dry weight of cells per min" (Jetten et al., 2009). This fact can explain the slow growing rate of 11-20 days of Anammox bacteria to some extent resulting in a competitive weakness in comparison with faster growing microorganisms used in wastewater treatment (Table 2, chapter 3.1.3). Therefore, a single stage reactor operation of Anammox bacteria with ammonium oxidizing bacteria (AOBs) requires an adapted reactor management and process engineering to promote Anammox biomass and surpress the accumulation of AOBs (chapter 3.1.3).

However, research proposes that Anammox can also use ferrous iron (Fe^{2+}) and various organic compounds instead of ammonium as electron donors for their metabolism (Strous et al., 2006; Jetten et al., 2009), including carboxylic acids as acetate and propionate. The electron acceptor nitrite can be substituted by Fe^{3+}, nitrate or manganese oxides (Strous et al., 2006). When nitrate is used, it is reduced to NH_4^+ via NO_2^- which are afterwards metabolised to N_2. Therefore, Anammox bacteria can facultatively act as denitrifiers. In

comparison to their conventional role in the nitrogen cycle (Figure 6) as shortcut of the nitrogen depletion through the direct conversion of ammonium and nitrite into dinitrogen gas and a small amount of nitrate, facultative denitrifying Anammox reverse their biochemical process and use the product – nitrate – as substrate. Anyhow, the conversion rate of nitrate to the intermediate nitrite proceeds at a slow rate representing only 10% of conventional Anammox conversion rate (Kartal et al., 2007).

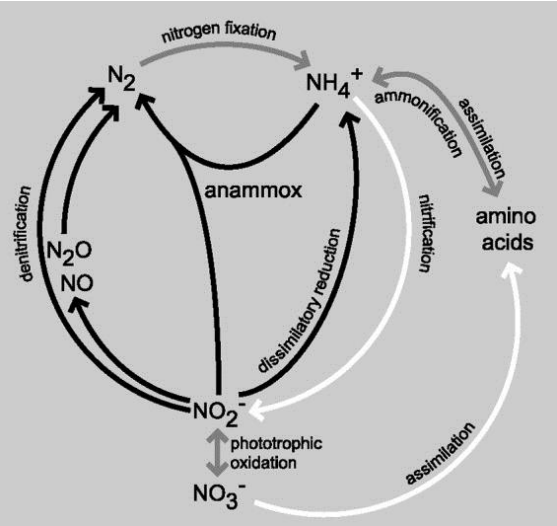

Figure 6: The nitrogen cycle and relevant microorganisms (van Niftrik and Jetten, 2012).

3.1.3 Deammonification and the application of the Anammox reaction

In conventional wastewater treatment, nitrogen is removed biologically over nitrification and denitrification. In the first step, ammonium, which is - besides organic nitrogen - the principal nitrogen compound in raw sewage, is aerobically converted to NO_2^- and NO_3^- through chemolithoautotroph microorganisms (nitrosomonas and nitrobacter). In the second step, facultative aerobe heterotrophic denitrifiers use nitrate under anoxic conditions as electron acceptor to metabolise carbon compounds. To guarantee that a sufficient amount of microorganisms is present in the reactor, the sludge residence time has to be higher than the growing rate of the MOs. This fact makes the reciprocal value of the growing rate – the sludge age- the main design parameter. To reach a sufficient nitrogen removal capacity, a sludge age of >= 20 days for nitrification and >=25 days for combined nitrification and denitrification representing the mean residence time of the sludge in the system are suggested (DWA-A 226, 2009).

To reach nitrogen removal through deammonification, approximately half of the influent ammonium has to be converted into nitrite. This first step aerobically conducted by ammonia-oxidizing bacteria (AOB) is combined with the second anaerobe step of Anammox bacteria oxidizing the remaining ammonium with nitrite as electron accepor to dinitrogen gas. This two-step process of AOB and Anammox is called deammonification (Wett, 2007). Compared to standard nitrification/denitrification and nitritation/denitritation up to 60% of oxygen demand can be saved (Figure 7) and only 10% of the C_{org} demand of nitrification/denitrification is needed in deammonification. This leads to a substantial cost reduction (Jardin and Hennerkes, 2012).

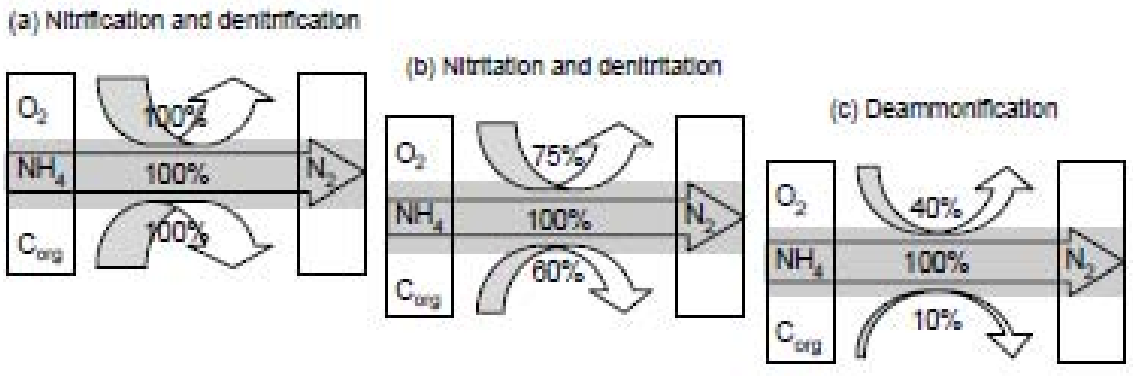

Figure 7: Comparison of O_2 and C_{org} demand of different ammonium removal processes (Wett 2007).

Deammonification can either be conducted in a two-step process with nitritation in the first stage (e.g. SHARON, van Dongen et al., 2001) and Anammox in a second stage or in a single stage partial nitritation/Anammox process (PNA; CANON, Third et al., 2005). A crucial point in the implementation of a PNA process is the suppression of full nitrification on the one hand and optimal process conditions e.g. long sludge retention time and therefore high sludge age for the Anammox bacteria on the other (Jardin and Hennerkes, 2012). Due to the fast growing rate of nitrifiers (Table 2), the sludge age of 20-25 days erases the possibility of wash out of nitrite oxidisers. They either have to be suppressed by intermittent aeration – which has an inhibitory effect on them – or the "lower oxygen half-saturation of the ammonia oxidisers as compared with the nitrite oxidisers" (Jardin and Hennerkes, 2012) has to be used. Another regulation possibility is temperature. While Anammox possess an increasing growing rate at 30 °C, the growing rate of heterotrophic MOs decreases rapidly when the temperature is increased to 30 °C (Table 2). This promotes the growth of Anammox in comparison to heterotrophic MOs and therefore deammonification is commonly applied at mesophilic conditions.

Table 2: Growing rates μ for autotrophic and heterotrophic microorganisms used in wastewater treatment (according to Ertl, 2008).

Microorganism	**$\mu_{maxnetto}$ [d^{-1}]**	**$\mu_{maxnetto}$20°C [d^{-1}]**	**$\mu_{maxnetto}$30°C [d^{-1}]**
Heterotrophic MOs	$6.0*e^{-0.0693*(T-20)}-0.4*e^{-0.0693*(T-20)}$	5.60	2.65
Nitrosomonas	$0.47*1.103^{(T-15)}$	0.77	2.05
Nitrobacter	$0.78*1.06^{(T-15)}$	1.04	1.87

The DEMON® reactor configuration, which is also used in this study, is a PNA process conducted in a SBR with pH based aeration control. It uses a combination of low oxygen concentrations and an elevated process temperature to favour Anammox bacteria (Wett, 2007). As nitritation leads to the production of H^+ ions during aerobic phases, the pH-value is lowered to the pre-determined threshold level and aeration is stopped. This leads to the depletion of DO and the use of nitrite accumulated during the aeration phase for ammonia oxidation through the Anammox bacteria. Through the Anammox reaction, alkalinity and pH recover until the pH-value reaches the upper threshold value which leads to the switching on of aeration. Reject water is fed continuously during the whole cycle except settling and withdrawal interval (Jardin and Hennerkes, 2012).

The implementation of deammonification for reject water treatment and the DEMON® reactor configuration at the WWTP Strass reduces the nitrogen load redistributed to the A-stage from 16.3 % to 1.5 % (Figure 8), proving the capability of deammonification to improve the system efficiency of WWTPs.

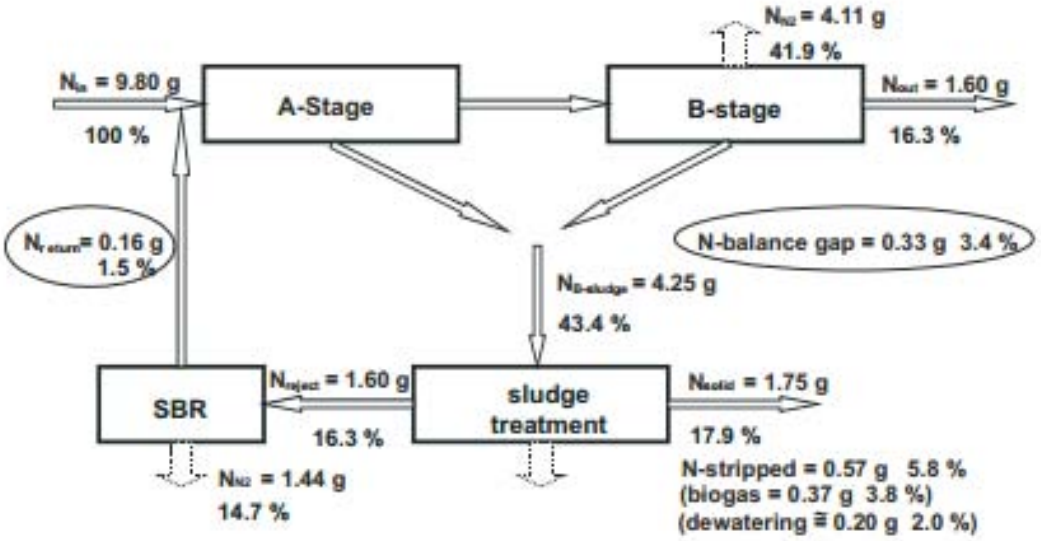

Figure 8: Nitrogen balance at WWTP Strass with side stream treatment of reject water. The A-Stage =high loaded activated sludge; B-stage = low loaded activated sludge; SBR = deammonficiation (Wett and Alex, 2003).

3.2 Inhibition of Anammox bacteria

Anammox bacteria are known for the slow doubling time of around 12 days and their susceptibility to many inhibitors/constituents occurring in wastewater (Jetten et al., 2001). Many inhibitors and their inhibition level have already been suspect of research work by Jin et al., 2013a and b; Jin et al., 2012; Zhang et al., 2014; Zhang et al., 2015; Dapena-Mora et al., 2007 and others. Summing up the conducted research, it can be concluded, that for most inhibitors no clear inhibition level can be found (Jin et al., 2012). While certain levels are seen as irreversible inhibitory in some references, the same level do not have a significant influence on the Anammox biomass in others or at least do not have such high or only reversible effects.

Throughout this chapter, several possible inhibitors for the Anammox process shall be discussed, such as

- Substrate inhibition
- Common wastewater constituents (phosphate, sulphide, O2, salinity, flocculants/coagulants)
- Other sources (heavy metals, antibiotics, organic matter,…)

3.2.1 Substrate inhibition

The Anammox process is often used for high-strength reject water from sludge dewatering and industrial wastewater which is rich in ammonium. Therefore, the possible inhibition of Anammox bacteria by ammonium is an important process parameter.

Although, nitrite and not ammonium is seen as main inhibiting substrate (Dapena-Mora et al., 2007; Jin et al., 2012; Strous et al., 1999), high ammonium concentration can have an impact on the Anammox metabolism (Table 3).

Table 3: Inhibitory concentrations of ammonium/free ammonia.

Reference	**Inhibitor**	**Concentration**		**Effect**
		mM (NH_4 or NH_3)	mg/L (NH_4-N or NH_3-N)	
Dapena-Mora et al., 2007	NH_4	55	770	IC 50
Tang et al., 2013	NH_4	530	7420	IC 50
Strous et al., 1999	NH_4	70	980	No effect
Fernández et al., 2012	NH_3	2.7	38	IC 50
Jaroszynski et al., 2012	NH_3	> 0.1 mM	1.4	inhibition
Waki et al., 2007	NH_3	< 1mM	14	No effect

While several authors agree that free ammonia is the inhibiting factor (Dapena-Mora et al., 2007, Fernández et al., 2012, Jaroszynski et al., 2012), Puyol et al. (2014) have identified a high pH as inhibiting factor rather than free ammonia. As the presence of free ammonia is pH dependent (pKa = 9.24) and a broad range of pH from 6.8 – 8.2 is used in Anammox research, the interdependency of pH and free ammonia has to be included in the ammonium inhibition research (Puyol et al., 2014). Hence, Puyol et al. (2014) did not discriminate whether high pH itself or the absence of sufficient bicarbonate supply (pKA=10.3) inhibited Anammox performance. At higher pH-values, bicarbonate is dissociated into CO_3^{2-} which is not available for Anammox metabolism. Jin et al. (2013a) could show that accurate levels of inorganic carbon/HCO_3^- enhance Anammox activity. While the addition of HCO_3^- improves

the nitrogen removal capacity (chapter 3.2.3), it also increases the pH-value and therefore leads to a raised free ammonia inhibition potential and pH-value. To determine the inhibitory potential of a substance, the IC_{50} value is a commonly used tool. This value describes the concentration of an inhibitor which leads to the loss of 50% of activity.

Nitrite is not only, as ammonium, one of the main substrates of the Anammox process, but also one of its most severe inhibitors (Carvajal-Arroyo et al., 2013). Although researchers agree, that the nitrite concentration has a strong effect on the metabolism and can cause irreversible inhibition (Carvajal-Arroyo et al., 2013; Bettazzi et al., 2010), the inhibitory concentration is controversially discussed. Table 4 compares several studies and clearly shows that the obtained concentrations vary significantly.

Table 4: Inhibition concentrations of nitrite.

Reference	**Concentration (NO_2-N)**		**Effect**
	mM	mg/L	
Carvajal-Arroyo et al., 2013	15.1 10.8	211.4 151.2	Almost complete inhibition IC 50
Strous et al., 1999		100	Complete inhibition
Egli et al., 2001	> 13.2	> 185	Complete inhibition/ inactivation
Dapena-Mora et al., 2007	25	350	IC 50
Lopez et al., 2008	7.14	> 100	Inhibition
Bettazzi et al., 2010	4.29 > 2.14	60 (spiked) > 30 (long term exposure)	Activity decrease Activity decrease
Isaka et al., 2007	> 20	> 280	Inhibition
Kimura et al., 2010	> 19.57 > 53.57	> 274 > 750	Inhibition Inhibition
Tang et al., 2010	27.14 27.86	380 390	31% TN removal efficiency 85% TN removal efficiency
Fernández et al., 2012	< 17.14	< 240	No inhibition

The inhibition effect of both, ammonium and nitrite, depends on the exposition duration and the structure of the biomass. Also, flocculent biomass and biofilm biomass react differently to substrate inhibition (Dosta et al., 2008). Besides the type (granules vs. biofilm) of biomass also its fitness state and time for adaption are relevant considering inhibition threshold levels (Carvajal-Arroyo et al., 2013; Bettazzi et al., 2010). Carvajal-Arroyo et al. (2013) proved that nitrite inhibition depends rather on surrounding factors such as absence of ammonium and a pH-value < 7.2 than on the absolute nitrite concentration. Also, active cells were not inhibited by nitrite levels which led to inhibition in underfed/starved cells.

Little research has been conducted on the effect of nitrate. As nitrate is a product of the Anammox metabolism, it can accumulate. Therefore, its inhibition potential should be measured. Nitrate effects are commonly measured during inhibition assays for ammonium and nitrite. Strous et al. (1999) did not observe any effect of nitrate on Anammox below a

concentration of 70 mM during a one week operation in a sequencing batch reactor. This does not agree with Dapena-Mora et al. (2007) who observed an IC_{50} of 45 mM in batch tests. Carvajal-Arroyo et al. (2013) found different behaviour for suspended enrichment cultures and granular Anammox enrichment: while the activity of suspended enrichment cultures decreased only by 15-20% when stressed with up to 20.5 mM nitrate, the granular Anammox enrichment lost 40-50% activity at the same concentrations. At 50 mM or higher nitrate concentrations, severe inhibition could be measured.

3.2.2 Common wastewater constituents as inhibitors

The Anammox process is prone to inhibition through several commonly occurring wastewater constituents such as phosphate, sulphide, dissolved oxygen and salinity (Jin et al., 2012; Carvajal-Arroyo et al., 2013; Dapena-Mora et al., 2007; van de Graaf et al., 1996). The determination of an inhibition concentration threshold is crucial to guarantee a stable process and therefore a sufficient activity. Therefore, conducted research on these constituents shall be presented in this subchapter.

The inhibitory effect of phosphate and sulphide has already been studied in the beginning of Anammox research (van de Graaf et al., 1996). For phosphate, the obtained data is rather general with 1 mM phosphate having no effect and 5 or 50 mM leading to a loss of activity (van de Graaf et al., 1996). More detailed results were published by Dapena-Mora et al. (2007) who obtained an IC_{50} of 21 mM phosphate. Carvajal-Arroyo et al. (2013) underlined this value with an IC_{50} of 25.3 +/- 5.9 mM for suspended culture and >100 mM for granular culture. Anyhow, the inhibition effect of phosphate is reversible (Wang et al., 2009).

As the toxicity of sulphide has by several researches been connected to unionized form (H_2S) (Carvajal-Arroyo et al., 2013), its inhibition capacity is often expressed in concentrations of H_2S rather than sulphide itself. The toxicity of sulphide probably arises from the interaction with heme proteins in cytochrome oxidase which reduces the heme iron in cytochrome (Pietri et al., 2011) and leads to a possible disturbance of the Anammox metabolism. While Carvajal-Arroyo et al. (2013) propose an IC_{50} of 0.03 mM H_2S for suspended and 0.10 +/-0.01 for granular biomass, van de Graaf et al. (1996) reported a stimulating effect of 2 mM sulphide in a fluidized bed Anammox reactor. Research conducted by Dapena-Mora et al. (2007) supports the findings of Carvajal-Arroyo et al. (2013) of low H_2S concentrations leading to strong inhibition with a value of 0.65 mM H_2S leading to a total loss of activity. Anyhow, Carvajal-Arroyo et al. (2013) observed complete inhibition at 0.32 mM H_2S for suspended enrichment cultures, while granular Anammox enrichment still had 24 +/- 4 % activity at a 0.9 mM H_2S. No consumption of H_2S was monitored.

In general, salinity is known to inhibit anaerobic biological treatment processes (Jin et al., 2012). As Anammox bacteria have been found in marine environment, they can be a promising tool to treat high-salinity wastewater. Research – summed up in Table 5 – shows that different salts have varying effects on the process, which enhances the hypothesis that certain ions, such as Na+ (Dapena-Mora et al., 2007), have stronger inhibitory effects than others. Hence, appropriate salinity concentrations (3-15 g/L NaCl) can promote the growth of granules and improve the retention (Fernández et al,. 2008). When given the possibility to acclimate to high salinity concentrations, the performance can be increased (Dapena-Mora et al., 2010).

Table 5: Research on salinity effects on Anammox biomass (adapted after Jin et al., 2012).

Reference	Concentration [g/L]	Used salt	Effect	Annotations
Dapena-Mora et al., 2007	< 8.78 13.46	NaCl	No effect IC_{50}	Non adapted biomass
	> 7.10 11.36	Na_2SO_4	Inhibitory effect IC_{50}	Non adapted biomass
	> 7.45 14.9	KCl	Inhibitory effect IC_{50}	Non adapted biomass
Kartal et al., 2007	10 45 60	90% NaCl and 10% KCl	Maximum SAA - 85% activity Activity lost	Non adapted biomass
	30 75 90	90% NaCl and 10% KCl	Maximum SAA Still active Activity lost	Adapted biomass (short-term batch-test)
Fernández et al., 2008	5 10	NaCl	Improved retention, SAA slightly reduced Improved retention, SAA increased	Non adapted biomass
Dapena-Mora et al., 2010	6 13.5	NaCl	Improved SAA IC_{50}	Non adapted biomass
	20	NaCl	Decrease of SAA	Adapted biomass

As most WWTPs use flocculants at some stage of the treatment process to remove colloidal (in)organic matter, it has to be tested whether this process affects Anammox. Dapena-Mora et al. (2007) observed slight effects of the flocculant “Floculex CS-49” on the SAA. 0 to 1 g/L flocculant were added. The maximum concentration reduced the SAA by about 30%. Nevertheless, the assayed concentrations neither reached the IC_{50} nor the IC_{100} (100% inhibition concentration) for nitrogen removal the Anammox activity did not suffer from a physical detrimental effect (Dapena-Mora et al., 2007). Except from this study, data on the effect of flocculants on Anammox is sparse. Therefore, further research should be conducted to prove, whether certain flocculants have an influence on the Anammox process. Especially considering the fact that most WWTPs use some kind of coagulant/flocculant within the process, influences of these substances have to be conducted to estimate possible system failures and difficulties. Hence, a multifactorial experiment executed within this thesis is dealing with the inhibition potential of a flocculant (chapter 4.3). Data obtained from research on aerobic granular biomass (Val del Río et al., 2012) highlight possible problems occurring from flocculants/coagulants such as worsening retention capacity and lower VSS concentrations.

Dissolved oxygen (DO) has been identified as critical factor for the Anammox process (Strous et al., 1997; Egli et al., 2001). Depending on the concentration of DO, reversible (< 2% air saturation (Strous et al., 1997)) or irreversible inhibition (> 18% air saturation (Egli et al., 2001) occurs. A more detailed study on the effects of DO on Anammox biomass (Carvajal-Arroyo et al., 2013) came up with an IC_{50} of 3.8 +/- 0.6 mg/L for a suspended enrichment culture and 2.3 +/- 0.03 mg/L for a granular enrichment culture. Concluding that low dissolved oxygen concentrations (<= 1 mg/L) effect the nitrogen removal capacity only minor (<20% activity reduction), but ambient saturation (8 mg/L) severely inhibit Anammox activity (Carvajal-Arroyo et al., 2013).

3.2.3 Other sources of inhibition ((heavy) metals, antibiotics, organic matter)

Some wastewaters contain high loads of heavy metals, which can affect the nitrogen removal capacity. Hence, the addition of certain heavy metals to synthetic wastewater used in the cultivation of Anammox is proposed. In general, the inhibitory capacity of heavy metals depends on the state of microbial growth and the biomass concentration (Wang et al. 2010). Therefore, the appropriate/tolerated heavy metals concentrations for Anammox growth have to be analysed. The research on the influence of metals on Anammox has been focused on three ions: zinc, copper(II) and iron.

The inhibition capacity of zinc has been studied by Daverey et al. (2014) and Lotti et al. (2012). Due to the different biomass concentrations used in the studies, the obtained IC_{50} values vary. Table 6 summarizes the results from the above mentioned studies and clearly shows that the IC_{50} depends on the exposure time as well as the biomass concentration.

Table 6: Anammox process inhibition by zinc.

Reference	**Exposure time [h]**	**IC_{50} [mg/L]**
Daverey et al., 2014	3	11.5
	6	14.8
	12	14.9
	24	6.9
Lotti et al., 2012	24	3.9
Kimura et al., 2014		12.5

Kimura and Isaka (2014) conducted a research on the effects of nickel, copper, cobald, zinc and molybdenum on Anammox activity. Besides the ones caused by molybdenum, all observed effects were reversible. The results of this study can be found in Table 7 and Figure 9.

Table 7: Overview on different heavy metals and their effect on the Anammox process.

Reference	Metal	Concentration [mg/L]	Effect
Lotti et al., 2012	Cu	1.9	IC_{50}
Kimura et al., 2014	Cu	6.5	IC_{50}
	Ni	7.9	IC_{50}
	Co	9.7	IC_{50}
	Zn	12.5	IC_{50}
	Mo	> 0.2	Strong irreversible influence

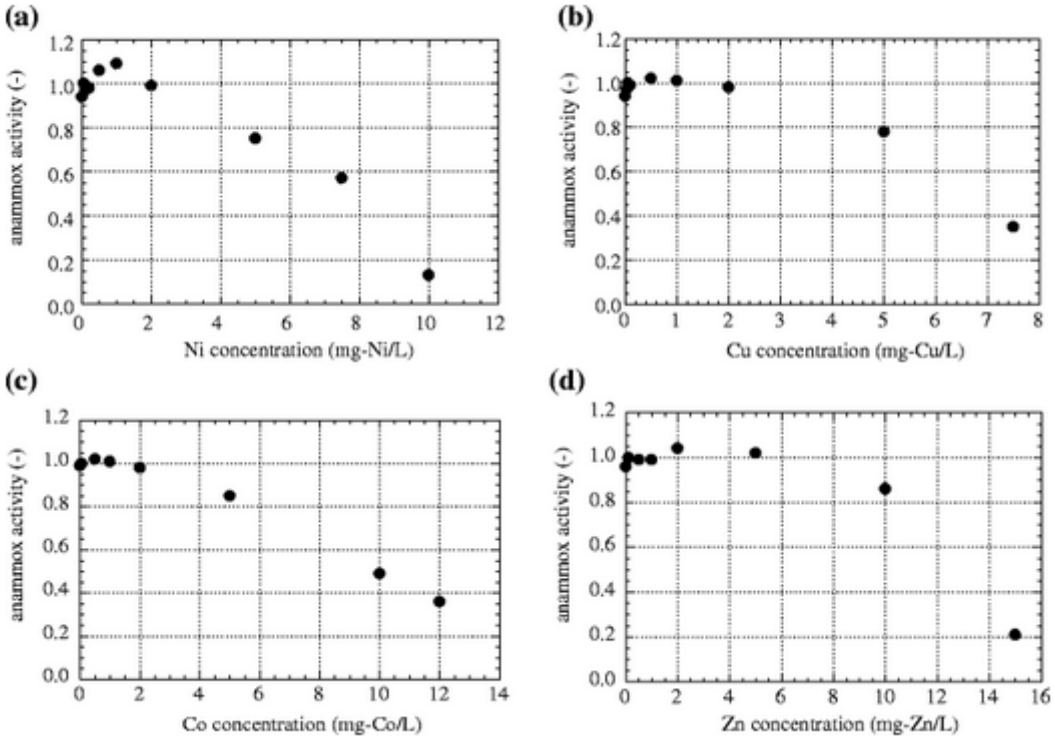

Figure 9: Effect of Ni (a), Cu (b), Co (c) and Zn (d) on Anammox activity. The average nitrogen conversion rate normal concentrations of each metal are set at 1.0 (Kimura et al., 2014).

Another metal ion which is crucial for the Anammox process is iron. As Anammox possess heme C, their metabolism is dependent on iron. Anammox bacteria can reduce Fe(III) to Fe(II) with organic matters as the electron donor (Zhao et al., 2014). At the presence of substrates for the Anammox process (ammonium and nitrite) the iron-reducing activity can be severely inhibited as the bacteria prefer nitrogen removal over the iron-reducing pathway. Therefore N_{tot} removal efficiency is not severely affected when Fe(III) is present (Zhao et al. 2014.

Zhao et al. (2014) used 0.25 mM Fe(III)-NTA, 1 mM Fe(III)-NTA and 1 mM Fe(III)-EDTA. It could be shown that Anammox are capable of reducing iron, but at the presence of nitrite, the iron reduction capability is severely inhibited. Anammox chose nitrite over Fe(III) for their metabolism. Oshiki et al. (2013) observed that the ferrous iron oxidation by Anammox bacteria is nitrate-dependent. Ren et al. (2014) revealed that the start-up time of the Anammox process can be shortened by adding zero-valent iron. Bi et al. (2014) improved the

start-up process by adding ferrous iron and shortened the process for 12 days (0.06 mM Fe^{2+}) and 20 days (0.09 mM Fe^{2+}).

Despite all these reported positive effects of the divalent ferrous ion the Anammox process, Qiao et al. (2013) found proof that Fe^{2+} concentrations of 0.18 mM (equals 10.05 mg/L) inhibit the activity of the Anammox biomass. Liu and Horn (2012) observed a drastic deterioration of the Anammox process when the Fe(II) and Fe(III) concentrations reached 1.3 and 0.4 mg/L. While Fe(II) in appropriate concentrations was highly beneficial for the Anammox bacteria, excess Fe(II) and Fe(III) had inhibitory effects on the planctomycetes.

Another possible source of inhibiton is (toxic) organic matter. As Anammox bacteria are chemoautotrophic organisms and use exclusively CO_2 as carbon source, the influent bicarbonate concentration is of high importance for the Anammox process and the cultivation of biomass (Jin et al., 2012). Providing inorganic carbon in a sufficient concentration can lead to an increase in activity and growth of the Anammox biomass (Liao et al., 2008). However, organic carbon/ organic matter can have a negative effect (Chamchoi et al., 2008). The influence of nontoxic organic matter on Anammox bacteria are believed to have their roots in two different mechanisms:

1) The out-competition of Anammox through heterotrophic bacteria. Heterotrophic bacteria have a faster metabolism than Anammox and therefore can replace Anammox biomass when having a sufficient organic carbon supply (Jin et al., 2012).
2) The change in Anammox metabolism/ the “metabolic pathway conversion inhibition” (Jin et al., 2012). This inhibition type is based on Anammox bacteria using organic matter instead of ammonium in their metabolism. Although Anammox bacteria are the dominant species, their nitrogen removal rate/ specific activity is decreased (Jin et al., 2012).

While nontoxic organic matter affects Anammox biomass in the two above mentioned ways, toxic organic matter can inhibit Anammox irreversibly “by microbial poisoning or enzyme inactivation” (Jin et al., 2012). Possible inhibitors are alcohol, aldehydes, phenol and antibiotics which are also common (industrial) wastewater constituents.

As antibiotics are widely used in many different fields such as industrial farming, aquacultures and human and veterinary medicine, filtering these substances from the wastewater or degrading them is an important tool to prevent accumulation.

Research proves that antibiotics have severe effects on the Anammox process. Table 8 indicates that antibiotics from different chemical sources lead to inhibition. More resent research has focused on broad spectrum antibiotics such as tetracycline and chloramphrenicol, but in total there has not yet been conducted much research in this field.

Table 8: Influence of different antibiotics on the Anammox process (edited after van de Graaf et al., 1996 and Jin et al., 2012).

Reference	Antibiotic	Concentration [mg/L]	Effect
Lotti et al., 2012	Sulfathioazole	650	IC_{50}
	Tetracycline	1100	IC_{50}
Zhang et al., 2014	Oxytetracycline	155-1731	SAA – 1.4% Heme C content: – 17.6-29.4% Reversible effect
Fernández et al., 2009	Tetracycline hydrochloride	100-1000	Strong inhibitory effect
	Chloramphrenicol	250-1000	Strong inhibitory effect
	Chloramphrenicol	20 (long term)	- 80% SAA
van de Graaf et al., 1996	Chloramphrenicol	200	- 68% SAA
	Ampicillin	400 800	- 71 +/-3% N-removal - 94 +/-4% N-removal
	Penicillin	1 100	- 17 +/-13% N-removal - 36 +/-10% N-removal
	$Hg^{II}Cl_2$	271	- 100 +/-4% N-removal
	2,4 - Dinitrophenol	37 368	- 53 +/-7% N-removal - 99 +/-2% N-removal
	CCCP	41	- 100 +/-3% N-removal

Besides the above mentioned inhibitors, process parameters as temperature, pH, hydraulic retention time, shear stress and others also have severe impacts on the Anammox process. These parameters are not part of the experiments conducted in this master thesis and therefore will be discussed elsewhere.

Summing up, the Anammox process can be influenced and inhibited by many factors. Some are reversible, others irreversible and interaction between certain factors occur. While the research on substrate inhibition is already in an advanced state, many other inhibitors have yet to be investigated in detail.

3.3 Monitoring of the Anammox process

To evaluate the fitness of Anammox bacteria, an appropriate monitoring scheme is mandatory. As the project focuses on the use of the Anammox process in small and medium scale WWTP without special laboratory equipment, the elaboration of a convenient, functional monitoring system is crucial. For monitoring of Anammox and their microbial and population characteristics several different approaches exist, targeting:

- Functional- and phylogenetic genes via quantitative polymerase chain reaction (qPCR) and fluorescence in situ hybridization (FISH)
- Characteristic cell membrane components (e.g. ladderanes)
- Characteristic molecules like heme, which are essential for specific enzymes
- The granular structure of Anammox biomass per se (Podmirseg et al., 2015)

The more elaborated microbiological analysing tools such as PCR, DNA-sequencing and FISH are used to determine the Anammox species rather than its performance. They target the unique cell structure and properties of Anammox bacteria which are discussed in detail in chapter 3.1.2. Therefore, and due to the fact that WWTP usually lack the equipment to use these methods, they are not appropriate for this study. These factors also hold true for the analysis of membrane components.

More relevant for a fast and reliable monitoring system are the characterisation of molecules, especially heme, and the analysis of the structure and nitrogen removal capacity of the Anammox biomass. Combined with standard methods for sludge analyses such as determination of SS, VSS, N-compartments,…, a approriate monitoring system can be implemented (Dapena-Mora et al., 2007; Podmirseg et al., 2015).

Due to the uncomplicated implementation of heme analysis and specific Anammox activity determination with standard laboratory equipment, these two methods have been chosen as target methods for the master thesis and are presented in this section and in chapter 4. . Heme analysis can be conducted with standard spectrophotometers (Sinclair et al., 1999) the implementation at WWTPs does not require additional equipment or special training of the personnel. Besides, the function of heme C as key enzyme in Anammox biomass (chapters 3.1.2 and 3.3.2) makes it a promising tool to obtain information on the fitness of the system.

Specific Anammox activity (SAA) assessment is a straightforward and fast approach to monitor the maximum achievable nitrogen removal capacity of Anammox bacteria. Therefore it provides information on the efficiency of the system and can help with optimization of the loading rate and system performance in total.

As both, heme analysis and SAA measurement, are promising uncomplicated but significant monitoring tools, their implementation and performance is evaluated within this study. Therefore they are explained in more detail in this section and in chapters 4.2and 4.2.2.

3.3.1 Specific Anammox activity (SAA)

As Anammox biomass is seldom used in a pure state but in symbiosis with other nitrogen metabolising bacteria, it is necessary to determine the nitrogen removal rate of solely Anammox. Especially when used in a single stage system, the total nitrogen removal efficiency gives only an insight into the total system activity, not in the Anammox activity. To get detailed and realtime information on Anammox activity can be an advantage in case of a possible system failure regarding Anammox bacteria fitness as a decrease in activity can be easily detected. In current large scale applications, the protection and growth of Anammox biomass is seen as important factor to guarantee a sufficient biomass concentration. Special equipment has been developed to prevent wash out of Anammox granules (Wett, 2007). Therefore, the concentration of Anammox biomass is quite high resulting in a possible underfeeding of the bacteria. Due to the risks arising through underloaded reactors – namely a reduced inhibition tolerance (Carvajal-Arroyo et al., 2013) – the knowledge of the actual maximum Anammox activity can be relevant to prevent a starvation/underloading of the reactor.

When referencing to the Anammox activity rate, the term “specific Anammox activity (SAA)” has been established. This term, expressed in g N_2-N/ gVSS/d or g N/ gVSS/d refers to the nitrogen removing capacity of the Anammox biomass during a defined time interval. It can be measured by analysing the manometric changes in a closed system during batch tests reflecting the N_2 production via the gaseous emission or through calculating the nitrogen mass balance when sampling the N-components NH_4, NO_2, NO_3 and N_2 and analysing them with standard methods such as spectrophotometry or chromatography. The concentrations of the different nitrogen compounds are measured and the SAA is calculated according to the stoichiometry of the Anammox process. This simple method is used by several authors such as Jin et al. (2013) and led to reproducable results and can be conducted through standard methods (spectrophotometry, ion chromatography, HPLC). Nevertheless, it makes frequenting sampling of the batch vials necessary.

Dapena-Mora et al. (2007) and Yang and Jin (2012) have developed a method to measure exclusively Anammox metabolism through manometric pressure changes in a closed system. This method is used in this study and therefore its details are explained in chapter 4. The method is based on a test conducted with denitrifying bacteria (Dapena-Mora et al., 2007; Buys et al., 2000) and has been adapted for Anammox biomass. It was found to be appropriate for analysing Anammox activity under standardized conditions (70 mg/L NH_4-N and 70 mg/L NO_2-N) and also for inhibition test assays. Since its establishment it has been broadly used in Anammox research (Dapena-Mora et al., 2010; Dapena-Mora et al., 2007; Carvajal-Arroyo et al., 2013; Puyol et al., 2014). Table 9 gives an overview on the experiments using manometric pressure changes to determine SAA and the adaptions made to the standard protocols by various authors.

The most obvious variations are the used “liquor” and N-inflow concentrations. One study included in the list – Podmirseg et al. (2015) – did not measure the overpressure produced in the vials but simply sampled the liquid phase through a syringe and measured solely the N-compounds. For a sufficient detection of N-conversion rate, samples have been taken every 30 minutes, which leads to a rather high sampling effort compared to sampling only in the beginning and at the end of a short term batch experiment.

Table 9: Overview on SAA measurements.

Reference	Total volume [ml]	Liquid volume [ml]	liquor	pH	Buffer system	Gas for gasifying	gasifying of liquor	T [°C]	Shaker [rpm]	NH4 and NO2 conc.	VSS [g/L]	Duration [h]	Max overpressure [mbar]	Max SAA
Dapena-Mora et al., 2007	38	25		7.8	Phosphate K_2HPO_4 & KH_2PO_4 (0.75 g/L & 0.14 g/L)	Ar	yes	30	150	42; 56; 70 for both	1	6	~150	0.28 g N_2-N $gVSS^{-1}$ d^{-1}
Dosta et al., 2008	38	24	Syn. WW	7.8	phosphate	Ar		10 - 45	150	70	1			
Fernández et al., 2009	38	25	Syn. WW	7.8	Phosphate K_2HPO_4 & KH_2PO_4 (0.75 g/L & 0.14 g/L)	Ar		30	150		1	6	~ 95	~ 0.3 g N_2-N $gVSS^{-1}$ d^{-1}
Scaglione et al., 2009	1000	1140	"original" reactor water	7.7-8.1	HCl (1 mol/L) if necessary	N_2		35	?			20	~ 120	0.1 g N_2-N $gVSS^{-1}$ d^{-1}d
Carvajal-Arroyo et al., 2013	160	100	"basal mineral medium" incl. HCO3	7.4-7.5	Carbonate	He/CO_2 80/20		30	115	48.78 NH4-N 164.22 NO2-N	0.6			0.242 – 0.618 g N_2-N $gVSS^{-1}$ d^{-1}
Yang and Jin, 2013	160	120	Syn. WW	7.5	0.1 mol/L HCl	Ar		35	180	100				
Zhang et al., 2014	According to Yang and Jin, 2013													0.12 g N_2-N $gVSS^{-1}$ d^{-1}
Podmirseg et al., 2015	100	100	"mixed liquor samples"	7.2	10 mM phosphate buffer	N2		30	Slow (?)	100 NH4-N 70 NO2-N	1.77 – 4.69	4	Not measured	0.01 – 2.76 g N_2-N $gVSS^{-1}$ d^{-1}

3.3.2 Heme analysis

One of the most characteristic features of Anammox bacteria is their red colour. The colour varying from brownish to carmine (Figure 10) is presumed to be a result from the high heme C concentration which can be found in Anammox sludge (Tang et al., 2011).

Heme C is an important factor for several Anammox bacterial enzymes (chapter 3.1.2) and plays a key role in Anammox metabolism. Through its indispensable part in these enzymes, its turnover rate provides information on the relative activity of the bacteria (Chen et al., 2012). Besides, it is involved in the energy metabolisms of Anammox bacteria, such as hydrazine synthesis (HZS) and oxidase (HZO) and hydroxylamine oxidoreductase (HAO) (Jetten et al., 2009). Therefore, heme analysis is often conducted when characterizing Anammox bacteria to gain additional information on the fitness of the Anammox biomass.

Two enzymes responsible for nitrogen turnover through the Anammox process – hydrazine hydrolase and hydrazine oxidoreductase – both include heme in their molecular structure. As the synthesis of these two depends on a sufficient supply with heme and Anammox are capable of storing heme to a certain amount for enzyme production, heme concentration can give information on the activity and nitrogen removal efficiency of the Anammox bacteria (Jetten et al., 2009).

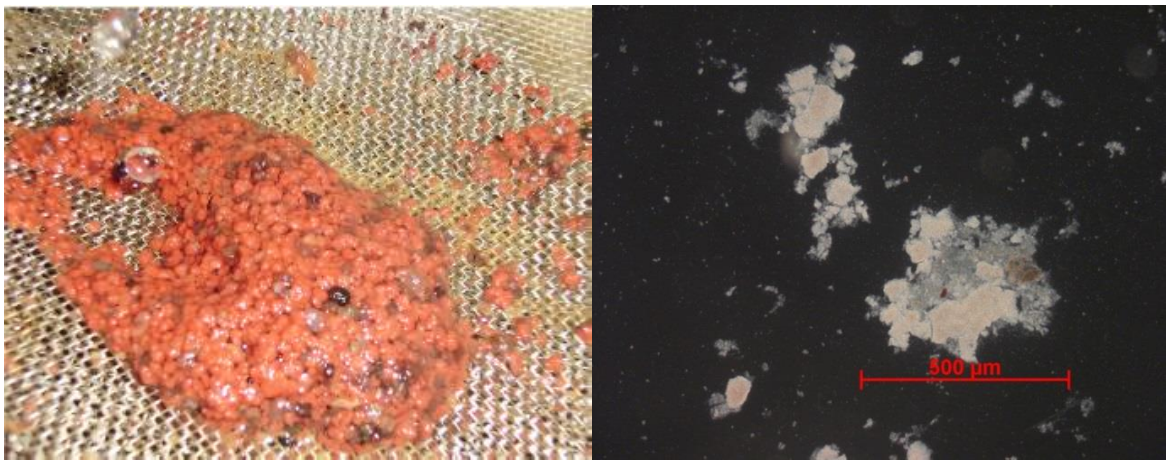

Figure 10: Enriched ANAMMOX sludge (Tang et al., 2011) and ANAMMOX granule (own microscopic picture).

The quantification of heme is understood to be a convenient tool to describe the health and performance of Anammox consortia (Podmirseg et al., 2015, Tang et al., 2011). It is, next to the specific Anammox activity (SAA), one of the main tools to characterize the recovery performance after inhibition (Jin et al., 2013, Zhang et al., 2014, Bi et al., 2014). Hence, the heme C content change can be time delayed and not always follows the same pattern as the specific Anammox activity (SAA) (Chen et al., 2012, Zhang et al., 2014). Especially Zhang et al. (2014) experienced a time delay in heme C recovery in comparison to SAA and varying concentrations which were not synchronous to SAA changes.

The heme C concentration obtained in the studies varies highly with maximum values of 6.8-10.3 mmol g-VSS^{-1} (Tang et al., 2011) for enriched biomass to 0.33 mAU/L (milli-Adsorption Units, Podmirseg et al., 2015) in a biological low rate stage.

In principal, two sources for heme analysis are used in most papers: after Sinclair et al. (1999 or 1997) and Berry and Trumpower (1987). Mostly, it is not specified which analytical method (spectrophotometry, fluorescence) was used. A detailed description of the heme quantification method used in this study is given in chapter 4 “Materials and Methods”.

4. Material and methods

As this master thesis was conducted within a research project focusing on deammonification in a PNA-reactor, several methodological approaches were necessary to describe not only Anammox biomass, but also the reactor performance as a whole. Therefore, this thesis used three methodological approaches:

- Application of standard methods to determine the sludge characteristics and the total nitrogen removal capacity of the whole system (AOBs and Anammox)
- Monitoring methods specifically characterising Anammox biomass (Heme analysis and SAA)
- 2^3 multifactorial inhibition experiment to test the applicability of monitoring methods targeting Anammox bacteria

The standard methods applied were also used to evaluate the accuracy of the Anammox monitoring tools during the inhibition experiment through analysing their correlation (chapter 4.4)

To implement these steps, Anammox inoculum sludge was withdrawn from the WWTP Strass i. Zillertal (see chapter 4.6 for detailed plant description) and a 3 L lab scale PNA reactor was installed at the technical hall of the institute (chapter 4.5). This reactor served as basis for the research within this thesis.

4.1 Standard methods for sludge characterisation and treatment performance

Besides the monitoring methods focusing on Anammox, standard methods were used to describe the sludge. These methods include

- SS & VSS
- N – compounds through spectrophotometric measurement

4.1.1 Suspended solids and volatile suspended solids

For suspended solids (SS) and volatile suspended solids (VSS), 100 ml of sample were taken in duplicate and thoroughly mixed. The analysis was performed with filtered samples which were dried at 105°C for 2 hours for determination of SS. To determine the VSS amount, the dried samples are processed in a muffle furnace at 550°C for 1h.

The measurements were conducted according to DIN 38409 (1987) and DIN EN 12879 (2001).

4.1.2 Plate Reader and Spectrophotometer for Ammonium Measurement

The N – compounds were measured with standard cuvette tests in a Hach Lange photometer (Lange Lasa 50) in the case of NO_2-N and NO_3-N. NH_4-N was either measured spectrophotometrically with a standard UV-Vis spectrophotometer according to DIN-norms or with a Tecan Reader. All spectrophotometric measurements are based on Lambert-Beer's law on the direct linear relationship of concentration and absorption.

The protocols for these measurements can be found in the appendix.

The method for NH_4-N measurement was established on a UV-Vis spectrophotometer and a plate reader. All steps were conducted according to the protocol (Appendix 9.3), which led to a standard calibration curve as in Figure 11.

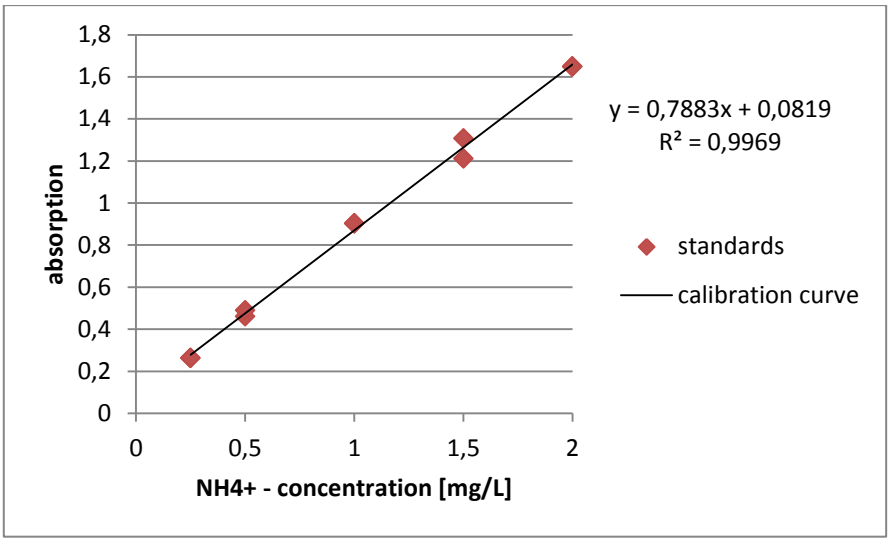

Figure 11: Calibration of the spectrophotometer.

In addition to the calibration at the spectrophotometer, a calibration (Figure 12) was conducted at a Tecan Reader (Perkin Elmer Multimode Plate Reader EnSpire 2300) with 24 Well plates.

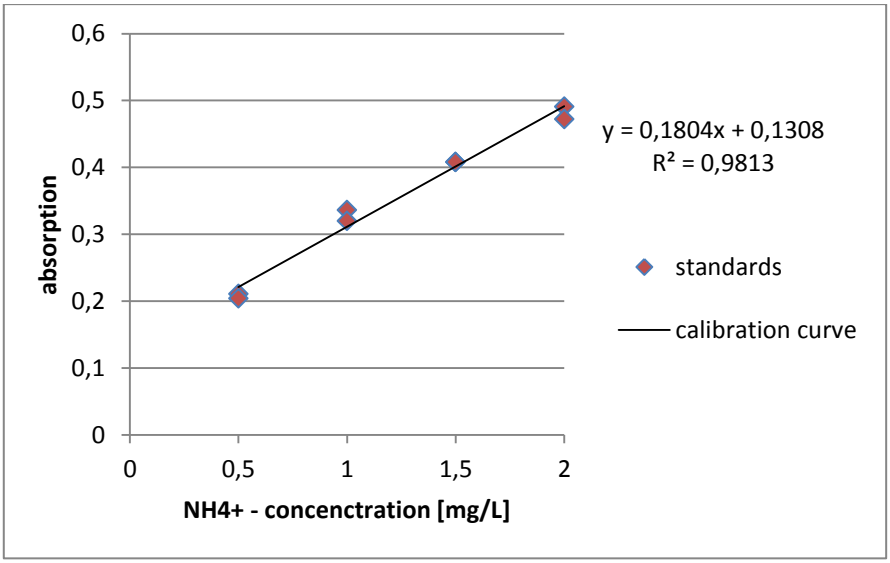

Figure 12: Standard calibration at Tecan Reader.

4.2 Monitoring methods targeting Anammox biomass

To gain information on the status of the Anammox biomass, additional methods supplementing commonly used tools were introduced. Under the premise that methods had to be applicable directly at WWTPs, a literature review on existing monitoring methods was conducted. The found methods were assessed according to:

- Simplicity in implementation
- Necessary equipment
- Costs
- Expenditure of time

The method assessment (chapter 5.1.1) led to the decision to establish heme analysis and SAA. These two methods are therefore explained in detail in the following chapters.

4.2.1 Heme analysis

As has been discussed in chapter 3.3.2, heme is a major compound in the Anammox cell biomass. Therefore, the analysis of the heme Content can give information on the fitness of the bacteria. For heme analysis, two methods were established: analysis through UV-Vis spectrophotometry and through fluorescence photometry.

UV-Vis spectrophotometry for heme detection

Determining the heme Concentration through spectrophotometry is the most common method (Sinclair et al. 1999). It was introduced by Paul et al. (1953) and mentioned in Fuhrhop and Smith (1975). The assay measures "the reduced-minus-oxidized difference spectrum of the pyridine hemochrome" (Sinclair et al. 1999). A major drawback of the method is "the use of pyridine and the requirement for a sensitive scanning spectrophotometer" (Sinclair et al. 1999). As spectrophotometry is sensitive to turbidity and particles, the obtained results can scatter highly when using unfiltered samples.

Fluorescence photometry for heme detection

Except photometry, which is proposed to be the fastest and most used tool to analyse heme Content (Sinclair et al. 1997), fluorescence spectroscopy can also be used. This method "is more sensitive than the pyridine hemochrome, but needs to be optimized for each tissue" (Sinclair et al. 1999). Due to this optimization necessity, the method had to be calibrated specifically for Anammox biomass. The calibration steps can be found in chapter 5.1.

The fluorescence assay described by Sinclair et al. (1997) is based on research by Sassa (1976) and Morrison (1965).

When following the standard protocol (chapter 9.1), the device specific settings were defined (Table 10). These settings were used throughout the whole master thesis

Table 10: Fluorescence spectroscope parameters

Excitation	402	nm
Emission	500-700	nm
Volt	800	
Excitation slit	20	nm
Emission slit	20	nm
Velocity	120	nm/min
Peaks	596 and 652	nm

4.2.2 Specific Anammox activity (SAA) determination

For the determination of SAA, two methodological approaches were tested: a manometric measuring system as introduced by Dapena-Mora et al. (2007) and Yang and Jin (2012) and a chemical system using the stoichiometry of the Anammox reaction and calculating the net turnover of Anammox bacteria.

The method Dapena-Mora et al. (2007) and Yang and Jin (2012) a is based on test conducted with denitrifying bacteria (Buys et al., 2000) and has been adapted for Anammox biomass by Dapena-Mora et al. (2007).

Completely closed vials with a total volume of 250 ml and a liquid volume of 200 ml were used to perform the batch assays. The biomass concentration at the beginning was 0.5 g VSS/L, the pH value was not adjusted and the process temperature was 35°C. The gaseous phase was washed with N_2 gas to exclude O_2. To determine the nitrogen turnover rate, overpressure sensor were attached to the bottles to measure the N_2 production rate.

In a second step, the overpressure sensors were replaced by sampling with syringes through the rubber seal every 30 min for 4 hours and analyse the liquid phase for NH_4-N, NO_2-N and NO_3-N. UV-Vis spectrophotometric analysis of the N-compounds and stoichiometric calculation was chosen.

4.3 Experimental set up for inhibitor assays

To determine the effect of different inhibitors on Anammox biomass, a 2^3 factorial design was applied: nitrite concentration (F1), $Fe(III)Cl_3$ concentration (F2) and polymer concentration (F3).

The tested inhibitors, their concentration levels and the factor combinations can be found in Table 11and Table 12.

To exclude influence of temperature and shear stress, all experiments were conducted at 35°C and 150 rpm in 24 vials with a total volume of 350 ml and a liquid volume of 300 ml (Figure 13).

Figure 13: Set up of the 24 batch flasks in the incubator. All flasks were equipped with needles to release pressure and to be able to draw samples with a syringe.

Table 11: Tested inhibitors and their concentration levels

Factor	Low level	High level
NO2-N	70 mg/L	105 mg/L
$Fe(III)Cl_3$	0 mg/L	1.5 mg/L
Polymer (Poly Separ® PK 1455, Separ Chemie)	0 mg/L	300 mg/L

Table 12: Set up configuration as used for the 8 treatments with 3 triplicates.

vial #	NO_2-N	Fe(III)Cl2	polymer
1	+	+	+
2	+	-	+
3	+	+	-
4	+	-	-
5	-	+	+
6	-	-	+
7	-	+	-
8	-	-	-

The activity and fitness of the biomass was monitored by SAA and heme analysis (fluorescence spectroscopy). Both, SAA and heme were measured every 4th day after feeding. The vials were fed with synthetic wastewater according to van de Graaf et al. (1996) and Dapena-Mora et al. (2007) with a NH_4^+-N concentration of 140 mg/L and a NO_3^--N concentration of 100 mg/L. The NO_2^--N concentration varied according to the experimental assay.

Before starting the experiment, the biomass was characterised through determination of SS and VSS concentrations. The enriched sludge had a SS concentration of 34 g/L and VSS of 30.6 g/L which results in a VSS of 90%. The inoculum was withdrawn from the enriched biomass cultivated at the WWTP AIZ Strass i. Zillertal, Tyrol.

The head space of the vials was washed with dinitrogen gas. After reaching a constant process temperature of 35°C, the substrates and inhibitors were added to all vials and the experiment was started.

The detailed experimental set up is described in the appendix (chapter 9.2).

4.4 Statistical analysis

The statistical analysis of all data was carried out with Microsoft Excel and R. Excel was used for basic analysis such as calculating SS and VSS and heme C concentration and NH_4-N-concentration from raw data produced by the Tecan Reader. For heme C concentration and NH_4-N-concentration calculations, linear integration of the standard calibration function was used.

Advanced statistical analysis such as calculating the total nitrogen depletion rate, SAA and correlation between the monitoring methods was conducted with R, using the packages “reshape2”, “gridExtra”, “GGally”, “plyr” and “data.table”. Graphs were plotted with the package “ggplot2“.

For the detailed analysis of the independent and identically distributed (iid) data following normal distribution, a linear model with ordinary least square (OLS) analysis was chosen. As the depletion rate N was the variable of interest, it was used as dependent variable. Due to the dependency of the depletion rate on inhibitors and substrates, it is justified to use a linear model to describe its behaviour (Puyol et al. 2014). To account for some possibly occurring inhibiting effects through factor combinations not measureable through the tested tools and variables, it is justified to introduce an intercept α. The tested factors and factor interactions are treated as independent variables, leading to a mathematical representation of the model as following:

$$N_i = \propto + \beta_1 * NO_2 - N + \beta_2 * Fe + \beta_3 * Polymer + \beta_4 * NO_2 - N * Fe + \beta_5 * NO_2 - N * Polymer + \beta_6 * Fe * Polymer + \beta_7 * NO_2 - N * Fe * Polymer$$

Table 13: Model indices and variables

i	cycle index 1, 2, 3
$\propto$	the model intercept for all data
$\beta_1 * NO_2 - N$	nitrite – nitrogen level
$\beta_2 * Fe$	Fe(III)Cl_3 level
$\beta_3 * Polymer$	polymer level
$\beta_4 * NO_2 - N * Fe$	Interaction between nitrite – ntirogen and Fe(III)Cl_3 levels
$\beta_5 * NO_2 - N * Polymer$	Interaction between nitrite – nitrogen and polymer levels
$\beta_6 * Fe * Polymer$	Interaction between Fe(III)Cl_3 and polymer levels
$\beta_7 * NO_2 - N * Fe * Polymer$	Interaction between nitrite – nitrogen, Fe(III)Cl_3 and polymer levels

As output data, estimates of the input variables and their p-values are obtained. The p-values for each input variable tests whether the coefficient (in this case the tested influencing factor) is equal to zero, which would mean that it has no effect on the output data. A low p-value - < 0.5 – indicates that the factor as a significant influence on the output (the nitrogen depletion rate). The estimates themselves give the mean absolute change in the output (dependent) variable for one unit change in the independent variable (the influencing factor). Therefore it gives an isolated view on the influence of one specific variable on the output.

4.5 Set up of the 3L PNA laboratory scale reactor

To monitor the Anammox biomass, a 3L PNA-sequencing batch reactor (SBR) was used. The reactor was run as thermostat at 35°C (Figure 14 and Figure 15) and all process steps (feeding, stirring, aeration, withdrawal) were automatized. The process parameters pH, ORP and O_2 were measured online.

The inoculum sludge needed for the PNA-reactor was withdrawn from two sources:

- Activated sludge containing nitrifiers from the SBR pilot plant situated at the technical hall of the institute used for the treatment of wastewater for research purpose
- Activate sludge containing Anammox biomass and nitrifiers from the SBR for sidestream treatment at the WWTP AIZ, Strass i. Zillertal, Tyrol.

The nitrogen loading rates and the control parameters (pH, DO) at the start up were set according to the actual treatment conditions of the reactor at the WWTP AIZ. The full scale Anammox reactor at the treatment plant of AIZ Strass i. Zillertal is described in the next chapter.

Figure 14: 3L PNA reactor set up at the technical hall of the Institute.

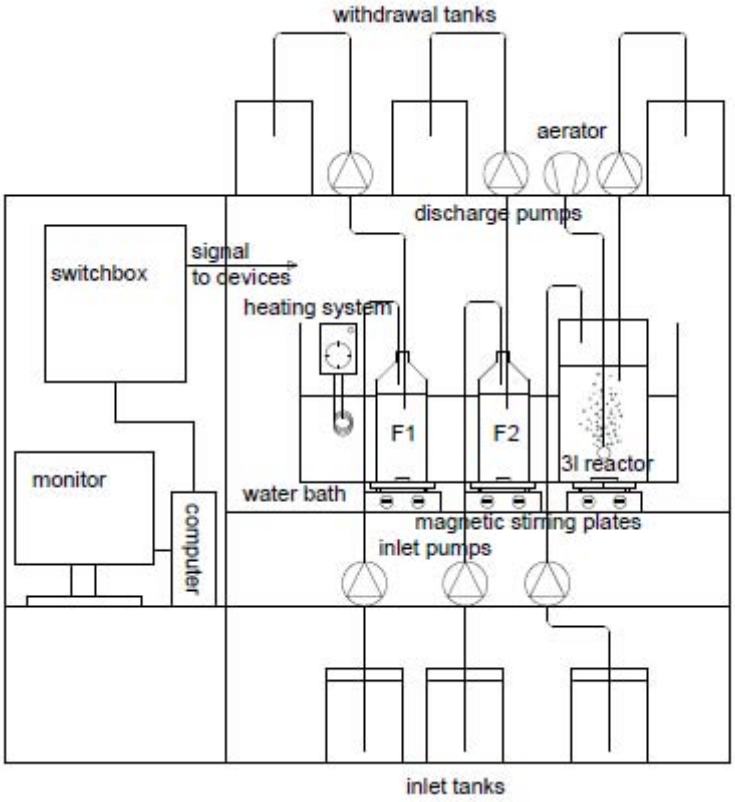

Figure 15: Process schematic of the 3 L PNA-reactor and two 1,5 L anaerobic bottles (F1 and F2) as installed at the technical hall of the Institute.

4.6 Anammox inoculum sludge source

The inoculum sludge (Figure 17) for all pilot reactors used in this study was withdrawn from the WWTP AIZ Strass i. Zillertal, Tyrol at 28.04.2015 and 20.01.2016. The WWTP AIZ uses a PNA reactor in the DEMON® configuration for sidestream treatment of reject water. The WWTP is designed for 167.000 PE with a daily inflow Q = 28.270 m³/d, the PNA-SBR for sidestream/reject water treatment has a total volume of 520 m³ (Figure 16). The PNA reactor has a cycle period of 8 h and is characterized by a tight pH-range controlling aeration of Δ 0.01 (Innerebner et al., 2007). Due to the low DO concentrations, nitrification and Anammox turnover take place at the same time.

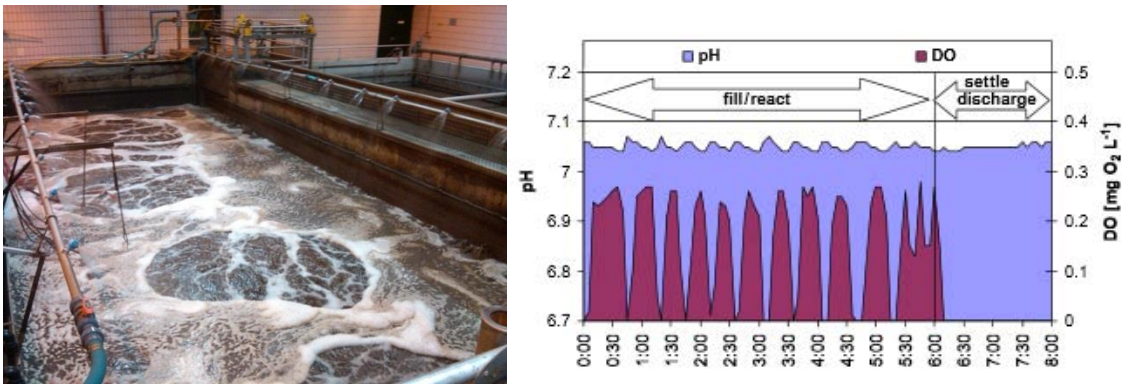

Figure 16: a) 520 m³ sidestream Anammox PNA reactor at the WWTP AIZ Strass during aeration b) intermittent aeration in the first 6 h of the SBR cycle (Innerebner et al., 2007).

Figure 17: Anammox sludge after one week of storage without any treatment.

5. Results and discussion

5.1 Establishment of methods

5.1.1 Assessment of existing methods

As already discussed in chapters 0 and 4.2, existing monitoring methods had to be evaluated according to their applicability at medium scale (municipal) WWTPs in Austria. Highly elaborated laboratory methods such as qPCR, FISH and CC (Coulter counter) were no option as they exceed cost and time capacities. Several methods were evaluated for advantages and drawbacks (Table 14). Gravimetric analysis (GA), which is based on the determination of granular sludge mass in comparison to SS content, and particle tracking (PT) – based on image analysis of particle sectional area and number -, are not able to give information on non-granular biomass. Due to the high shear forces in the lab scale PNA reactor and the thereby reduced granular structure of the biomass, these two methods were no monitoring options. This led to the decision to establish specific SAA and heme analysis (HA), as these two can be used for granular and non granular biomass. Nevertheless, especially HA had to be optimized for Anammox biomass to improve the dissolving of heme c through the dissolving agent and increase the reliability of the results through a decreased variance and – at the same time - simplify the method to enhance time and cost efficiency. The optimization of SAA focused especially on monitoring costs and time sufficiency through simplification of the method and decrease of laboratory analysis afford. The necessary calibration steps for both methods are discussed in the following chapters.

Table 14: Comparison of monitoring methods for Anammox biomass according to literature and own experience. GA=gravimetric analysis, PT=particle tracking, HA=heme analysis, SAA=specific Anammox activity (adapted after Podmirseg et al., 2015).

Method	Advantages	Drawbacks	Appropriate for non-granular biomass	Time per sample needed	Non-basic lab-equipement needed	Price per sample (materials	Source
GA	Fast, cheap, little euqipement needed	Not applicable for small fractions, unspecific	no	5-10 min	Analytical sieves, drying balance or oven	< 0.50 €	Podmirseg et al. 2015
PT	Granular size distribution, biomass area; fast, exact and cheap	Red granule colour necessary	no	10 min	Flat-bed scanner, image analysis software	< 0.50 €	Podmirseg et al. 2015
HA	Fast, exact, cheap	Euqipement needed	yes	15 min	Fluorometer, Precellys bed mill, water bath	1-2 €	Own experience
SAA	Not only presence, but activity measured	Possibly biased through dentirifiers	yes	5 h	Shaker, incubator	20 €	Own experience

5.1.2 Fluorescence heme analysis

In comparison to UV-Vis spectrometry for heme analysis, fluorescence spectrometry has to major advantages: the higher sensitivity and the use of fewer and easier manageable chemicals (Sinclair et al., 1999). While the sample preparation for UV-Vis spectrometry includes several dissociation steps – one of them is a dissolving step with pyridine, which should be handled only under a hood and has an acrid odor -, fluorescence analysis relies only on dissociation through oxalic acid. Therefore, fluorometric measurement is faster, more sensitive and easier applicable than UV-Vis analysis and was chosen over UV-Vis spectrophotometry; the research was based on a protocol proposed by Sinclair et al. (1999).

Fluorescene heme analysis has already been used be several researchers (Table 15): Morrison (1965) conducted several experiments to determine the appropriate heating time and temperature for obtaining the highest possible intensity when analysing heme via fluorescence spectrometry. An important factor was that the oxalic acid solution should be added rapidly maximum 10 minutes before heating. Otherwise, the fluorescence intensity of the samples decreased proportionally to the time they were left at room temperature with oxalic acid added.

The experiments also showed that the maximum fluorescence intensity yield was reached after 30 minutes of heating at 120°C (Morrison, 1965). To obtain this result, Morrison (1965) varied both, heating time (from 98°C to 125°C) and heating interval (from 0 to 60 minutes).

Sassa (1976) adapted the protocol proposed by Morrison (1965) slightly by changing the heating temperature from 120°C to 100°C and by changing the formula of the standard solution. Nevertheless, Sassa (1976) still added bovine serum albumin to guarantee the stability of the standard solutions.

The fluorescence heme assay this research is based on - proposed by Sinclair et al. (1999) - includes minor changes to the previously mentioned assays: the protoporphyrin standards are produced with 100% DMSO as dissolving agent and diluted in 40% (vol/vol) DMSO without adding of bovine serum albumin. Otherwise, Sinclair et al. (1999) propose to use the method described by Sassa (1976) (Table 15).

Table 15: Comparison of fluorescence heme assays as established in previous research.

Source	Heme C standard	Solvent	Additional reagents	Linear measuring range	Heating temperature and time
Morrison (1965)	Red blood cells washed in isotonic saline; hemoglobin	2M oxalic acid	0.1M phosphate buffer, 7.4pH, containing 0.05% albumin	0 –0.5 mg haemoglobin per ml oxalic acid	120°C for 30 minutes
Sassa (1976)	Hemin solution	2M oxalic acid	1% (wt/vol) bovine serum albumin	10^{-9} – 10^{-6} M per tube	100°C for 30 minutes
Sinclair et al. (1999)	Protoporphyrin or heme standard solution	2M oxalic acid	100% and 40% (vol/vol) DMSO	10^{-9} – 10^{-6} M per tube	100°C for 30 minutes

As first step, the protocol proposed by Sinclair et al. (1999) was used for pilot tests. Two peaks were obtained for a standard heme solution using 100 % DMSO (Figure 18).

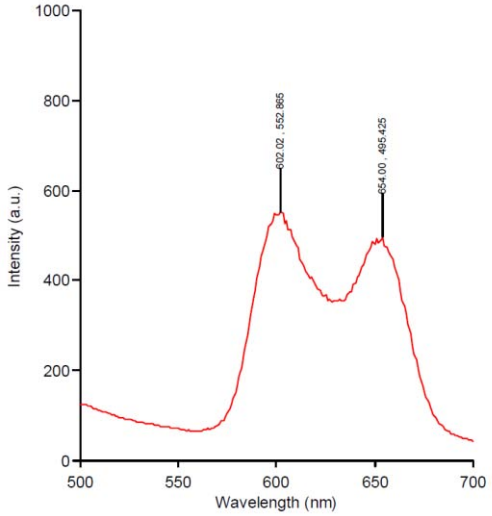

Figure 18: Intensity graph over wavelength as produced by the fluorometer for a 15 mg/L ECC standard.

Although the first peak at 602 nm led to a higher intensity, the second peak at 654 nm was chosen for further analysis as this peak is claimed to be less susceptible to interferences through background noises (Sinclair et al., 1999). This could be proved through a lower variance in the results when comparing the two peaks and was also shown by the coefficients of determination obtained by the two peaks throughout the pilot calibration: while peak 1 produced R^2=0.9224, peak 2 had an R^2=0.924. As the coefficient in both cases was below 0.95, the calibration was repeated and an optimization of the method was conducted. The low coefficient could, on one hand, have been produced by the flocks in the stock solution, on the other hand by storing of the heated samples. Sinclair et al. (1999) proposed 100% DMSO as dissolving agent for the production of a heme C standard stock solution (1mg Cytochrome C /ml Diymethylsulfoxide). As heme C source cytochrome C from equine heart (Sigma Aldrich) was used. The dissolving of the cytochrome C standard in the proposed agent led to flocculation and precipitation of cytochrome C particles in two pilot tests as can be seen in Figure 19. Various methods to increase the solubility (stirring, heating, ultrasonic bath) were used, but none of them produced a satisfying result. Therefore, the propositions of this protocol seemed to be incorrect or at least not appropriate for cytochrome C from equine or bovine heart and further experiments had to be conducted, to find out whether a different solvent was more sufficient.

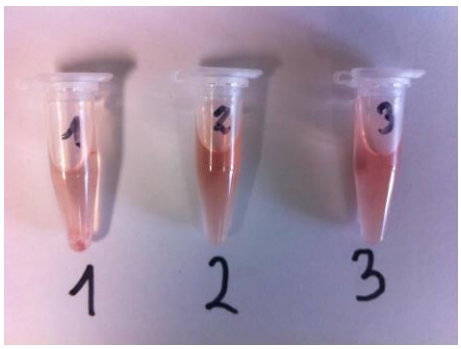

Figure 19: 100% (v/v) DMSO (1), 60% (v/v) DMSO and 40% (v/v) DMSO as dissolving agents for a heme C standard stock solution with equine cytochrome C as standard

Although Sinclair et al. (1999) based their protocol on Sassa (1976) – who increased the concentration of albumin in the standard solutions from 0.05% (Morrison, 1965) to 1% (wt/vol) – it is surprising, that they did not propose the use of albumin to secure the heme C concentration. Morrison (1965) stated that “in the absence of added albumin, dilute haemoglobin standards cannot be stored and even fresh standards give erratic results”. Sinclair

et al. (1999) did not state whether they have chosen DMSO as dissolving agent in the standards to decrease this effect or if the decision on the dissolving agent was driven by other factors.

Another point of discussion is the usage of DMSO as dissolving agent. As DMSO is known to interfere with the heme pathway in cells (Sassa, 1976), its influence on the heme C concentration has to be kept in mind. When diluting samples from the 3L PNA reactor with 60% DMSO, a change of colour could be observed within a day of storage: the brownish sludge flocks (nitrifiers, heterotrophs and inorganic matter) decreased in size and the granular Anammox biomass turned more reddish. As this effect already can be seen after a rather short time interval, biomass samples which have been diluted with DMSO should not be stored for a longer time interval before measuring their heme C content to decrease measuring errors.

The effect of adding albumin to the standards as done at Morrison (1965) and its influence on the results has not been tested. Nevertheless, only fresh standards (not older than one day) were used for all measurements to decrease a possible error occurring through the missing of albumin. It could not be proven that the absence of albumin led to erratic results when using fresh standards, as the cytochrome C standards led to a sufficient calibration line and standard deviation (Figure 25).

A more crucial point in the laboratory procedure than the addition of albumin is the adding of the 2M oxalic acid: after the acid is added, the samples have to be heated as fast as possible as the oxalic acid eliminates fluorescence capacity at room temperature (Morrison, 1965). According to Morrison (1965), “the loss in ultimate fluorescence of the pre-incubation mixture of heme protein and oxalic acid is almost linear during the first 30 minutes at room temperature and amounts to approximately 15%...”. Therefore, a preparation of samples with oxalic acid for measuring at a later stage cannot be conducted. When the samples have already been heated, they can be measured within a time span of 24 hours, as the solution is stable within this time interval at room temperature (Morrison, 1965). The fact that 2M oxalic acid is close to saturation (Sinclair et al., 1999) - which can lead to precipitation at room temperature– should not have a negative influence on the obtained fluorescence values: Morrison (1965) observed that 1.8M to 2M oxalic acid are most effective in removing the iron from the heme moiety and in accordance to this of porphyrin derivate fluorescence. Therefore, a slightly lower oxalic acid concentration resulting through precipitation should not negatively affect the results. The later research/ protocols (Sassa, 1976, Sinclair et al., 1999) on fluorescence heme analysis did not propose a time interval within which the samples should be measured. Nevertheless, all samples which have been measured during this research have been measured within 12 hours after heating. The effect of measuring samples at a later stage (over 24 hours) has not been tested.

The results of the pilot tests led to the conclusion that the dissolving agent and the heating time had to be optimized to develop an accurate fluorescence heme analysis. This led to the establishment of the following procedure to determine the appropriate sample preparation for the method:

1) Finding of an accurate dissolving agent
    a. Testing of DMSO – solutions (20% [v/v] to 100% [v/v])
    b. Comparison with USF – water as dissolving agent
2) Testing of two different standards to recheck the results
    a. Cytochrome C from equine heart (Simga Aldrich)
    b. Cytochrome C from bovine heart (Sigma Aldrich)
3) Determining the appropriate heating time
    a. 10 min to 240 min
4) Calibration and determination of the accurate measuring interval
    a. Finding of the limit of determination
    b. Finding of interval with acceptable variance in the results

As DMSO in principle increases the solubility of heme C (Sinclair et al., 1999), three dissolving agents were tested: 100 % DMSO, 40 % DMSO (v/v) and 100 % USF-H_2O. The 100 % DMSO showed the expected result: flocculation occurred and the particles could not be destroyed by

standard methods (stirring, ultrasonic bath, heating). As flocks would highly decrease the reliability of the results, 100 % DMSO was excluded as possible solvent.

The dilution with 40 % (v/v) DMSO led to no obvious flocculation. Centrifugation of the solution produced no pellet; therefore this method was used to create standard solutions and a calibration was conducted. 100 % USF-H_2O also dissolved the cytochrome C standard completely and without flocks and was therefore used to obtain a second calibration.

Comparing the calibration of 100 % USF-H_2O and 40% (v/v) DMSO as dissolving agents for heme C, the DMSO solution showed higher intensity (Figure 20). Standards with no clear peaks when using USF-H_2O as solvent were above the measuring range when using 40 % (v/v) DMSO. The 40% (v/v) DSMO solution was also able to outperform the 100% DSMO. Therefore, DMSO was chosen as dissolving agent, but the concentration had to be optimized. The DMSO concentration was increased to the highest possible percentage without flocculation to ensure a high dissolving capacity for cytochrome C while keeping a sufficient accuracy of the method.

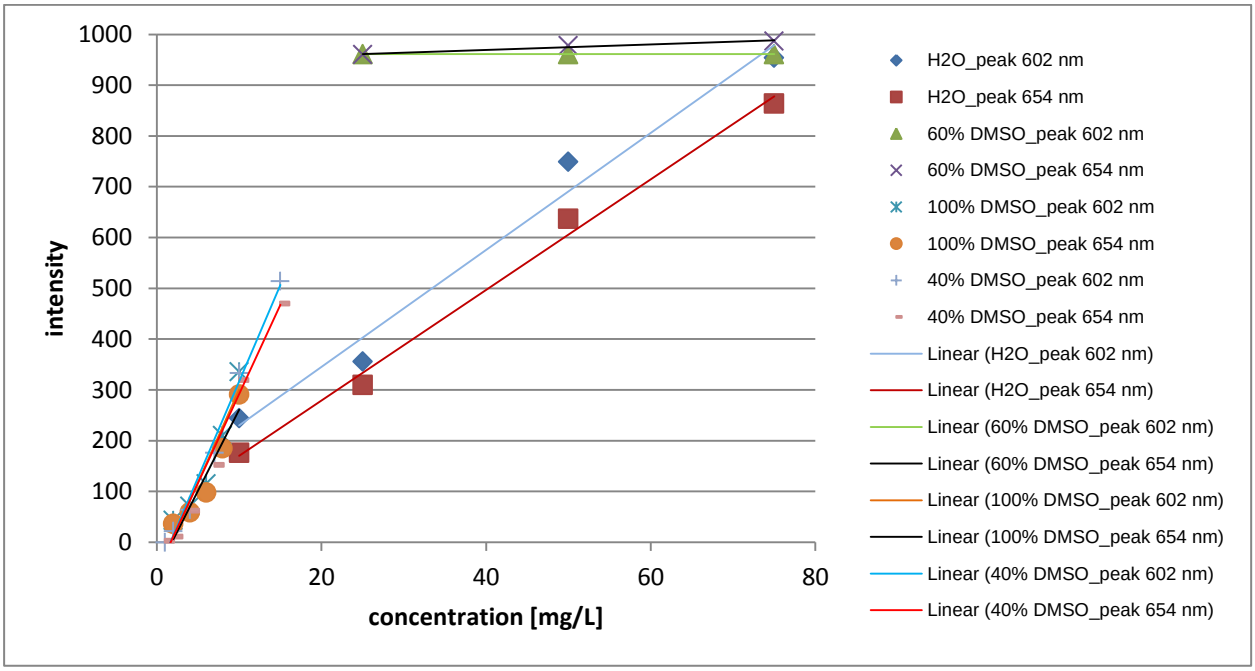

Figure 20: Comparison of the standard calibration for different solvents with 100 % DMSO, 40% DMSO and 0% DMSO (USF-H_2O).

To find this optimal DMSO concentration, several experiments with cytochrome C (CC) from equine heart (ECC) were conducted (Figure 21, Figure 22). The experiments led to the result that a DMSO concentration (v/v) of approximately 60% (exactly 61%) possesses the best dissolving capacity.

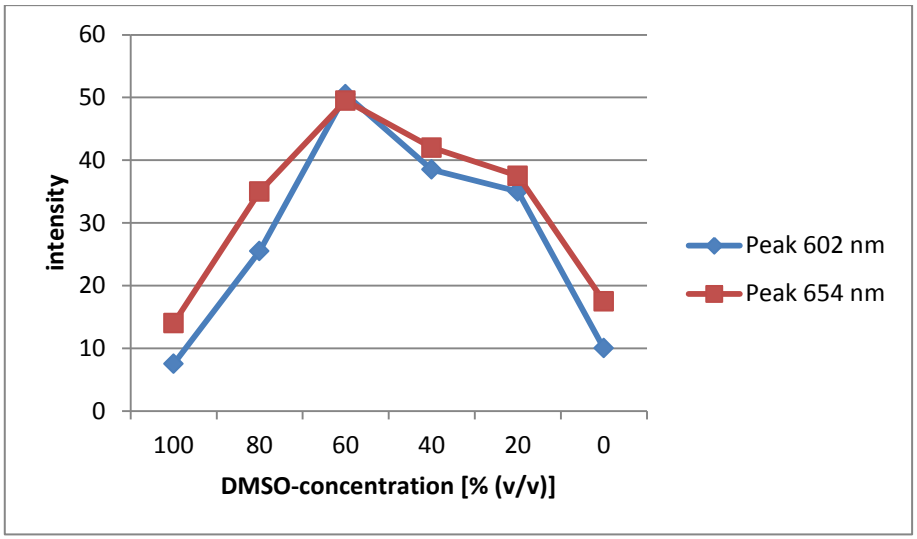

Figure 21: Determination of appropriate DMSO concentration with centrifugation (4mg/L ECC standard). Six different DMSO concentrations were compared.

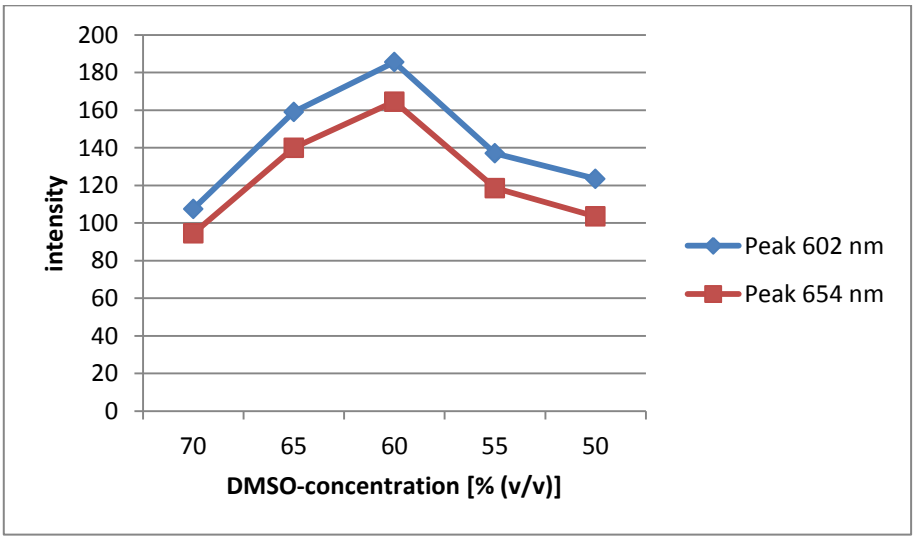

Figure 22: 8mg/L standards without centrifugation. 5 standards in 5 % intervals were tested. 8 mg/L CC-standards were used.

To ensure this assumption, cytochrome C from bovine heart (BCC) was introduced as second standard and the measurements were repeated. Although BCC also possessed a clear peak in intensity when solved in 60% (v/v) DMSO, ECC and BCC showed slightly different behaviour in fluorescence intensity, as can be seen in Figure 23.

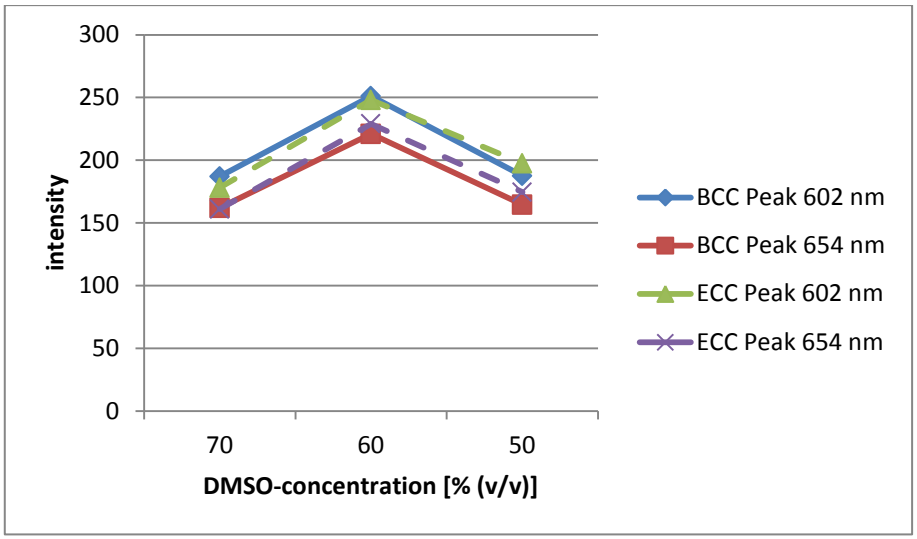

Figure 23: Comparison of BCC and ECC standards. For both heme sources 8 mg/L Cytochrome C standards were used and centrifuged prior to the experiment. 3 different DMSO-concentrations were tested.

To determine the optimal heating time (at 100°C), several time steps and cytochrome C concentrations were tested. Exemplarily, experiments with an 8 mg/L ECC standard are summed up in Figure 24. The experiments resulted in an optimal heating time of around 30 minutes. Below 30 min, the intensity highly decreased. Above 30 min, a slight decrease in the intensity could be observed. Therefore, the heating time of 30 minutes also proposed by Sinclair et al. (1999) was used for further analyses.

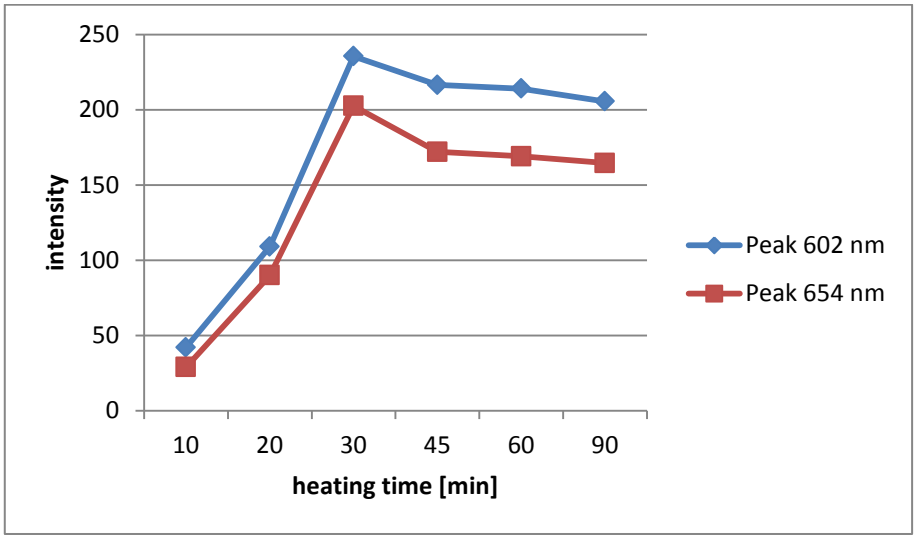

Figure 24: Complete data set of the heating experiments with 8 mg/L ECC-standards.

Although Morrison (1965) already conducted research on the effect of the heating time on the fluorescence intensity, it was chosen to redo this experiment to monitor whether a slight increase of heating time could lead to improved results. Comparable to Morrison (1965) who has concluded that an additional heating after 30 min does not lead to further increase in fluorescence; it was observed that 30 minutes produce the highest fluorescence values. While fluorescence stayed stable in Morrison's research, the standards used in this research showed a peak at 30 minutes followed by a decrease of fluorescence at longer heating times (Figure 24: Complete data set of the heating experiments with 8 mg/L ECC-standards.). The reason for these differing observations could either lie in the different dissolving agents used (bovine plasma albumin – phosphate buffer (Morrison, 1965) versus 60% DMSO (this research)) - or in the differing heating temperatures. Morrison focused his research on 120°C of heating

temperature while this study used 100°C as proposed by Sassa (1976) and Sinclair et al. (1999).

Overall, the establishment of the fluorescence heme analysis led only to an adaption of the standard protocol, considering:

- The change from 100% (v/v) DMSO to 60% (v/v) DMSO as dissolving agent for standard solution.
- Dilution of the samples not with USF-H_2O but with 60% (v/v) DMSO at all dilution steps to increase the dissolving capacity.

All other steps were kept as proposed by Sinclair et al. (1999), leading to a finally established protocol as following:

- Collect tissue samples from reactor
- Dilute tissue samples with **60%** (v/v) DMSO solution
- Collect tissue culture cells in 6 x 50 mm-disposable glass tubes by centrifugation or place small volume (up to 50 µl) in the tubes
    - Protein amount should be < 10 µg/tube. The assay is linear from 1nM to 1µM (or 0.5pmol to 0.5 nmol) heme per tube.
    - The assay is most conveniently performed using a cell holder that holds four 6 x 50 mm tubes. Alternatively, samples can be transferred into disposable plastic cuvettes for fluorescent measurements
- Add 0.5 ml of 2M oxalic acid solution to mix thoroughly
- Put sample tubes in a heating block set at 100°C for 30min. Do not heat blanks.
    - Blank and sample tubes contain tissue and oxalic acid, but the blanks are not heated.
- Read fluorescence of porphyrin using **402** nm excitation and **654** or **602** nm emission, after determining that these represent the actual peaks with the instrument used.
    - The **654** nm emission peak has lower fluorescence than the 602 nm peak, but also has less scatter and other interference.
    - Actual peaks vary from instrument to instrument.
- Subtract blank values (parallel unheated samples in oxalic acid) from samples.
- Calculate heme C content based on a standard heme curve over the range of 0 to 1 nmol heme per tube, or calibrate with 1mg/ml heme standard solution or a protoporphyrin standard.

Heme C analysis was established at a fluorescence spectrophotometer (Cary Eclipse Fluorescence Spectrophotometer, Agilent Technologies, US) with a sufficient $R^2 = 0.9968$ for the relevant peak (Figure 25) using a Precellys bead mill (Precellys 24, Bertin Technolgies, FR) for homogenisation. As the heme C content is dependent on the concentration of Anammox biomass, it is expressed as mg heme C/ g-VSS. The detailed established laboratory protocol can be found in the appendix (9.1.5) For data analysis the peak at 654 nm was used as it yielded a higher R^2 at the final calibration (Figure 25).

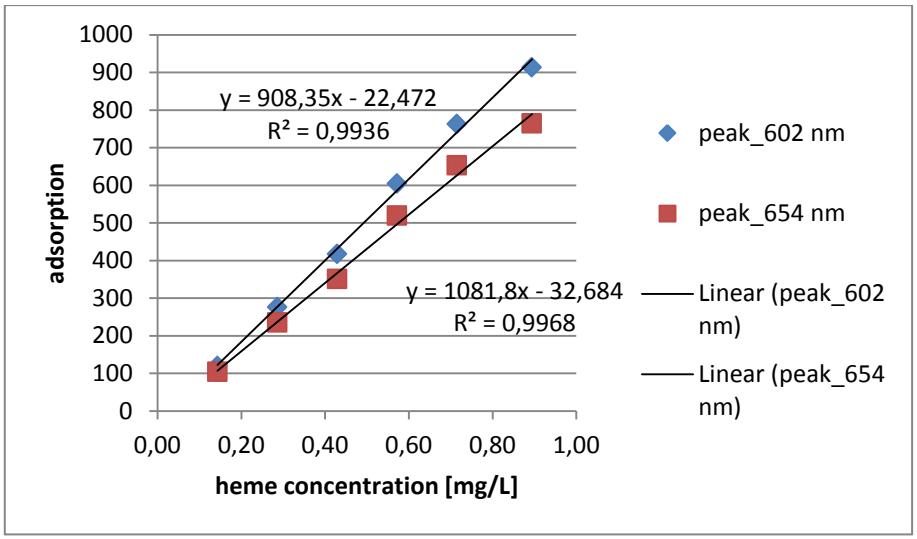

Figure 25: Standard calibration for heme C with cytochrome C from equine heart as standard.

5.1.3 Specific Anammox activity (SAA) measurement

In principal, SAA can be measured in two ways: wet chemically over the stoichiometry of the Anammox process or the overpressure produced when N_2 gas accumulates. As a manometric measuring system would decrease the time and cost expenditure, it was decided to introduce this system as proposed by Dapena-Mora et al. (2007).

Manometric measuring of SAA is compared to a wet chemical measurement cost and time saving as the analysis effort is drastically reduced. Several authors (Dapena-Mora et al., 2007, Dosta et al., 2008, Yang and Jin, 2012) were able to establish a sufficient manometric measuring system.

250 ml Schott Duran bottles were equipped with a manometric sensor capable of measuring up to 200 mbar pressure increase. For the installation of the sensor, the bottles' caps got drilled and were afterwards equipped with a rubber seal. The system's tightness was checked through pilot tests with yeast. Several yeast – glucose-solution combinations were used to evaluate the set up. As substrate media synthetic wastewater with 70 mg/L NO_2-N and NH_4-N was used (Dapena-Mora et al., 2007, Dosta et al., 2008). Two alterations were made in comparison to Dapena-Mora et al. (20079 and Dosta et al. (2008): To exclude pH fluctuations, a phosphate buffer system was established and the pH set to 7.8, to guarantee a sufficient buffer capacity, 1.5 g/L sodium bicarbonate.

Unfortunately, no tight system set up could be reached with the equipment available at the laboratory and the overpressure could not be maintained. The failure of the system used in this study could be justified by the missing thickness of the rubber seals which led to leakage after inserting the substrates with a syringe or with an untight connection of the sensors to the caps. While this study used 250 ml Schott Duran bottles, earlier research (Dapena-Mora et al., 2007, Dosta et al., 2008, Yang and Jin, 2012) mostly used a maximum of 100 ml total volume. Although none of the mentioned researchers describes the used vials, a volume of up to 100 ml suggests that headspace vials were used. As these vials are commonly equipped with sealed disposable caps, a tight system can be easier reached.

Therefore, SAA was established wet chemically as proposed by Podmirseg et al. (2015). The set-up of the system was kept as described above, yet instead of manometric measurement, spectrophotometric analysis of the liquid phase was used. Samples were drawn every 30 min to 60 min over an interval of 4 hours using the stoichiometry of the Anammox reaction. The samples were analysed with a Tecan Reader (Tecan Multimode Reader Infinite 200 PRO, Tecan Trading AG, CH; used for NH_4-N measurement) and with standard cuvette tests (Hach Lange, LCK 342 for NO_2-N and LCK 339 for NO_3-N; Lasa 50, Hach Lange, A). SAA is related to the VSS concentration and expressed as g-N/(g-VSS*d).

Nevertheless, the advantages of manometric SAA measurement – faster analysis with reduced work load and cost expenditure – argue for the establishment of this methodological approach. While wet chemical SAA determination sums up to material costs of approximately 50 € (10 cuvette tests á 3 €, chemicals for synthetic wastewater, eppis), manometric measurement does not need any cuvette tests, reducing the costs to 20 €.

The preparatory work conducted throughout this research – determination of appropriate synthetic wastewater, establishment of buffer system, testing of manometric sensors, decision on measuring intervals and total run time - allows a fast future establishment of this method when a thigh system is reached.

5.2 Multifactorial inhibition assay

To determine the practical applicability of the adapted monitoring, a multifactorial batch assay was developed. This allowed the testing of both methods at standard operation conditions and at the presence of inhibitors.

Other researchers conducting inhibition assays have mainly focused on the inhibition potential of industrial wastewater. Considering municipal wastewater and the influent standards for municipal WWTPs in Austria and the EU, the high inhibitor concentrations tested in previous research are of almost no relevance (Jin et al., 2013a and b; Jin et al., 2012; Zhang et al., 2014; Zhang et al., 2015; Dapena-Mora et al., 2007).

For the introduction of deammonification in broad application at communal WWTPs with <50,000 PE, possible inhibition caused by the influence of process additives or sub-optimal process conditions occurring in practice at such plants have to be analysed. Currently, the main application of deammonification – the treatment of reject water from sludge dewatering after mesophilic digestion – implies the necessity to test Anammox bacteria on their ability to deal with these influences.

Therefore, the experiment focused on the influence of nitrite accumulation during the deammonification process and commonly used additives for dewatering the digested sludge (iron chloride, polymers) in concentrations applied in practice at WWTPs.

Previous research focussed only at short-term effects of inhibitors through singular addition and SAA measurements (Carvajal-Arroyo et al. 2014, Dapena-Mora et al. 2007, Liu and Horn, 2012, Puyol et al., 2014). In contrast, the experiment conducted within this thesis focussed on long-term effects with a run time of 12 days. Hence, also accumulation effects of the tested inhibitors were monitored.

5.2.1 Nitrogen removal and VSS

In total, the treatment efficiency and total nitrogen removal rate decreased for most treatments. While treatments with low nitrite concentrations and singularly added inhibitors suffered only minor losses (1% for polymer and 4% for Fe(III)), the low nitrite, high polymer and Fe(III) vials showed an activity loss of 21%. Interestingly, also the blank (70 mg/L NO_2-N, no adding of Fe(III) and polymer) lost 10% of its nitrogen removal capacity over time. As NO_2-N was always completely consumed in the withdrawal samples, it can be assumed that the Anammox biomass at low level nitrite conditions suffered from starving which led to an overall decrease in its fitness and removal efficiency. Anammox bacteria are known to show a lower tolerance to inhibitors at starving conditions (Carvajal-Arroyo et al., 2014). This can be an important factor at reactor start up after a longer sludge transportation/storage period or at underloaded WWTPs, where the biomass is continuously underfed and therefore prone to inhibitors. Especially for small WWTPs facing high fluctuations in inflow volume and concentrations or plants which are supplied by a combined sewer, underloading could play a role in successful Anammox reactor operation.

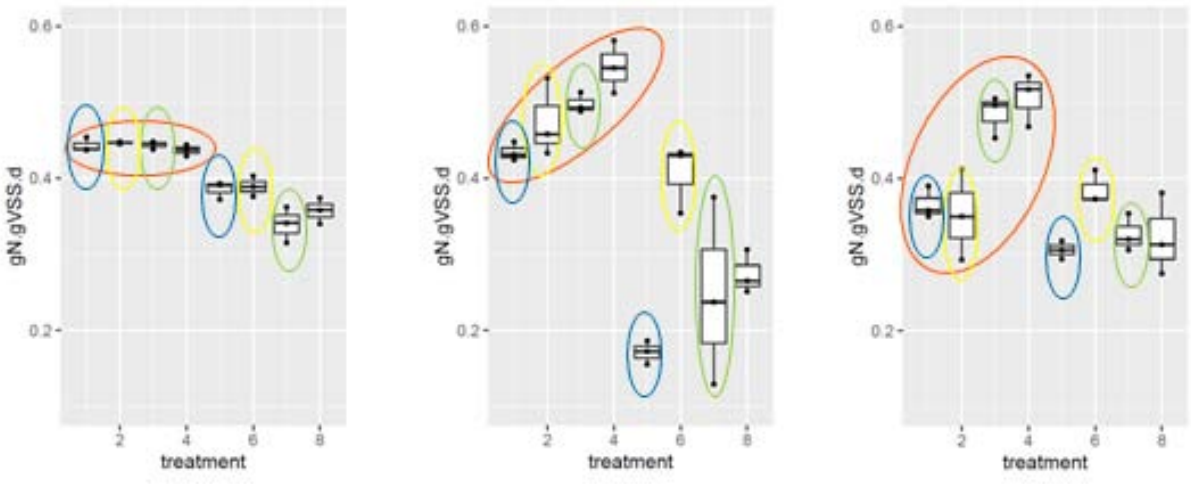

Figure 26: Total nitrogen removal capacity of all treatments for the sampling cycles. Cycle 1 (left), cycle 2 (middle), cycle 3 (right) ■ = High polymer and Fe(III)Cl_3 levels, ■ = high nitrite level, ▪■ = high polymer level, ■ = high Fe(III)Cl_3 level

In total, treatments with a higher NO_2-N concentration performed better than those with lower nitrite content (Figure 26). While the first cycle was rather uniform for all treatments, the influences of the different treatments got observable within the runtime of the experiment. Especially treatments 3 and 4 (high nitrite/high Fe(III)Cl_3/low polymer and high nitrite/low Fe(III)Cl_3/low polymer) were able to outperform the other treatments. Treatments 1 and 2 could not use their advantage of higher NO_2-N concentration and did not show a significantly better performance than treatments with low nitrite levels. As the used NO_2-N is higher than the inhibitory concentration proposed by several authors (chapter 3.2.1, Lopez et al., 2008, Strous et al., 1999), this result could be based on a possible underloading of the vials throughout the experiment.

The cumulative plots (Figure 27) clearly show that all treatments were able to deplete the whole added NO_2-N. Nevertheless, the nitrate concentration did not behave as stochiometrically expected. This leads to the assumption that either denitrification occurred, which is unlikely as most denitrifiers are heterotrophs and no organic carbon was available, or that Anammox bacteria used nitrate as electron acceptor when nitrite was fully consumed. This metabolic pathway was already observed by Kartal et al. (2007).

For treatment 5 and 7, cycle 2 showed a problematic behaviour: NH_4-N concentration seemed to increase rather than decrease. Combined with the rapidly decreasing nitrate concentrations in these treatments, it was assumed that Anammox bacteria functioned as facultative denitrifiers, depleting NO_3^- to NH_4^+ and NO_2^- and converting it to N_2 gas. This behaviour has been observed in previous research (Kartal et al., 2007).

The ability of Anammox bacteria to function as facultative denitrifiers (Kartal et al., 2007) and their possible use of Fe^{3+} as electron donor complicates the calculation of an appropriate nitrogen mass balance based solely on Anammox activity. Especially treatments 2, 4, 6 and 8 showed a stoichiometrically adequate accumulation of NO_3-N throughout the experiment of 8% to 13% of the supplied NH_4-N. While Treatments 1 (7%), 3 (6%) and 7 (6%) still accumulated nitrate quite sufficiently, treatment 5 reached only a NO_3-N concentration of 2% of the supplied NH_4-N, meaning that almost all NO_3-N added with the synthetic wastewater was consumed.

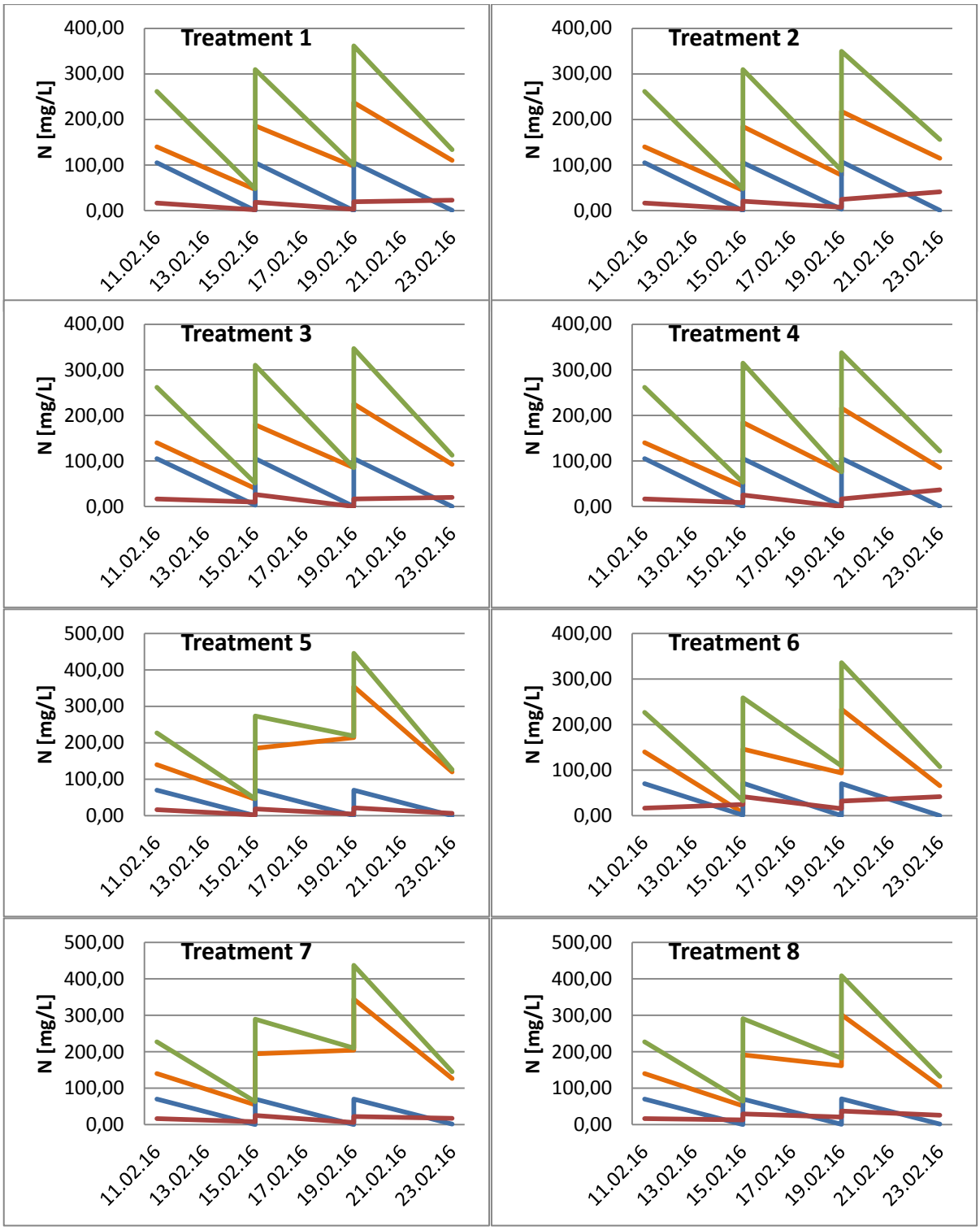

Figure 27: Cumulative sums of N_{tot}(green line), NH_4-N (orange line), NO_2-N (blue line) and NO_3-N (red line).

Treatments with polymer led to a floating coagulate and sludge flocks sticking to the glass wall which could not be suspended into the liquid phase of the vial. Therefore, nitrogen depletion as well as heme C content and SAA might be underestimated for these treatments.

Although the experimental runtime exceeded the proposed doubling time of Anammox biomass, (approximately 11-20 days) no biomass growth could be observed. On the contrary, VSS decreased (rapidly). This trend can on the one hand be explained by the die-off of nitrifiers which were not supplied with oxygen and could not maintain their metabolism, on the other hand by starvation and inhibition conditions for the Anammox biomass. As the substrate supply

was not sufficient for the whole cycle time, Anammox had to use the whole energy to maintain their metabolism and could not invest it in growth (Chen et al., 2012). At sufficient growth and maintenance levels, a linear correlation between substrate removal/conversion rate (NO_2^- and NH_4^+), product formation rate (NO_3^-) and VSS accumulation can be observed (Chen et al., 2012).

5.2.2 Heme C content and influences of the inhibitors

The relevance of heme C on the Anammox metabolism and nitrogen turnover rate (Chen et al., 2012) allows heme c analysis to be a promising monitoring tool. Hence, heme C has not yet been tested as continuous monitoring parameter at the presence of possible inhibitors or normal wastewater constituents in long-term experiments.

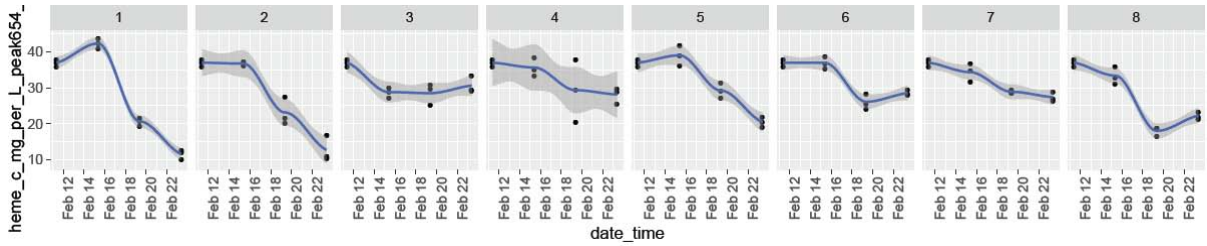

Figure 28: Development of heme C content over 12 days of experiment for each treatment in relation to the VSS content in the vials.

During the experimental run time, all treatments lost heme C content (Figure 28). Especially treatment 1 and 2 suffered from severe heme C concentration losses (-69% for treatment 1 and -66% for treatment 2). Heavy heme C content losses were also observed at treatment 5 (-45%) and treatment 8 (-40%). The lowest concentration deterioration was seen at treatment 3 (-17%), followed by treatment 6 (-23%).

For the decision upon the $Fe(III)Cl_3$ level to be tested, literature data on threshold levels was used. To minimize the risk to severely inhibit Anammox biomass already at the first cycle, a $Fe(III)Cl_3$ concentration which was already inhibitory according to some research (Liu and Horn, 2012) but below the Fe(II) inhibitory level observed in another paper (Qiao et al., 2013) was chosen. Despite the fact, that the effect of Fe(III) and Fe(II) on Anammox differ and supposedly some Anammox species react differently to Fe, the observed positive effect of Fe(III) is incompatible with the research by Liu and Horn (2012). Nevertheless, Liu and Horn (2012) have not specified their used Anammox bacteria in microbiological terms. In this study, also no microbiological analysis defining the Anammox species was conducted. Earlier research done with the sludge at WWTP AIZ Strass i. Zillertal, Tyrol led to differing results: An analysis conducted in 2007 resulted in a 92% similarity to Candidatus Brocadia anammoxidans (Innerebner et al., 2007). However, younger research (Park et al., 2010) found several Anammox species in sludge originating from Strass, with bacteria related to Kuenenia stuttgartiensis representing the principal. During operation of the experimental reactor Park et al. (2010) observed a shift in the bacterial community over Candidatus Brocadia fulgida to Candidatus Brocadia sp.40. Therefore, no secured statement on the bacterial community can be made which makes a definite statement on the inhibitory concentration of Fe(III) difficult, as research analysing the effect of iron on Anammox bacteria (Qiao et al., 2013) did use Candidatus K. stuttgartiensis and not the species presumably present at the inoculum used in this study.

Although also treatments with high $FeCl_3$ concentration lost high amounts of their initial heme C content, the linear models fitted to the results showed a significantly positive influence of higher $FeCl_3$ concentration on the heme C concentration of the biomass expressed by p-values <0.5 for cycles 2 ($p=0.05$) and 3 ($p=0.001$). This effect is based on the necessity of iron for Anammox biomass growth and heme C synthesis. The increase in heme C content through moderately increased Fe(III) concentrations in the reactor/vial indicates that Fe(III) enhances heme C synthesis and therefore also hydrazine dehydrogenase activity and Anammox bacteria growth.

Nevertheless, the increase in positive effect of $FeCl_3$ lasted only until cycle 3. Afterwards, the effect started to decrease. As literature reports that an increased iron level is only beneficial until certain threshold levels and leads to a decrease in Anammox activity when this value is exceeded (Qiao et al., 2013), the decreasing effect might suggest the reaching of or getting close to the threshold for this Anammox species which would then lay at 6 mg/L Fe(III). The result found in this study lays severally beneath the threshold level obtained by (Qiao et al., 2013) at 10.05 mg/L Fe(II), but as Fe(II) is assumed to be less inhibitory than Fe (III) (Liu and Horn, 2012), it is reasonable. Compared to Liu and Horn (2012) who observed a drastic deterioration of removal capacity when reaching a Fe(III) level of 0.4 mg/L, a contradicting results was found.

Also the other two tested factors, NO_2-N and polymer, significantly enhanced heme C concentration when added singularly, expressed by positive estimates in the fitted linear model (Table 16). In combination especially NO_2-N and polymer but also NO_2-N and Fe resulted in a heme C deterioration. The NO_2-N/polymer interdependency might have been a result of the physical limitation for NO_2-N degradation in the presence of a polymer when its concentration exceeded a certain threshold value. In total, the linear model fitted the heme C concentrations and the analysed inhibitors showed a sufficient goodness of fit with R^2 of 0.6413 to 0.9475. While significant influences of the tested factors on heme C content were not observed in the first sampling cycle expressed by p-values exceeding the significance level 0.5, their significance increased as the experiment proceeded. For the last sampling cycle, several inhibitors (combinations) had high levels of significance (Table 16, Figure 28).

Table 16: Linear model description for heme c concentration prediction through the tested factors.

	cycle 1		cycle 2		cycle 3		cycle 4	
Factor	**estimate**	**p-value**	**estimate**	**p-value**	**estimate**	**p-value**	**estimate**	**p-value**
Intercept	37 ± 2.082	0	29 ± 4.3	0	-4.7 ± 7.8	1	10.4 ± 4.1	0.01
NO_2-N	2.1E-17 ± 3.9E-3	1	1.1E-2 ± 8E-3	1	5.4E-2 ± 1.4E-2	0.001	2.8E-2 ± 7.7E-3	0.001
Fe	1.6E-15 ± 4.9E-1	1	2.8 ± 1	0.01	5.7 ± 1.8	0.001	1.8 ± 1.0	0.5
polymer	1.4E-17 ± 9.8E-3	1	2.9E-2 ± 2E-2	1	0.1 ± 0.04	0.001	0.2 ± 1.9E-2	0
NO_2-N:Fe	-3.5E-18 ± 9.2E-4	1	-6.2E-3 ± 1.9E-3	0.001	-9.3E-3 ± 3.4E-3	0.01	-2.1E-3 ± 1.8E-3	1
NO_2-N:polymer	-3.3E-20 ± 1.8E-5	1	-3.9E-5 ± 3.8E-5	1	-2.3E-4 ± 6.9E-5	0.001	-3.4E-4 ± 3.6E-5	0
Fe:polymer	9.5E-18 ± 2.3E-3	1	-1.2E-2 ± 4.7E-3	0.01	-1.1E-2 ± 8.7E-3	1	-1.8E-2 ± 4.6E-3	0.001
NO_2N:Fe: polymer	-2E-20 ± 4.3E-6	1	3E-5 ± 8.9E-6	0.001	1.6E-5 ± 1.6E-5	1	2.5E-5 ± 8.6E-6	0.001
R^2	**1.9E-29**		**0.8342**		**0.6413**		**0.9475**	

5.2.3 SAA and influences

The development of SAA was characterised by a drop at the third cycle of the experiment (Figure 29). Nevertheless, SAA recovered quite fast and cycle 4 had an improved SAA in comparison to cycle 3.

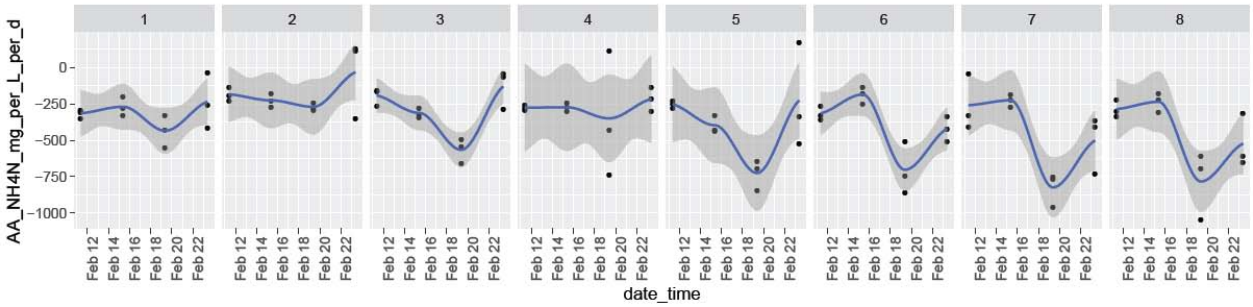

Figure 29: Development of SAA at the four sampling dates. SAA are given as decrease in mg total nitrogen concentration per L and d.

When plotted over VSS, SAA showed a rather drastic increasing trend for several treatments and a rapid decrease for others. Especially treatments with low nitrite concentration could improve their SAA by up to 95% (Table 17). In principal, nitrite had a negative effect on SAA resulting in a decreasing SAA for all four treatments with high nitrite concentrations. Comparable to heme, the interdependency between NO_2-N and polymer had the worst overall effect. Due to the high variances in the SAA results, the fitted linear models did not reach a sufficient quality to predict further influences. This was also confirmed by the relatively low R^2 of 0.3464 to 0.6762 (Table 18). The standard deviation of the estimates in most of the SAA models was statistically not acceptable (

Table 18, Figure 29).

Table 17: Comparison of SAA performance expressed in percental change from cycle 1 to cycle 4.

Treatment	Relative SAA of cycle 4 to cycle 1
1	-28%
2	-100%
3	-21%
4	-20%
5	+77%
6	+35%
7	+7%
8	+95%

Table 18: Linear model description for SAA prediction through the tested factors.

Factor	cycle 1		cycle 2		cycle 3		cycle 4	
	estimate	p-value	estimate	p-value	estimate	p-value	estimate	p-value
Intercept	-6.1E2 ± 3.3E2	0.05	-3.2E2 ± 2.3E2	1	-4.0E3 ± 1.1E3	0.001	-3.0E-3 ± 1.1E-3	0.01
NO_2-N	9.0E-2 ± 6.1E-1	1	-0.5 ± 0.4	1	4.8 ± 2.0	0.01	3.7 ± 2.1	1
Fe	-29.5 ± 76.9	1	35.8 ± 53.4	1	1.1E2 ± 2.5E2	1	-68.1 ± 2.7E-2	1
polymer	-1.8 ± 1.5	1	0.1 ± 1.1	1	-0.4 ± 5.0	1	-1.0 ± 5.4	1
NO_2-N:Fe	9.1E-2 ± 1.4E-1	1	-7.4E-2 ± 10.0E-2	1	-0.3 ± 0.5	1	0.2 ± 0.5	1
NO_2-N:polymer	3.9E-3 ± 2.9E-3	1	5.7E-4 ± 2.0E-3	1	2.3E-3 ± 9.4E-3	1	4.2E-3 ± 1.0E-2	1
Fe:polymer	0.6 ± 0.4	1	-0.8 ± 0.3	0.001	0.2 ± 1.2	1	1.7 ± 1.3	1
NO_2N:Fe: polymer	-1.3E-3 ± 6.8E-4	0.05	1.2E-3 ± 4.7E-4	0.01	-1.2E-4 ± 2.2E-3	1	-3.3E-3 ± 2.4E-3	1
R^2	**0.3464**		**0.6762**		**0.5801**		**0.516**	

Overall, SAA measurement did not produce as reliable results as expected. The variance within the treatments was rather high which decreased the ability to predict N-removal through SAA which is proven by the low R^2 values and high standard deviations for the estimates in the obtained linear models (Table 18). Anyhow, the experiment showed that SAA reacts faster to influencing factors than heme C concentration and the treatments' mean values showed an overall trend of high NO_2-N levels having a negative effect on SAA.

5.2.4 SAA, heme and N-removal

To estimate the usability of SAA and heme as monitoring tools for daily use, their capability of prediction the nitrogen removal efficiency and therefore the fitness of the biomass had to be analysed. If SAA and heme should be implemented in standard operation, they have to provide additional information on the system status of the bioreactor.

The linear model fitting heme C content and SAA against N-removal efficiency (Table 19) resulted in a significant relation between higher heme C concentration of the biomass and N removal capacity for cycles 2 and 4. SAA was not able to significantly predict N-removal efficiency of the treatments in most cycles. Only in cycle 2, a significant relation was observed (p=0.01). The reason for the insignificancy of SAA probably was the high variance in SAA data within the treatments. Altogether the linear model had no high quality of fit as R^2 never exceeded 0.4932. Therefore, it is proposed to measure SAA in further experiments manometricly to decrease the sampling error.

Table 19: Linear model description for the nitrogen removal efficiency model at cycle 4 with an R^2=0.4932.

Monitoring tool	estimate	t value	p
Heme	0.003379±0.00111	3.048	0.00635
SAA	-0.000023±0.00004	-0.668	0.51160
Heme:SAA	2.691e-06±1.549e-06	1.737	0.09781

For daily use, these results underlines that, the combination of common monitoring such as nitrogen depletion rate measurement with monitoring tools focusing on Anammox biomass is appropriate to gain insight into the total system in a PNA reactor. Although the fast fluctuation of SAA is important for prompt information on the reactors state and occurring inhibitions to

prevent reactor failure, it can overestimate the inhibiting effect if measured instantly after an inhibition without adaption possibilities.

5.2.5 Effects and interactions of the tested inhibitors

The experiment did show that process additives, especially in certain factor combination, have a significant influence on the performance of Anammox biomass (Table 20).

Table 20: Linear model description for nitrogen removal through the tested factors.

Factor	cycle 1		cycle 2		cycle 3		cycle 4	
	Estimate	p-value	estimate	p-value	estimate	p-value	estimate	p-value
Intercept	0.3 ± 0.02	0	0.3 ± 0.03	0	0.4 ± 0.1	0	-3.0E3 ± 1.1E3	0.01
NO_2-N	1.7E-4 ± 4.2E-5	0.001	2.5E-4 ± 6.3E-5	0.001	3.6E-4 ± 1.1E-4	0.001	3.7 ± 2.1	1
Fe	-3.9E-3 ± 5.3E-3	1	-1.2E-3 ± 7.9E-3	1	5.3E-3 ± 1.4E-2	1	-68.1 ± 2.7E2	1
polymer	4.3E-4 ± 1.1E-4	0.001	7.4E-4 ± 1.6E-4	0	1.1E-3 ± 2.9E-4	0.01	-1.0 ± 5.4	1
NO_2-N:Fe	7.4E-6 ± 9.9E-6	1	-4.9E-7 ± 1.5E-5	1	-1.7E-5 ± 2.7E-5	1	0.2 ± 0.5	1
NO_2-N:polymer	-8.0E-7 ± 2.0E-7	0	-1.5E-6 ± 3.0E-7	0	-2.2E-6 ± 5.4E-7	0	4.2E-3 ± 1.0E-2	1
Fe:polymer	-2.6E-5 ± 2.5E-5	1	-5.6E-5 ± 3.7E-5	0	-1.0E-4 ± 6.8E-5	1	1.7 ± 1.3	1
NO_2N:Fe: polymer	4.9E-8 ± 4.7E-8	1	9.2E-8 ± 7.0E-8	0	1.6E-7 ± 1.3E-7	1	-3.3E-3 ± 2.4E-3	1
R^2	**0.7246**		**0.7643**		**0.7377**		**0.516**	

The adding of polymers for coagulation/flocculation is a standard procedure in wastewater treatment. Nevertheless, research on the influence of polymers on Anammox biomass is sparse. The polymer used at a fullscale WWTP (Poly Separ® PK 1455, Separ Chemie) is a positively charged hydrophilic compound and comparable to the flocculant used by Dapena-Mora et al. (2007) who investigated the influence of concentrations used in industrial wastewater treatment (0-1 g/L) and observed a SAA decrease at a concentration of 1 g/L flocculant of approximately 35 %. Comparable to their research, the formation of biomass conglomerates was observed. Anyhow, Dapena-Mora et al. (2007) could not observe a physical detrimental effect of the tested flocculant on the Anammox biomass.

In this experiment, the conglomerates tended to float which could lead to withdrawal of biomass and therefore decrease VSS and removal capacity of a reactor. In addition, bulking sludge destabilizes reactor performance which could affect the total reactor performance and stability negatively. Beside the observation of this behaviour, a reduced N-removal capacity was monitored.

Similar effects were observed for aerobic granular biomass (Val del Río et al., 2012) when the wastewater was spiked with a coagulant (2.5 mg/L polychloride of aluminium) and a flocculant (polyelectrolyte Chemifloc ®). The substitutes led to a decreased VSS content, a higher diameter of the then fluffy and filamentous granules and therefore to an accumulation of flocs and lowered sinking and biomass retention capacity through higher SVI. All these effects were also observable at this study.

The slight inhibitory effect of the polymer observed in this study probably resulted from a physical limitation through hindering biomass to get in contact with the liquid phase rather than from a chemical effect of the flocculant on the biomass. This would also explain the effect that the combined adding of NO_2-N and polymer led to a significantly negative interdependency. The reduced contact of Anammox cells and substrates led to a reduced NO_2-N removal capacity as the cells could not be supplied with substrates sufficiently. This interdependency occurred not

only for nitrogen removal efficiency, but also for heme C content and SAA. Also, vials treated with an NO_2-N: polymer spiked synthetic wastewater suffered from a decreased VSS content.

Therefore, it can be stated that the addition of polymer has a significantly negative effect on the Anammox biomass and treatment performance when the reactor is highly loaded and NO_2-N levels are high. If AOBs outperform Anammox or aeration is kept at a high level, the lowered treatment performance at the presence of a flocculant could result in a nitrite accumulation and lead to an inhibition over nitrite if the system failure occurs over a longer period.

The second tested additive, Fe(III) has already been investigated by different researchers (Qiao et al. ,2013; Bi et al., 2014; Liu and Horn 2012; Zhang et al. 2012). It has been stated by several investigators that the adding of iron (Fe(II) or Fe(III)) up to a certain threshold level enhances the heme production and therefore also the total Anammox metabolism and removal efficiency (Qiao et al. ,2013; Bi et al., 2014; Liu and Horn 2012; Zhang et al. 2012). This was confirmed by this study. A slight increase of iron (in this case Fe(III)) concentration in the batch vials led to an increased heme C concentration and was significantly positively correlated with the removal capacity of the system. However, the positive trend of Fe(III) enhancement decreased at the last sampling cycle. This could be due to reaching or at least approaching the threshold value for optimal Fe(III) concentration (Qiao et al., 2013, chapter 3.2.3).

Comparing the results with earlier research, the main difference is the use of Fe(III) instead of Fe(II) in this study. The decision on using $Fe(III)Cl_3$ instead of $Fe(II)Cl_2$ was made to reproduce the conditions at a full scale WWTP, where high concentrations of $Fe(III)Cl_3$ in sludge treatment are used. It was assumed that the excess Fe(III) resulting from this treatment inhibited the Anammox biomass in earlier experiments conducted in this project. At this WWTP approximately 29.20 mg/L $Fe(III)Cl_3$ are added to sludge treatment which equals 10.24 mg/L Fe(III). As this concentration is 25 times higher than the reported threshold level for exceed Fe(III) by Liu and Horn (2012), the experiment was conducted with 1 mg/L Fe(III) concentration added at each feeding. Although this concentration already was 2.5 times higher than the assumed threshold value and accumulated during the experiment, the Anammox process was not inhibited, but enhanced. Both, nitrogen removal efficiency and heme C content increased throughout the experiment when treated with Fe(III) rich synthetic wastewater. The drastic nitrogen removal rate deterioration when Fe(III) concentration was increased from 0.75 mg/L to 2.19 mg/L as reported by Liu and Horn (2012) was not observed in this research. On the contrary, the increase of Fe(III) concentration led to an enhanced performance.

The observed effect of Fe(III) correlates with research conducted by Bi et al. (2014) and Qiao et al. (2013) who set the optimal concentrations for Fe(II) at values 2.6 to 5.2 times higher than those reported by Liu and Horn (2012). Although these recommendations were given for Fe(II), the behaviour of Fe(III) seems to follow a similar pattern.

The positive effect of Fe(III) could lay in the support production of heme C at the presence of adequate iron concentrations which guarantees the synthesis of heme-proteins and improves their activity (Bi et al., 2014; Qiao et al., 2013). Anyhow, additional or excess iron (Fe(II) and Fe(III)) can lead to a severe inhibition and would not be favourable for the heme C content in the bacterial cells.

As the combined adding of nitrite and Fe(III) led to a significantly negative effect according to the measured heme C concentration in the Anammox biomass, Fe(III) threshold levels might not be constant but changing with other influencing factors. The iron reducing capacity is reportedly inhibited at the presence of nitrite (Zhao et al., 2014), therefore high nitrite levels could hinder the production of heme C in the cells and iron respiration. This could lead to an accumulation of Fe(III) in the system and the reaching of the threshold value for Fe(III) which would result in an Anammox biomass inhibition. In the treatment of iron rich wastewaters, attention should be given to the nitrite level to hinder accumulation of excess iron and to prevent iron inhibition.

In comparison to Fe(III), a high nitrite concentration had no significantly negative effect on the nitrogen removal efficiency, but possessed a positive correlation with heme C content and

nitrogen depletion rate when added singularly. This might be justified by the fact that biomass fed with higher total nitrogen or NO_2-N content had more substrate to process and therefore could obtain activity longer than less fed bacteria. When added with one of the other two inhibitory factors, nitrite significantly inhibited heme C production. This might, as already discussed, originate in the hindering of iron respiration when added together with iron and in the physical limitation of substrate depletion when combined with the polymer. In contrast to heme C concentration, SAA was negatively affected by the high nitrite concentrations. As a nitrite concentration of > 100 mg/L has been observed as inhibitory (Strous et al., 1999), SAA could have been reduced through a reversible inhibition of the biomass, leading to an acute shock directly after feeding.

6. Conclusions and outlook

The assessment of existing monitoring tools revealed that highly elaborated methods focusing on microbiological analysis of the biomass characterize the biomass exactly, but are not feasible for standard monitoring at WWTPs. These methods require expensive and sensitive laboratory equipment and precise analytical work which cannot be provided at middle scale plants. These methods such as qPCR, FISH and CC require expensive and sensitive laboratory equipment and precise analytical work which cannot be provided at middle scale plants. Anyhow, a microbiological characterization of the biomass in the system at some points is proposed as the community structure highly influences the capability of the biomass to deal with inhibitors according to literature. How fast the community composition changes and if it reaches a steady state after long term operation should be evaluated in further research to provide suggestions on the required frequency of microbiological community structure analysis.

The evaluation and comparison of simpler monitoring approaches showed that methods analysing the Anammox biomass through their granular structure – such as gravimetric analysis and particle tracking - were not applicable at lab scale reactors with high shear forces as the bacteria's granules were constantly destroyed and did not form up to sizes well measureable with these methods.

As heme C analysis and specific Anammox activity (SAA) are capable of monitoring both, non-granular and granular biomass, these two methods have been chosen for the establishment of a monitoring scheme. To reduce the cost and time expenditure connected with these methods and increase their reliability, they were adapted for the application with Anammox biomass.

Heme C analysis was established at a fluorometer as this method is more sensitive than an UV-Vis spectrophotometric analysis and includes fewer work steps. The standard protocol (Sinclair et al., 1999) was optimized for Anammox biomass through changing the dissolving agent and adapting the homogenisation method. To obtain absolute, comparable heme C concentration values related to VSS content, a calibration with equine and bovine cytochrome C was conducted. To exclude errors arising through storage effect, it is proposed to analyse only fresh samples or freeze samples if they cannot be immediately analysed. These improvements led to the establishment of a sensitive, time and cost efficient (approximately 15 min /sample, 1-2 € /sample) monitoring tool displaying not only active, but also (currently) inactive biomass. In addition, it gives information on the community structure: the concentration of heme C in accordance to VSS allows a conclusion on the Anammox fraction and gives information if it is outgrown by AOBs.

SAA, on the other hand, is capable of measuring the maximum nitrogen depletion performance of momentarily active biomass at reasonable time and cost effort (5 h /sample, 20 € /sample). Therefore, it gives information on acutely inhibiting conditions and the present status of the system. As it produces, in contrast to standard nitrogen depletion rate calculations, exclusively data for the Anammox biomass, it can be used to determine whether Anammox or AOBs are the limiting consortia in a PNA system. Although a manometric measurement of SAA through overpressure sensors could not be established, this system has many advantages in comparison to wet chemical analysis (lower cost and time expenditure, no stoichiometric calculation necessary, fewer chemicals used). Comparable to manometric measuring bottles of BOD_5, manometric SAA measurement could be easily applied at a WWTP if a flushing station with dinitrogen gas and an incubator are available. Preparatory work such as decision on a synthetic wastewater, a buffer system and sampling interval has been conducted and the establishment of this method will be pursued.

The combination of SAA, which gives an overview on the status quo of a system and acute problems, and heme C analysis, which reflects not only acute, but also earlier influences which led to inactivation of the biomass, provides a holistic picture on the systems state, unutilized

capacities and arising problems. This led to an adapted monitoring protocol including the establishment of a SAA protocol and an adapted heme C analysis protocol.

The applicability of SAA and heme C analysis as monitoring scheme for Anammox biomass was verified through a 2^3 multifactorial batch assay. Throughout this experiment, SAA showed that fluctuations in the inflow media and inhibition effects are quickly reflected by this method. It also gives a more detailed insight into the effects of inhibitors on the biomass than standard nitrogen mass balancing as it monitors intermediate effects on the maximum activity. SAA displays inhibition faster than standard nitrogen balancing and enables accurate and prompt counter steering to reduce (ir)reversible effects on the system. For middle (and small) scale WWTPs, this fast and accurate information of the systems reaction to fluctuations in the inflow is crucial, as these plants face faster and higher changes in inflow volume and composition than large scale WWTPs.

The capability of heme C analysis to measure not only active, but also inactive biomass supplements the information gained by SAA through giving information on reversible inhibited bacteria. The batch experiment proved that heme C concentration changes slower than SAA, indicating long term and accumulated effects rather than acute inhibition. Nevertheless, it correlates well with the nitrogen depletion rate.

Although the fast fluctuation of SAA through the adaption of biomass to the inhibitors led to a reduced correlation of SAA and the other monitoring factors, the established monitoring scheme (chapters 5.1.2 and 5.1.3) confirmed that the combination of a high NO_2-N concentration and polymer addition has the worst effect on Anammox bacteria (-100% SAA, -66% heme C concentration). Fe(III)Cl_3 in limited concentrations (up to 4.5 mg/L) possesses a positive effect on the Anammox biomass.

The tested inhibition factors polymer and Fe(III)Cl_3 are commonly used at WWTPs as additives for sludge dewatering. The data obtained through the 2^3 multifactorial inhibition experiment shows that these two substances affect the reactor stability significantly. While Fe(III)Cl_3 enhances the biomass' performance at concentrations usually applied at WWTPs, the addition of polymers leads to a destabilization through physical limitation of substrate uptake and conglomerate formation increasing sludge withdrawal. To determine concrete threshold levels for polymer and Fe(III)Cl_3 addition, further research should be conducted. This research should also include microbiological determination of the bacterial community as Anammox species possess, based on their different natural environments, varying tolerance towards inhibitors. Furthermore, the adaption capacity should be investigated further as this could allow the treatment of inhibitory wastewater if a sufficient adaption time is granted.

7. Summary

Due to economic and environmental concerns, not only the treatment efficiency of a wastewater treatment plant (WWTP) in terms of nitrogen and carbon removal, but also energy efficiency and environmental sustainability of the system have become major driving forces for design and process improvement (Hernández-Sancho et al. 2011; Schaubroeck et al. 2015).

To reach the goal of energy consumption reduction or even energy self-efficiency, the implementation of a deammonification/Anammox driven nitrogen removal for ammonium rich wastewater in the side stream can lead towards the goal of energy consumption reduction or even energy self-efficiency (Wett et al. 2007). This process, which is characterized by partial nitritation combined with anaerobe ammonium oxidation, uses the capability of the 1999 discovered Anammox bacteria (Strous et al. 1999) to convert ammonium and nitrite directly to dinitrogen gas.

Nevertheless, the Anammox process and possible inhibiting factors are yet not fully understood. This lack of knowledge leads to difficulties in the introduction of deammonification at middle scale WWTPs. As these plants do not possess personnel capacity to run a complex monitoring system or adapt the process engineering accordingly to the specific needs of both, Anammox biomass and the nitrifiers. Especially keeping equilibrium between sufficient nitritation and anaerobe ammonium oxidation presents a difficulty for implementation.

The “DEKO”-project (deammonification for middle scale wastewater treatment plants in Austria), in which this thesis is embedded, focuses on these problems hindering deammonification from being implemented at a broader scale at communal WWTP. Besides the optimization of process engineering, the development and implementation of an adapted, time- and cost-efficient monitoring presents a major field of interest in the project.

Therefore this thesis focused on the development and implementation of a sufficient monitoring scheme applicable directly at the WWTPs. To reach this goal, several steps had to be conducted:

- Literature review on available methods
- Evaluation of existing methods and decision on two methods through an assessment according to
    - Simplicity in implementation
    - Necessary equipment
    - Costs
- Method calibration
    - Adaption of the methods to perform best with Anammox biomass
- Verification of the accuracy of the two chosen methods through a 2^3 multifactorial inhibition batch assay

The monitoring scheme developed for Anammox biomass was supplemented by standard methods to determine the sludge characteristics and the total nitrogen removal capacity of the whole system (AOBs and Anammox) as SS, VSS and N-compounds analysis. The standard methods applied were also used to evaluate the accuracy of the Anammox monitoring tools during the inhibition experiment through analysing their correlation.

To implement the monitoring scheme at a running system, Anammox inoculum sludge was withdrawn from the WWTP Strass i. Zillertal and a 3 L lab scale PNA reactor was installed at the technical hall of the institute. This reactor was used as fundament for the research executed within this thesis.

Starting in March 2015, all necessary steps were implemented at the laboratory of the Institute of Sanitary Engineering and Water Pollution Control. After the literature review and pretesting several methods (particle tracking, specific Anammox activity (SAA), UV-Vis-heme analysis and fluorometric heme analysis), the decision was made to calibrate and implement only

fluorometric heme analysis and SAA during this thesis as the combination of these two tools promised to give a holistic view on the status and activity of the Anammox biomass.

Both methods were calibrated; especially heme analysis needed an intense calibration optimized for this kind of biomass. The calibration of heme analysis included several steps such as:

- Adaption of dissovling agent concentration
- Comparison of two standards
- Determination of appropriate heating time

Finally, a sufficient calibration could be reached and the method could be established. at a fluorescence spectrophotometer (Cary Eclipse Fluorescence Spectrophotometer, Agilent Technologies, US) with a sufficient $R^2 = 0.9968$ for the relevant peak using a Precellys bead mill (Precellys 24, Bertin Technolgies, FR) for homogenisation. As the heme C content is dependent on the concentration of Anammox biomass, it is expressed as mg heme C/ g-VSS. The detailed established laboratory protocol can be found in the appendix (9.1.5)

Although it was planned that SAA was measured manometricly through overpressure sensors recording the pressure change when N_2 is produced, SAA assessment was finally conducted over UV-Vis spectrometry measurement of the N-compartments in the liquid phase as no tight system for SAA analysis could be established. Therefore, SAA was established through wet chemical analysis of the nitrogen compounds. 250 ml Schott Duran bottles were filled with synthetic wastewater and 0.5 g /L VSS biomass. The bottles were flushed with N_2 and substrates were added. Samples were drawn every 30 min to 60 min over an interval of 4 hours using the stoichiometry of the Anammox reaction. The samples were analysed with a Tecan Reader (Tecan Multimode Reader Infinite 200 PRO, Tecan Trading AG, CH; used for NH_4-N measurement) and with standard cuvette tests (Hach Lange, LCK 342 for NO_2-N and LCK 339 for NO_3-N; Lasa 50, Hach Lange, A). SAA is related to the VSS concentration and expressed as g-N/(g-VSS*d).

After the establishment of both methods, they were applied in a 2^3 multifactorial inhibition batch assay focussing on inhibitors commonly present at communal WWTPs in Austria. Therefore, nitrite, Fe(III)Cl_3 and a polymer in two concentrations were chosen for the experiment. As Fe(III)Cl_3 and polymers are used in sludge treatment, they can occur in rather high concentrations in reject water and possibly can affect the side stream deammonification system.

As the experiment had a run time of 12 days and included 4 cycles, long term effects of the inhibitors could be shown. To determine the (cumulative) effects of every inhibitor and inhibitor combinations, linear models were fitted to the data.

When evaluating the output data, it could be shown that different inhibitor combinations lead to varying results: although nitrite has a positive affect when added solely, it interacts negatively with the polymer in regards of nitrogen depletion and heme C concentration. Fe(III)Cl_3 enhances both, heme C concentration and nitrogen depletion, concluding that an accurate Fe(III)Cl_3 concentration is beneficial for the system.

Regarding the two tested methods, the experiment showed that heme C concentration has a slower reaction time than SAA at the presence of inhibitors. Therefore, SAA measurement can be seen as accurate tool to indicate acute process difficulties, while heme C gives an overview on cumulative effects in long term. Heme C analysis is also a sufficient method to observe the Anammox bacteria concentration in the community as it measures all Anammox bacteria, if active or inactive. The capability of heme C analysis to measure not only active, but also inactive biomass supplements the information gained by SAA through giving information on reversible inhibited bacteria.

Summing up, during this thesis a monitoring scheme for Anammox biomass consisting of heme C analysis and SAA measurement supplemented by standard methods was developed. This scheme is capable of giving a holistic view on the status of a deammonification system.

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9. Appendix

9.1 Fluorescence Spectophotometry

9.1.1 Pilot tests (10. & 11.9.2015)

To determine the accuracy of heme analysis by UV-Vis spectroscopy, a fluorescence heme analysis is conducted. The used protocol is an alternative protocol of the standard protocol (Sinclair et al. 1999).

According to the original protocol, a heme standard solution with 1mg/ml DMSO heme should be produced. As heme C source cytochrome C from equine heart (Sigma Aldrich) is used. When dissolving the Cytochrome C in DMSO, flocculation occurs and not all of the cytochrome C is dissolved. Various methods to increase solution (stirring, heating, ultrasonic bath) are used, but none of them produces a satisfying result. Therefore, experiments have to be conducted, whether a different solvent is more sufficient.

As DMSO in principle increases the solubility of heme C, 3 different solvents are tested: 100 % DMSO, 40 % DMSO (v/v) and 100 % RO-H_2O. The 100 % DMSO shows the expected result: flocculation occurs and the particles can't be destroyed by standard methods (stirring, ultrasonic bath, heating). As flocks would highly decrease the reliability of the results, 100 % DMSO is excluded as possible solvent.

The dilution with 40 % (v/v) DMSO leads to no obvious flocculation. Centrifugation of the solution leads to no pellet, therefore this method is used to create standard solutions.

As already seen in previous tests when calibrating UV-Vis spectroscopy, cytochrome C is dissolved easily and completely in 100 % RO-H_2O. Nevertheless, the capability of water to extract heme C is questionable. This solution also is used as stock solution for different heme C standards used in calibration Figure 30 shows a typical graph produced by the fluorometer.

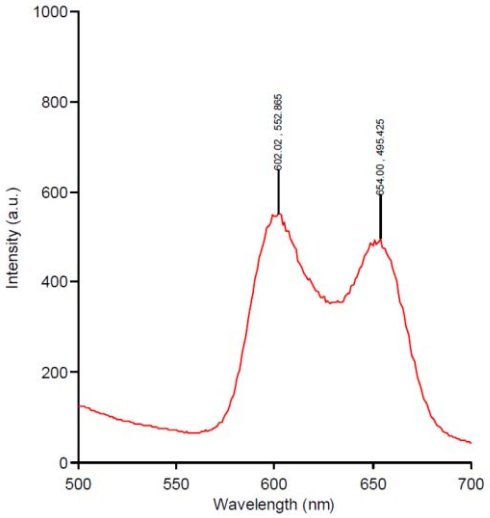

Figure 30: Intensity graph over wavelength as produced by the fluorometer for a 15 mg/L CC standard. Peak at 602.02 nm with 552.865 and peak at 654 nm with 495.425

Comparing the calibration of 100 % RO-H2O and 40% DMSO diluted heme C, the DMSO solution shows way higher intensity. Standards which almost show no peaks when using RO-H2O as solvent are over the range when using 40 % DMSO. Therefore, the DMSO solution should be chosen as solvent. The DMSO concentration should be increased to the highest possible percentage without flocculation. Also, the use of DMSO in the standard heme analysis with UV-Vis spectroscopy should be discussed.

As can be seen in Figure 31, standard solutions with DMSO show higher dissolving and therefore heme quantification capacity than the standard solution with pure RO-water. Nevertheless, the standard calibration with RO-water standards (experiment 2) show a better regression curve with a coefficient of determination R^2=0.994 compared to the 100% DMSO solution with R^2=0.8601, both for peak 1. Peak 2 shows for both methods a higher precision. The use of peak 2 for further analysis is also recommended by the basic protocol, as it is not that susceptible for interferences. The high differences between the 100% DMSO, which is expected to solute cytochrome C better than the 40% DMSO, can be explained through the insufficient dissolving of cytochrome C in the 100% DMSO. Also, the standards for the 1st calibration with 100% DMSO were produced and heated with 2M oxalic acid a day previous to the measurements. Therefore, there is a high possibility that not all of the heme C was still reduced at the time of the measurements.

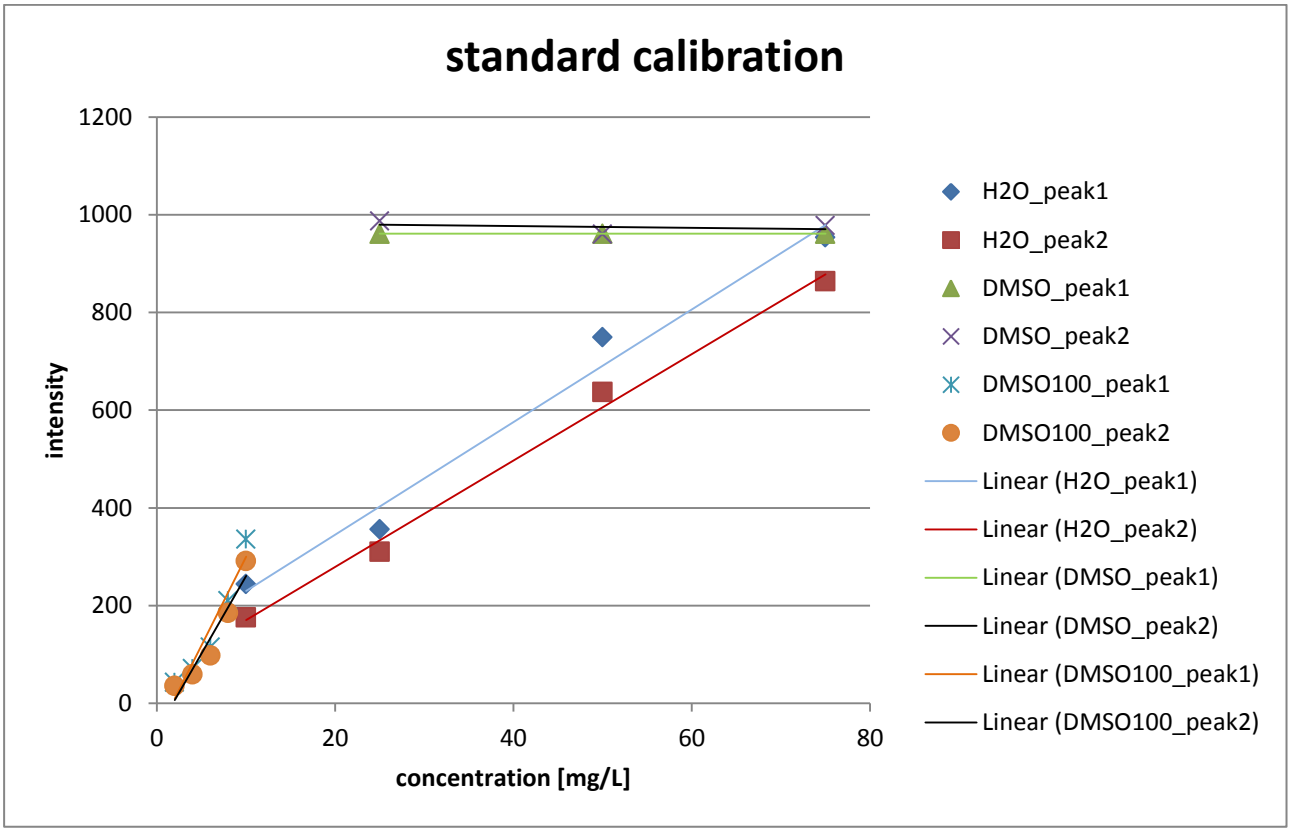

Figure 31: Comparison of the conducted pilot tests. 3 different solvents are used: 100 % DMSO, 40% DMSO and 0% DMSO

A new calibration is necessary as the DMSO standards mostly are out the range. Standards with higher dilution must be produced and used for further experiments. Another crucial point is the storage temperature of the vials: if the analysis isn't run directly after the preparation of the blank samples, they mustn't be stored at room temperature for longer time. Otherwise, the blank samples also show fluorescence.

In pilot test 3, more standards with 40%DMSO are calibrated. As the 2M oxalic acid always shows sedimentation, it is reheated directly before the experiment. Through the heating, all oxalic dehydrate is dissolved and therefore a higher concentrated oxalic acid can be used. Nevertheless, this procedure leads to a problem: when the samples cool down after boiling, the oxalic acid precipitates rapidly. This leads to difficulties with the measurements. In the following measurements, it will be continued to heat the 2M oxalic acid once and use the supernatant for all measurements.

To obtain more detailed and precise data of the heme Content of the Anammox biomass, the standard heme analysis by UV-VIS spectroscopy is complemented by fluorescence heme analysis.

In the first pilot test, the standard protocol was completely followed. When dissolving the Cytochrome C out of equine heart, which is used as heme C standard, with 100% DMSO flocculation occurred and could not be undone with neither stirring nor ultrasonic bathing. Nevertheless, the produced standards where used for the first try out. The standard stock solution was diluted from 1:100 to 1:500 accordingly. Another problem is the 2M oxalic acid. As already stated in the protocol, the solution is close to saturation and the oxalic acid –dihydrate can only be dissolved completely by heating.

The first pilot test led to the following machine-specific parameters for further analysis:

Table 21: Fluorescence spetroscope parameters.

Excitation	402	nm
Emission	500-700	nm
Volt	800	
excitation slit	20	nm
emission slit	20	nm
velocity	120	nm/min
peaks	596 and 652	nm

As can be seen in Figure 32, two peaks are obtained. While the first peak leads to a higher intensity, the second peak is not so susceptible to interferences. This fact is also shown by the coefficients of determination obtained by the two peaks: while peak 1 shows R^2=0,9224, peak 2 has R^2=0,924. Anyhow, the coefficient is rather low and the calibration should be repeated. The low coefficient could, on one hand, be produced by the flocks in the stock solution, on the other hand by the fact that the heating with 2M oxalic acid for 30 min was done on the day before the actual measurements. Therefore, it is possible that not all of the heme C was still reduced.

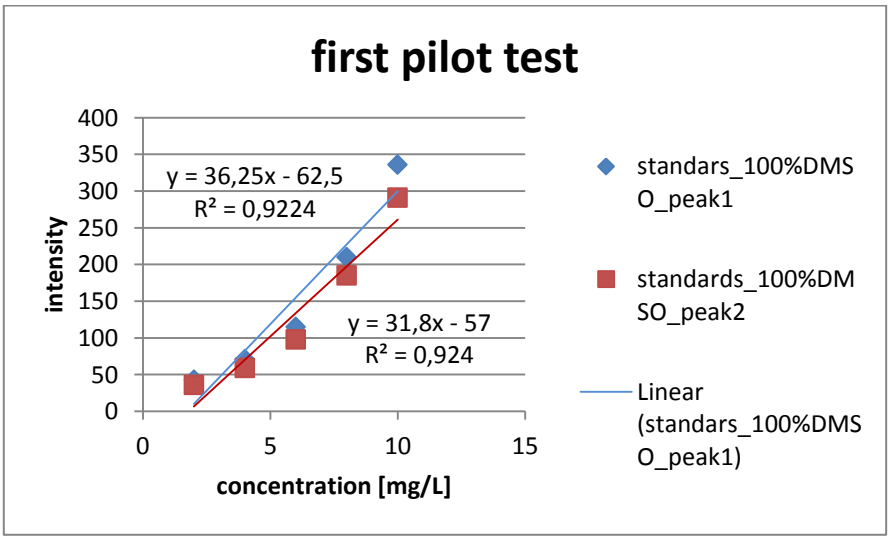

Figure 32: First calibration with 100% DMSO as solutant.

For the second pilot test, three different experiments were conducted:

1) Stock solution with 1mg Cytochrome C/1ml 100% DMSO
2) Stock solution with 1mg Cytochrome C/1ml RO-H_2O

3) Stock solution with 1mg Cytochrome C/1ml 40% DMSO

Table 22: Standard dilution scheme.

standard	Cytochrome C		Heme C		dilution
	mmol/L	mg/L	mmol/L	mg/L	1:
STD 1	0,0008	10	0,0008	0,72	100,00
STD 2	0,0020	25	0,0020	1,79	40,00
STD 3	0,0040	50	0,0040	3,58	20,00
STD 4	0,0061	75	0,0061	5,36	13,33
STD 5	0,0081	100	0,0081	7,15	10,00
STD 6	0,0101	125	0,0101	8,94	8,00
STD 7	0,0121	150	0,0121	10,73	6,67
STD 8	0,0141	175	0,0141	12,52	5,71
STD 9	0,0161	200	0,0161	14,31	5,00
STD 10	0,0000	0,5	0,0000	0,04	2000,00
STD 11	0,0001	1	0,0001	0,07	1000,00
STD 12	0,0002	2	0,0002	0,14	500,00
STD 13	0,0003	4	0,0003	0,29	250,00
STD 14	0,0006	7	0,0006	0,50	142,86
STD 15	0,0012	15	0,0012	1,07	66,67

In Table 22 the standard dilution scheme can be seen. Standards STD1-STD9 have been used for experiment 2), while standards STD2-STD6 have been used in experiment 3). As experiment 3) clearly showed that DMSO highly increases the solubility of heme C in oxalic acid and therefore the fluorescence, standards STD10-STD15 have been prepared after pilot test 2 for pilot test 3.

Experiment 1) was conducted to prove that the flocculation which occurred in the first pilot test did not occur up to bad practice. This could be proven: the Cytochrome C showed immediate flocculation when coming in contact with 100 % DMSO. Therefore, this stock solution was not used for any further steps in the experiment.

As using pure RO-H_2O as dissolvent showed satisfying results with the UV-VIS spectroscopy, in experiment 2) the capacity of USF-H_2O to dilute and solve Cytochrome C should be tested. During the experiment, no difficulties concerning flocculation occurred and a good linear regression model could be obtained. The standard stock solution was diluted from 1:100 to 1:5.

As Figure 32 shows, this experiment had a coefficient of determination R^2=0,981 for peak 1 and R^2=0,994 for peak 2.

To gain a solution with a solution capacity as high as possible, Cytochrome C was diluted with 40 % DMSO in experiment 3). No flocks occurred and the stock solution was diluted according to the scheme used for the RO-H_2O standards. As can be seen in Figure 32, this dilution set up was too low for the 40 % DMSO standards: all measured standards were above the detection level (green and black line in figure 2).

This pilot test clearly showed that DMSO increases the detectability of heme C. Therefore, 40 % DMSO was chosen as dissolvent for all further measurements. A sufficient calibration for this method has to be conducted. To prove that flocculation only occurred in 100 % DMSO, all 3 stock solutions were centrifuged at 4000rpm for 3 minutes. Only in the stock solution from experiment 1 sedimentation could be seen. Stock solutions 2 and 3 did not produce a pellet.

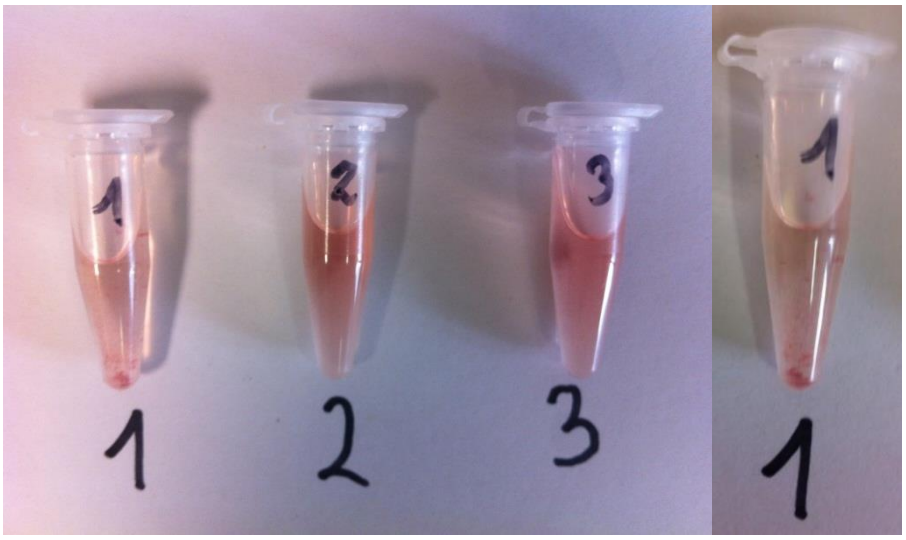

Figure 33: Centrifuged samples with 100% (1), 40% (2) and 0% (3) DMSO.

Interestingly, the position of the peaks changed between pilot test 1 and 2. Peak 1 shifted from 596 to 602 and peak 2 from 652 to 654.

To increase the reliability of the calibration, two further calibrations with 40 % DMSO will be conducted. All standards were produced from the same stock solution to guarantee same prerequisites.

9.1.2 Determination of the appropriate DMSO-concentration

Protocol 23.9.2015 and 24.9.2015

The aim of today's work was to determine the optimal DMSO-concentration to solve Cytochrome C.

6 different concentrations from 100 to 0 % DMSO (v/v) were tested.

Two setups were planned:

1) Analysis without centrifugation -> could lead to diverse results, as not all of the Cytochrome C is solved
2) Analysis with centrifugation

When dissolving the Cytochrome C in 60 % and 80 % DMSO solutions, flocks occurred. For the 60 % solution, all flocks disappeared in approximately 5 minutes without stirring or any other dissolving tool. The flocks in the 80 % DMSO solution mostly disappeared yet some stayed and

could not be destroyed. Centrifugation showed a minimal amount of remaining flocks. Concentrations below 60% did not show any flocculation as can be seen in Figure 34.

Figure 34: View into the centrifuge (left), eppis with standards from 40% [v/v] to 0% DMSO after centrifugation.

As the 60 % DMSO solution produced the highest peaks and had no flocculation, 60% DMSO is the starting point for determining the optimal concentration.

The results from the 100% DMSO solution showed a high diversity, which can be explained by the flocks: The presence of a flock can heighten the fluorescence immediately.

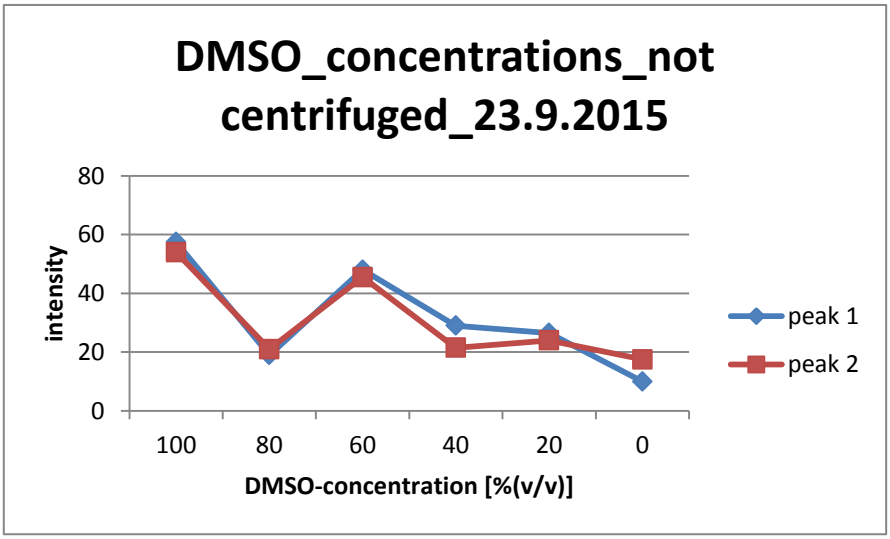

Figure 36: Determination of appropriate DMSO concentration with 4mg/L Cytochrome C standards. The standards were not centrifuged. 6 different DMSO concentrations were compared. Peak 1 occurred at 602 nm, peak 2 at 654 nm. CC-standards from old stock solutions were used.

When analysing the centrifuged samples, the theory, that 100 % DMSO measurements are highly influenced by flocks could be proven(Figure 36 and Figure 21): The peaks dropped from 57,5 respectively 54 to 7,5 and 14. Therefore, all DMSO concentrations which produce flocks are no possibility for further measurements. The 80 % DMSO samples should no difference in the reached peaks. But still, the occurrence of flocks makes this concentration unreliable.

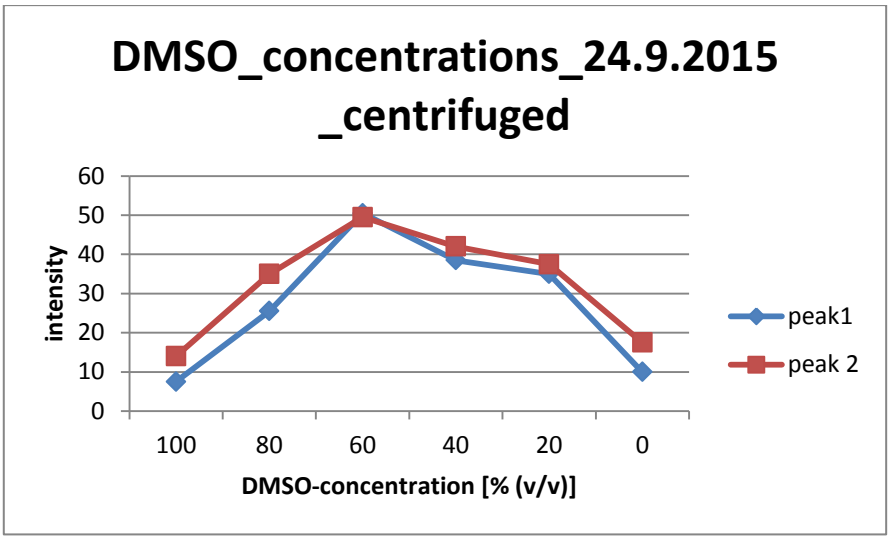

Figure 37: Determination of appropriate DMSO concentration with centrifugation (4mg/L Cytochrome C standard). 6 different DMSO concentrations were compared. Peak 1 occurred at 602 nm, peak 2 at 654 nm.

The 60 % DMSO solution again showed the best results. To find the best dissolving agent, stock solutions from 70 % to 50 % DMSO content were produced in 5 % steps and tested. Again, all stock solutions were produced at 1 mg Cytochrome C/1 ml DMSO-stock-solution in the accurate concentration. Afterwards, the stock solutions were diluted with USF-H_2O to 500 mg Cytochrome C/1 ml DMSO-solution. To increase the peak, the upcoming experiments will be conducted with 8 mg/L Cytochrome C standards.

As the two peaks are at 602 nm and 654 nm, the measurement interval was shortened to 550-680nm to save time.

As described beforehand, all standard solutions (diluted with 70 % - 50 % DMSO accordingly) were measured both, centrifuged and not centrifuged. All steps were conducted as previously described. The measurement without centrifugation showed that the appropriate dilution is close to 60%-DMSO, as can be seen in Figure 22.

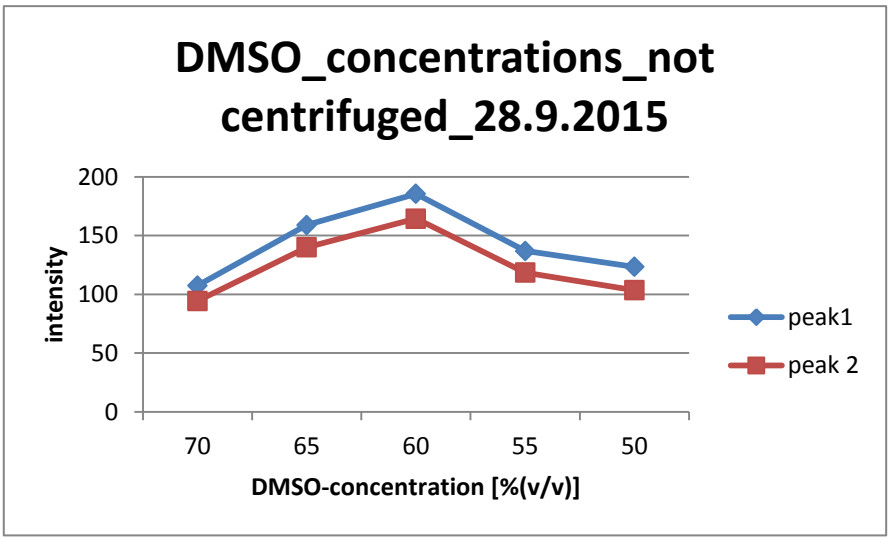

Figure 38: 8mg/L standards without centrifugation. 5 standards in 5 % intervals were tested. Peak 1 occurred at 602 nm, peak 2 at 654 nm. 8 mg/L CC-standards were used.

Unfortunately, the experiment with the centrifuged standards was disturbed by a malfunction of the water bad. The assumption is that the 100 °C needed were not reached over the whole heating period due to a low filling level (the water bad produced an error prompt). Therefore, the first test led to confusing results (Figure 39) and had to be repeated. As the peak close to 60 % was already assumed, a 59 % DMSO and a 61 % DMSO standard were included in the repetition.

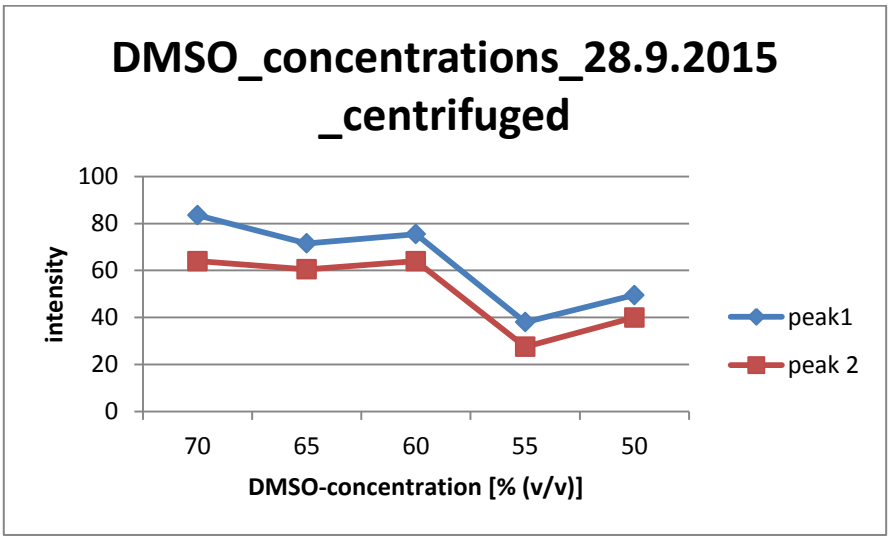

Figure 39: Experiment with disturbances. The water bad did not stay at 100 °C for the whole heating period. Peak 1 occurred at 602 nm, peak 2 at 654 nm. 8 mg/L CC-standards were used.

Through the inclusion of 59 %-DMSO and 61 %-DMSO standards it could be stated that the maximum intensity is reached with a concentration between 65 % and 60 % DMSO (Figure 40). To proof this assumption, further experiments have to be conducted.

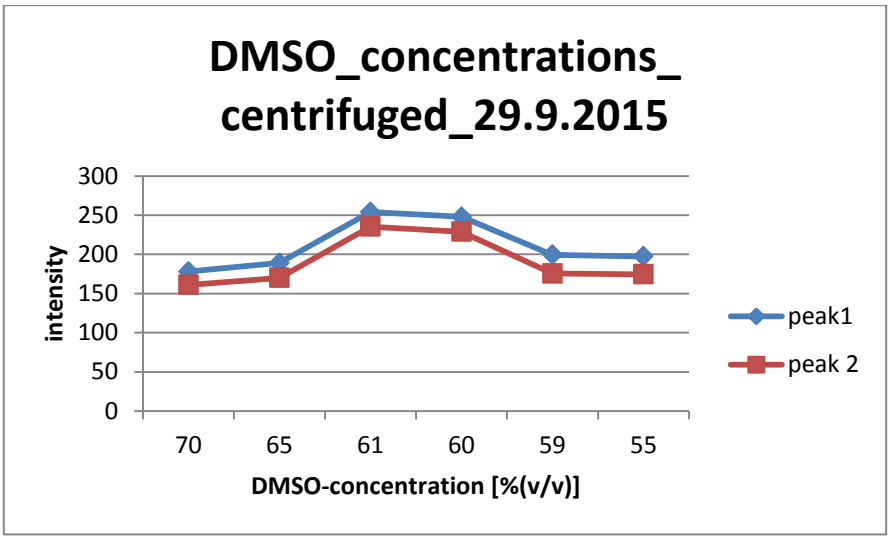

Figure 40: Inclusion of 59% and 61% DMSO-standards into the experiment. 8 mg/L CC-standards were used. The peaks occurred at 602 nm and 654 nm respectively.

9.1.3 Cytochrome C from bovine heart (BCC)

To analyse whether cytochrome C from a different sources leads to results deviant from the ones measured with cytochrome C from equine heart, bovine heart was chosen as second source. The cytochrome C from bovine heart (molar weight M=12327) was ordered from Sigma Aldrich (C2037). Compared to cytochrome C from equine heart (Sigma Aldrich, C2506; M=12384) it has a slightly different molar weight. As we are dealing with extremely small concentrations and volumes, the differing molar weight leads to a negligible change in volumes when producing the standards.

As first experiment different DMSO concentrations were tested to find the concentration with the highest dissolving capacity and reliability. Experimenting with the DMSO-concentration for the cytochrome C from equine heart **(ECC)** has shown that the peak in intensity is reached between 60 % (v/v) and 65 % (v/v) DMSO. Therefore, only three DMSO-concentrations (50 %, 60 % and 70 % (v/v) were tested in both centrifuged and not-centrifuged stage to determine a first trend for cytochrome C from bovine heart **(BCC).** All steps conducted with Cytochrome C (CC) from equine heart have to be done with CC from bovine heart as well. To guarantee comparability, the experimental setup from ECC was also used for BCC, as can be seen in Table 23.

Table 23: Experimental setup for the calibration of BCC

Centrifugation time and velocity	15000 rpm for 10 min
Standard stock solution	1mg BCC / 1ml DMSO-solution
Tested Standard	8 mg/L BCC
Heating time	30 min
Oxalic acid	2 M
Sample volume	0,1 ml

Figure 41 and Figure 23 show that BCC seems to have similar behaviour as ECC. Both cytochrome C standards produce the highest intensity when dissolved in a DMSO solution with approximately 60 % DMSO (v/v).

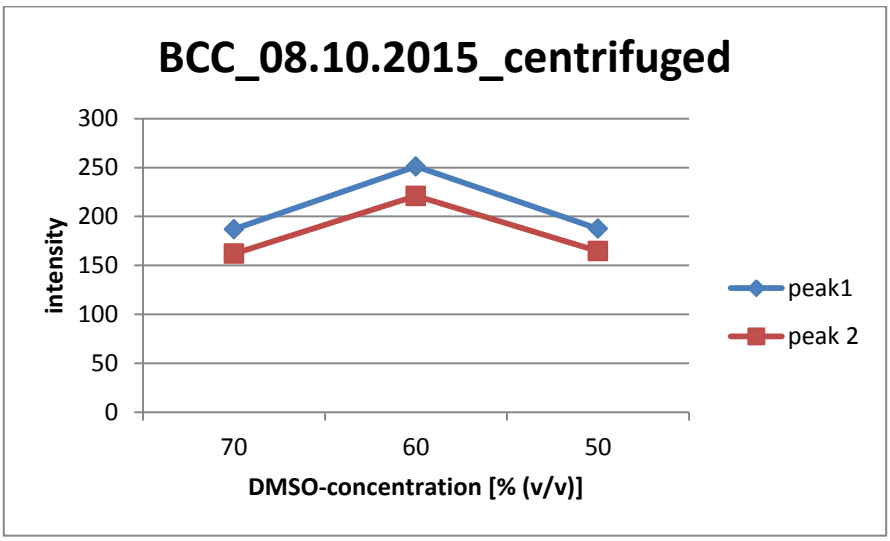

Figure 41: BCC standards (8 mg/L CC) with different DMSO-concentrations. The standards were centrifuged prior to the experiment. Peak 1 occurred at 602 nm, peak 2 at 654 nm.

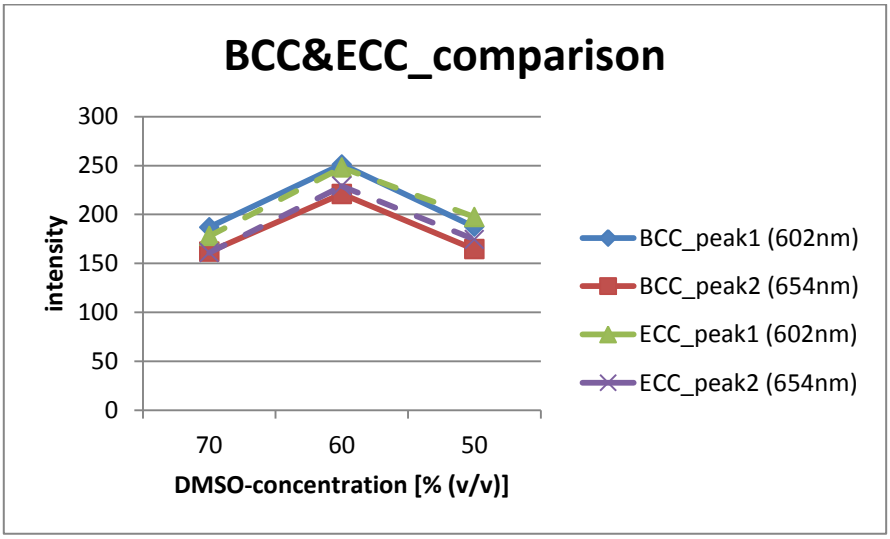

Figure 42: Comparison of BCC and ECC standards. For both heme sources 8 mg/L Cytochrome C standards were used and centrifuged prior to the experiment. 3 different DMSO-concentrations were tested.

9.1.4 Heating experiments

To determine the optimal heating time (at 100°C) for both Cytochrome C standards, the following times were tested:

Table 24: Used standards for heating experiments (%-value stands for DMSO-concentration).

Time interval [min]	Used standards (8 mg/L CC)
10	ECC 70%, ECC 65%, ECC 60%, ECC 55%, BCC 60%, BCC 65%
20	ECC 70%, ECC 65%, ECC 60%, ECC 55%, BCC 60%, BCC 65%
30	ECC 70%, ECC 65%, ECC 60%, ECC 55%, BCC 60%, BCC 65%
40	ECC 60%, ECC 65%, BCC 60%, BCC 65%
45	ECC 60%, BCC 60%
60	ECC 60%, BCC 60%
90	ECC 60%, BCC 60%

All heating experiments were conducted from 06.10.2015-19.10.2015. A major problem with the experiments was the filling level of the water bath: the bath suffered from high level losses. For heating times bigger than 30 min the starting filling level had to be rather high which led to floating of the samples. During two experiments the water bath was not able to hold 100 °C during the whole heating period.

As can be seen in figure 10, the first heating time experiment conducted on 14.10.2015 clearly shows the trend that heating times below 30 min (the time proposed by the standard protocol Sinclair et al. 1999) lead to a decrease in obtained intensity. Figure 43 only includes values for the 8 mg/L ECC in 60 % DMSO standard to increase the readability. All other standards showed a similar pattern.

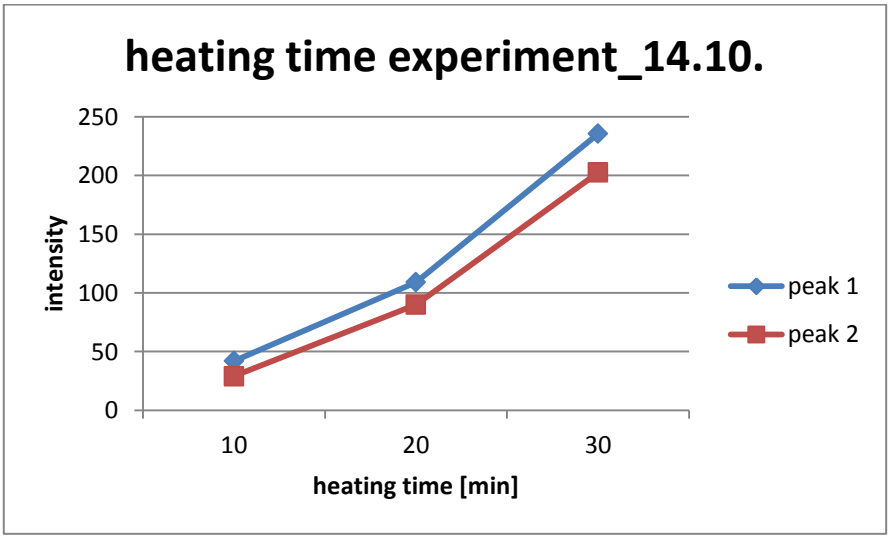

Figure 43: Comparison of different heating periods for 8 mg/L CC standards.

Due to the steepness of the graph in Figure 43, it was assumed that the values would still increase with an extension of the heating time. In Figure 44 can be seen that this assumption does not hold. Slowly, but clearly, the values begin to decrease with the increase of the heating time.

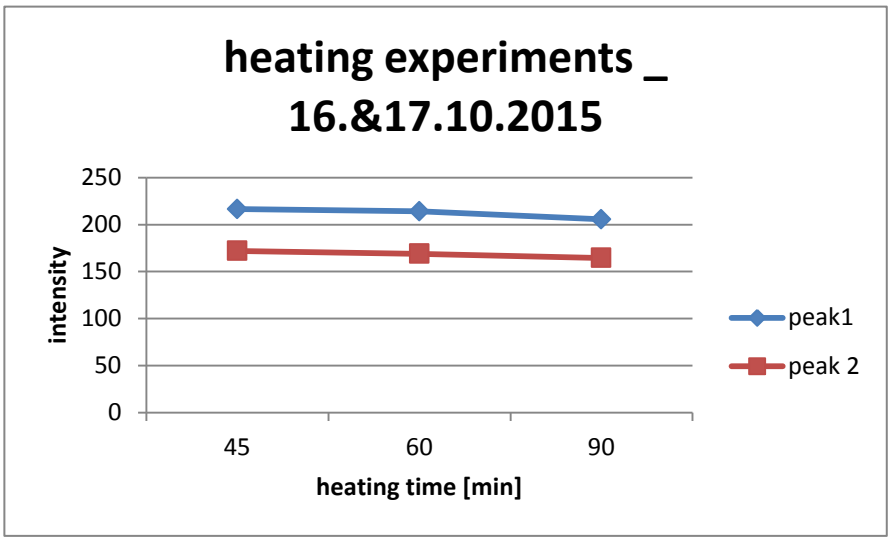

Figure 44: Further experiments on heating time with 8 mg/L CC-standards.

When combining Figure 43 and Figure 44 the obtained graph (Figure 45) gives the impression that the optimal heating time is around 30 minutes. Below 30 min, the intensity highly decreases. Above 30 min, the steepness of the graph is rather flat, but a decrease in the reached values can be seen.

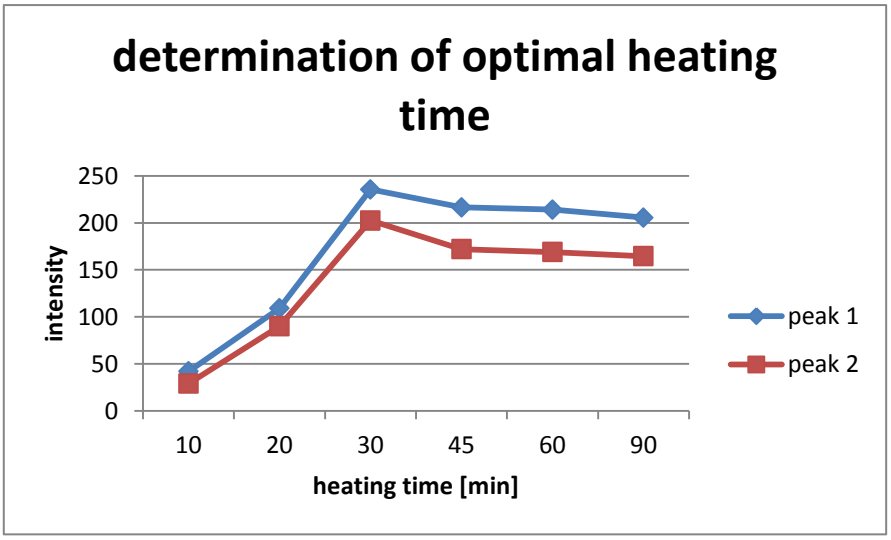

Figure 45: Complete data set of the heating experiments with 8 mg/L CC-standards. Peak 1 occured at 602 nm, peak 2 at 654 nm.

9.1.5 Established protocol

- Withdrawal of sample (15 ml) from a mixed reactor
- Centrifugation of sample (4000 rpm, 3 min)
- Pouring away of discharge, refilling to original volume with 60 % [v/v] DMSO
- Shaking of vial until completely mixed state is reached
- Withdrawal of 2 ml sample and inserting it into precellys vial equipped with 10 2,4 mm ceramic balls
- Homogenisation at 6000 rpm for 2 min with 5 repetitions

- 2 ml Probe in Röhrchen geben und gut verschließen
- Adding of 100 µl sample (in accurate dilution) into glass tube; dilution has to be produced with 60% [v/v] DMSO
- Adding of 1 ml 2 M oxalic acid
- Heating for 30 min at 100°C in a water bath
- Measuring of samples at fluorometer
- All samples have to be made in duplicate. A blank has to be produced (same procedure except heating) to subtract background noise

9.2 Experimental set up

Anammox bacteria are known for the slow doubling time of around 12 days and their susceptibility to many inhibitors/constituents occurring in wastewater (Jetten et al. 2001). Many inhibitors and their inhibition level have already been suspect of research work by Jin et al. 2013; Jin et al. 2012; Q.-Q. Zhang et al. 2014; Z.-Z. Zhang et al. 2015; Dapena-Mora et al. 2007 and others. Summing up the conducted research, it can be said, that for most inhibitors no clear inhibition level can be found (Jin et al. 2012). While certain levels are seen as irreversible inhibitory in some papers, the same level do not have a significant influence on the Anammox biomass in other research work or at least do not have such high or only reversible effects.
To determine the inhibitory effect of as many relevant factors as possible, a 2^3 factorial design is applied: nitrite concentration (F1), Fe(III)Cl_3 concentration (F2) and polymer concentration (F3). To exclude influence of temperature and shear stress, all experiments are conducted at 35°C and 150 rpm.
The high level concentrations of Fe(III)Cl_3 and the polymer are taken from the concentrations of the WWTP Tulln, Austria. When trying to run the 3L PNA laboratory reactor with the reject water from this WWTP, no stable system could be reached. Therefore, the concentrations of this WWTP are used to identify the main inhibitor in this reject water. As research by Dapena-Mora et al. (2004) suggests that polymer concentrations of up to 1 g/L do not reach the IC50 concentration, the original concentration used by the WWTP (22.5 mg/L) is increased. When 300 mg/L of polymer are used, this accumulates to 1200 mg/L until the end of the experiment.

Table 25: Inhibitors tested in the multifactorial experiment with the low level and high level concentrations.

Factor	Low level	High level
Nitrite	70 mg/L	105 mg/L
Fe(III)Cl_3	0 mg/L	2.85 mg/L
Polymer	0 mg/L	300 mg/L

Table 26: Set up configuration as used for the 8 treatements with 3 triplicates.

vial #	NO2	Fe(III)Cl2	polymer
1	+	+	+
2	+	-	+
3	+	+	-
4	+	-	-
5	-	+	+
6	-	-	+
7	-	+	-
8	-	-	-

9.2.1 Experimental procedure

Material

- 24 batch vials (250 ml bottles)
- Pressure sensors
- Stirring plate (150 rpm)
- Incubator (35°C)
- Inoculum sludge -> fresh sludge from WWTP AIZ Strass i. Zillertal
- Fluorescence photometer
- Drying oven and muffle chamber
- Synthetic wastewater

Preparation of sludge

To determine the appropriate sludge concentration, **SS and VSS** have to be determined before the experiment. According to VSS (30.6 g/L), an appropriate amount of sludge can be added to the vials in an end concentration of 1g VSS/L.

The samples are centrifuged (5min, 4000 rpm) and washed with synthetic wastewater without substrates (according to van de Graaf et al. 1996 and Dapena-Mora et al. 2004). To lower the O2 level <1 mg/L, the Na2SO3 is added. After washing, they are again centrifuged. Then synthetic wastewater (without substrates and inhibitors) is added and the samples are suspended into the assay vials.

Start-up of experiment

Before substrates and inhibitors are added, all vials are brought to a constant temperature of 35°C (approximately for 30 min) and the gaseous phase is washed with N2. After this acclimatisation, substrates and inhibitors are added according to the set up.

Analysing methods

- SAA -> every 4^{th} day (4 points)
- Heme analysis -> every 4^{th} day (4 points)

Sample taking

To conduct all analyses, 5ml of sample volume should be taken when heme analysis is done, 1ml should be sufficient for determining NH4-N.

The sample has to be taken in a completely mixed stage as heme Concentration is relative to VSS. After sampling, the vials are fed and the gaseous phase is washed with N2 again. After adapting pressure to air pressure, SAA can be measured.

If the samples are not measured directly after sampling, they have to be frozen. Otherwise erratic results could occur.

Duration of experiment and additional set up conditions

The experiment has a run time of 2 weeks (or shorter for some vials if the inhibitors kill the Anammox biomass).

9.2.2 Used protocols

Fluorescence heme analysis

Adapted protocol of Sinclair et al. (1999)

- Collect tissue samples from reactor
- Dilute tissue samples with **60%** (v/v) DMSO solution
- Collect tissue culture cells in 6 x 50 mm-disposable glass tubes by centrifugation or place small volume (up to 50 µl) in the tubes
    - Protein amount should be < 10 µg/tube. The assay is linear from 1nM to 1µM (or 0.5pmol to 0.5 nmol) heme per tube.
    - The assay is most conveniently performed using a cell holder that holds four 6 x 50 mm tubes. Alternatively, samples can be transferred into disposable plastic cuvettes for fluorescent measurements
- Add 0.5 ml of 2M oxalic acid solution to mix thoroughly
- Put sample tubes in a heating block set at 100°C for 30min. Do not heat blanks.
    - Blank and sample tubes contain tissue and oxalic acid, but the blanks are not heated.
- Read fluorescence of porphyrin using **402**nm excitation and **654** or **602** nm emission, after determining that these represent the actual peaks with the instrument used.
    - The **654** nm emission peaks has lower fluorescence than the 608 nm peak, but also has less scatter and other interference.
    - Actual peaks vary from instrument to instrument.
    - **At our instrument: peaks at 402 nm excitation and 654 and 602 nm emission**
- Subtract blank values (parallel unheated samples in oxalic acid) from samples.
- Calculate heme Content based on a standard heme Curve over the range of 0 to 1 nmol heme per tube, or calibrate with 1mg/ml heme standard solution or a protoporphyrin standard.
- Standard solution: Dissolve heme at 1mg/ml in DMSO. Determine concentration by spectrophotometry of a 1:300 (v/v) dilution in **60%** (v/v) DMSO, using a millimolar extinction coefficient of 180 at 400 nm. Store indefinitely at room temperature.

9.2.3 Synthetic wastewater

According to van de Graaf et al. 1996 and Dapena-Mora et al. 2004

Table 27: Synthetic wastewater as used in the multifactorial experiment.

Chemical	Amount [mg/L]
(NH4)2SO4	With end concentration of 70 mg/L
NaNO2	According to experimental set up
NaNO3	100
KHCO3	1250
NaH2PO4	50
MgSO4 * 7H2O	200
CaCl2 * 2H2O	300
FeSO4	6,25
EDTA	6,25
Trace element solution	1,25 **ml/L**

Table 28: Trace element solution (stored in toxic storage room at 4°C, van de Graaf et al. 1996)

Chemical	Amount [mg/L]
EDTA	15000
ZnSO4 * 7H2O	430
CoCl2 * 6H2O	240
MnCl2 * 4H2O	990
CuSO4 * 5H2O	250
NaMoO4 * 2H2O	220
NiCl2 * 6H2O	190
NaSeO4 * 10 H2O	210
H3BO4	14

9.2.4 Storage of samples

To analyse the N-parameters, a total sample volume of 1,4 ml is needed (200 µl for NH_4-N and NO_2-N each and 1 ml for NO_3-N). While NO_2-N and NO_3-N can be measured at a later stage, NH_4-N has to be measured instantly, especially if the sample is not filtrated.
To allow storage of NH_4-N, 1:2 H_2SO_4 can be added to the sample to lower the pH <2 (for 10 ml sample volume, 20 µl of 1:2 H_2SO_4 would be sufficient). This treatment is not possible, if NO_2-N should be measured from the same sample as this compound would be oxidized.
To decrease the work load and the error, all parameters will be measured from the same sample which is stored at -20 °C after centrifugation at 4000 rpm for 3 minutes. All samples are analysed as rapidly as possible, the maximum storage time does not exceed 1 week.

For heme analysis of highly enriched biomass with a heme Content of 6.8 mmol – 10.3 mmol/gVSS (Tang et al. 2011), a maximum of 0.15 mg – 0.01 mg VSS have to be in one tube to be in the linear range of the fluorescence heme assay. Taking into account that all samples have to be done in duplicate and that a blank is needed, approximately 0.5 mg VSS is needed in total for heme analysis. As the batch assays are conducted with 1g VSS/L, a 0.5 ml sample is sufficient for heme analysis.

For SAA measurement, a very tight sampling interval is necessary: ammonium is measured in 30 min – 60 min intervals after feeding for 4 hours (30, 60, 120, 180, 240 min). This leads to 5 measuring points for ammonium per day which have to be measured directly. The sampling point withdrawn with NO2-N and NO3-N measurement before feeding at the end of a cycle is frozen and therefore does not have to be measured instantly.

9.2.5 Laboratory protocol to the batch experiment

01.02.2016 and following days: All vials were equipped with caps with two holes for syringes. These holes were sealed with 3mm rubber mats which also function as seals for the caps.
The vials were numbered from 1-24 and equipped with colour codes to ensure an easy identification of the treatments.
On the 5th of February, the used biomass has been harvested from the storage tank (originally taken from the WWTP Strass i. Zillertal), centrifuged (2min, 4000 rpm), washed with synthetic wastewater (without substrates, but 100 mg/L NO_3^-N) and after a second run of centrifugation (2min, 4000rpm) suspended into synthetic wastewater at a concentration of 6.6 g VSS/L. The biomass was stored at room temperature until the start of the experiment.
The adding of Fe(III)Cl_3 decreased the original pH of the synthetic wastewater from 8.2 to 7.6, a carbonate buffer system with $NaHCO_3$ and H_2CO_3 was introduced to fix the starting pH at 7.8. The synthetic wastewater already included 1250 mg/L KHCO3 which had to be taken into account when calculating the necessary buffer capacity of the system.
On February 9th 2016, all treatment specific synthetic wastewaters were produced. For all eight settings the same stock solution was used.
To determine the appropriate Fe(III)Cl_3 concentration, a pre-test will be conducted on the 10th. All treatment synthetic wastewaters (blank to determine the SAA, ½ of planned Fe(III)Cl_3 concentration and total planned Fe(III)Cl_3 concentration).
The SAA obtained in the pre-test (in duplicate) is taken as basis for the batch experiment. A blank in triplicate is run during the whole test interval, to monitor the development of the SAA of standard treatment under the set conditions. To guarantee that a sufficient amount of synthetic wastewater is available, 625 ml instead of the necessary 600 ml were produced for every treatment. The calculated concentrations can be found in

Table 29.

Table 29: Calculation of concentrations and volumes

liquid volume	300	ml					
feeding	50	ml					
biomass	250	ml					
sample #	24						
feeding #	4						
needed volumes			dilution necessary				
biomass	6000	ml	:6	42	ml biomass		
syn. WW	4800	ml		208	ml syn. WW		
HRT	24	days					
WW/treatment	600	ml					
				5 L (mg)	2,5 L	1,25 L	0,625 L
NH4-N conc	**840**	**mg/L in feeding media**		**2100**	1050	525	262,5
NO2-N conc 1	**420**	**mg/L in feeding media**		2100	**1050**	525	262,5
NO2-N conc 2	**630**	**mg/L in feeding media**		3150	**1575**	787,5	393,75
Fe(III) conc	**3**	**mg/L in feeding media**		15	7,5	**3,75**	1,875
polymer	**300**	**mg/L in feeding media**		1500	750	375	**187,5**
(NH4)2SO4	**3960**	**mg/L in feeding media**		**19800**	9900	4950	2475
NaNO2 conc 1	**2070**	**mg/L in feeding media**		10350	**5175**	2587,5	1293,75
NaNO2 conc 2	**3105**	**mg/L in feeding media**		15525	**7762,5**	3881,25	1940,625
Fe(III) conc	**8,55**	**mg/L in feeding media**		42,76	21,38	**10,69**	5,35
polymer	**300**	**mg/L in feeding media**		1500,00	750,00	375,00	**187,50**
Fe(III)conc	40%	**solution**					
total need	21,38	**mg tot**					
total volume	53,45	**ml tot**					
=>	**80**	**ml tot**					
Fe(III)conc tot	**32**	**mg tot**					
	2%	**deviation -> acceptable**					

To guarantee standardized and comparable starting conditions, the pH of all treatments is adjusted to 7.8 by adding of HCl. To establish a sufficient buffer system, the synthetic wastewater used by Dapena-Mora et al. (2004) is enriched with 1.75 g $NaHCO_3$.
The synthetic wastewater for all treatments was stored in 1L PET-bottles at 4°C and brought to room temperature before adding to the bottles.

11.02.2016
Today, the stored biomass (centrifuged and suspended in synthetic wastewater at 6,6 g-VSS/L) was suspended into the bottles to a volume of 250 mL. The headspace was washed with dinitrogen gas to exclude oxygen; in the liquid phase oxygen was depleted with Na_2SO_3. All vials were put into the shaker in the incubator and brought to the operation temperature of 35°C (approximately for 30 min). When process temperature was reached, the spiked synthetic wastewater (depending on treatments 1-8) was added and the shaker started (150 rpm).
The experiment started at 11:30, samples were drawn according to the sampling plan at 12:00, 12:30, 13:30, 14:30 and 15:30.
All samples were centrifuged and frozen (-20°C).
When adding the treatments including polymers, swimming sludge occurred immediately. This could lead to flushing out of sludge at normal WWTPs. The effect of swimming sludge/polymer on VSS could be interesting to monitor.

15.02.2016
2nd day of SAA measurement. No obvious changes in any bottle; as already described, bottles with polymer tend to build clumps. The clumps can be destroyed through increasing rpm.
All measurements were conducted as planned. Bottle nr. 6 has a low filling volume (no obvious reason can be identified).

19.02.2016
3rd day of SAA measurement. In vial nr. 13 a change of colour can be observed. All other bottles do not have any obvious changes. Standard procedure has been followed; pH, electrical conductivity and temperature were measured.

23.02.2016
4th day of SAA measurement. Start of measurement at 10:15. pH has been measured. Vial 2/4 was not fed (no synthetic wastewater left due to spilling out). Therefore, vial 2/4 has to be excluded from the analysis of measurement 4.
NO2-N and NO3-N values of the withdrawal and the 3rd measurement were measured.

24.02.2016
Preparation of NH4-N measurements -> adding of blank solution to formerly frozen samples for dilution -> further storage in fridge.

26.02.2016
Today all suspended solids measurements were prepared according to APHA standards. The SS will be evaluated on Monday. The last NO2-N and NO3-N samples have been analysed. Therefore, NO2-N and NO3-N measurements are completed.

In total, the analysis of all samples lasted until 11.3.2016.
For all measured parameters, excel-files were elaborated to directly calculate the actual mg/L concentration (if applicable, some parameters have other standard units) for each sampling point and vial. For heme, the standard calibration was repeated and a new calibration curve was obtained. The heme samples were all dissociated in DMSO with the precellys bead mill at the same day. Therefore, some samples were longer stored in DMSO which could lead to an increase in observed heme Concentration.

9.3 Ammonium Measurement Protocol

This technique improves the efficiency when making several replicates and dilution series. To guarantee the reproducibility, all flasks have to be homogenized and mixed thoroughly. Especially before conducting the photometric analysis, a recurring mixing should be done. As can be seen in the standard calibration curve, the method is highly linear according Lambert-Beer's law. The Protocol can also be used for ammonium nitrogen measurement on a UV-Vis spectrophotometer. For this measurement, the sample and reagent media volumes have to be increased accordingly.

Material:

- 24 Well Plate
- Tecan Reader

Chemicals:

- Blank solution
- Reagant solution
- Salicyclate-citrat-solution
- Ammonium-Nitrogen-standard solution

Fabrication of chemicals:

- Blank solution
    - 10 ml 0,5M H_2SO_4 diluted to 500ml with USF-water
        - For 500 ml of 0,5 M H_2SO_4 add 14 ml 96% H_2SO_4 and fill it up with water
- Reagant solution
    - 3,2 g caustic soda pads
    - 0,2 g Sodium dichloroisocyanurate
    - 100 ml flask filled with USF-water
    - Homogenize
- Salicyclate-citrat-solution
    - 13 g Sodium salycilate
    - 13 g Sodiumcitrate-Dihydrate
    - 0,097 g Sodiumnitroprusside
    - Weigh in seperately
    - Put in 100 ml flask; add USF-water
    - Homogenize through stirring
- Ammonium nitrogen – standard solution
    - Stock solution:
        - 0,5647 g Ammoniumhydrogencarbonate
        - 1 l flask
        - Prepare water
        - Add 20 ml 0,5 M H_2SO_4
        - Homogenize
    - Working solution
        - 5 ml into 250 ml flask
        - Fill up with 0,01 M H_2SO_4
            - 0,278 ml H_2SO_4 in 500ml H_2O

Standards -> 1 ml volume

Table 30: Dilution scheme for standard dilution.

	Blank solution [ml]	Standard working solution [ml]	Dilution

STD 0,5 mg	0,75	0,25	1:4
STD 1,0 mg	0,5	0,5	1:2
STD 1,5 mg	0,25	0,75	1:1,33
STD 2,0 mg	0	1	1:1

To guarantee that the samples are in the correct interval after dilution, a proposed dilution working solution scheme is given in the next table. The following dilutions can be made according to the exemplary table for 70 mg/L NH_4-N.

Table 31: Dilution scheme for sampling according to their assumed concentration.

starting concentration	starting dilution	10	ml	dilution 1	dilution 2	dilution 3
mg/L	1:	ml sample	ml blank solution	1:	1:	1:
1500	1000	0,010	9,990	1000	1330	2000
1000	800	0,0125	9,9875	800	1064	1600
500	250	0,040	9,960	250	332,5	500
250	200	0,05	9,95	200	266	400
200	100	0,1	9,9	100	133	200
150	100	0,1	9,9	100	133	200
100	80	0,125	9,875	80	106,4	160
90	50	0,2	9,8	50	66,5	100
80	50	0,2	9,8	50	66,5	100
70	50	0,2	9,8	50	66,5	100
60	50	0,2	9,8	50	66,5	100
50	25	0,4	9,6	25	33,25	50
40	25	0,4	9,6	25	33,25	50
30	25	0,4	9,6	25	33,25	50
20	10	1	9	10	13,3	20
10	10	1	9	10	13,3	20
5	2,5	4	6	2,5	3,325	5

Samples with a starting concentration of approximately **70 mg/L** NH4-N

➔ Create a working solution in a 1:50 dilution

Table 32: Example for a dilution scheme of a sample with prospected 70 mg/L NH_4-N concentration.

70 mg/L start with 1:50	Blank solution [ml]	Sample working solution [ml]	Dilution
Dilution 1 ~ 0.7 mg	0,5	0,5	1:100
Dilution 2 ~ 1.05 mg	0,25	0,75	1:66.5
Dilution 3 ~ 1.4 mg	0	1	1:50

Used wavelength: 650nm -> measure 4x per Well
Measure area: 0-2 mg NH4-N/L

Procedure:

- 5ml sample (in an accurate dilution) + 0,5 ml reagant solution + 0,5 ml salicylat citrat solution
- Mix thoroughly
- After 15-45 min, the samples can be measured with the photometer at 650 nm

All samples should be made in duplicates!

10. Curriculum Vitae

PERSONAL INFORMATION

street	Hippgasse 11/61
city	1160 Wien
phone	+436641158672
e-mail	karin.schitzenhofer@gmx.at
citizenship	Austria
place of birth	Zwettl
date of birth	22.07.1992

EDUCATION

Oct. 2013 – today

University of Natural Resources and Life Sciences, Vienna
Master Water Management and Environmental Engineering

- Master thesis at the Institute of Sanitary Engineering and Water Pollution Control
- Structural exercise in Rural Water Management and Irrigation

Oct. 2011- Oct 2015

University of Vienna
Bachelor Slavonic Studies

Oct. 2010 – Sept. 2013

University of Natural Resources and Life Sciences, Vienna
Bachelor Environment and Bio-Resources Management

- Environmental law and economics
- Waste management

Sept. 2002 – June 2010

Bundesgymnasium Zwettl
Matura passed with distinction

WORK EXPERIENCE

February 2016 - ongoing

University of Natural Resources and Life Sciences, Vienna
Student assistant at the Institute of Sanitary Engineering and Water Pollution Control; chemical and microbiological monitoring of Anammox lab scale reactors; development of batch assays

August 2015

June 2015- July 2015

Samek Dipl-Ing Civil Engineering Corporation, Langenlois
Intern; Technical drawing with AutoCAD for hydraulic engineering and road construction projects

July 2013	**TERRA Environment Engineering Corporation**, Vienna Intern; Building supervision, geological survey, pumping tests
Feb. 2013 – June 2013	**Arge AIWWF**, Vienna Team leader, fund-raising
	Arge AIWWF, Vienna Field staff, fund-raising
May 2012 – June 2012	**Socialdata**, Vienna Mobility survey with focus on electromobility; telephone and deepening personal interviews
Dec. 2011 – Jan. 2012; July 2012	**Association for the promotion of poppy seed and cereal breeding**, Zwettl; Analysis of the quality characteristics of grains
July 2011	**Agricultural Technical School Edelhof**, Zwettl Intern; Harvesting and analysing of grains and their quality characteristics; field and laboratory work
March 2011	**University of Natural Resources and Life Sciences, Institute for Transport Studies**, Vienna Interviews with visually handicapped people
July 2008	**Municipality** Rastenfeld Intern; office work

LANGUAGES AND COMPUTER LITERACY

German	Mother tongue
English	fluent, IELTS-Score: 8
Russian	level B1 (european framework of reference for languages)
Czech	basic knowledge
Computer literacy	GeoGebra, R (Statistics), LaTeX, ArcGIS, AutoCAD, GeODIN, CropWat, AquaCrop, EPANET

PERSONAL SKILLS

Communication skills	Open-minded, outgoing Volunteer in the university's "Buddy-Network" to support foreign students during their stay in Vienna
Organisational and managerial skills	Tactic, good time management Experience in leadership for a team of 10 employees

11. Affirmation

I certify, that the master thesis was written by me, not using sources and tools other than quoted and without use of any other illegitimate support.

Furthermore, I confirm that I have not submitted this master thesis either nationally or internationally in any form.

Place, date, name surname, signature