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**KINETIC CHARACTERISTICS AND MICROBIAL COMMUNITY ANALYSIS BY FISH IN ANAMMOX LAB-SCALE SEQUENCING BATCH REACTOR**

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Institute of Chemical Technology, Prague

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Assignment of Diploma Thesis

Student: Anh Thy Vo
Study Programme: Environmental Technology and Engineering
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Specialisation:

Subject of Diploma Thesis:
Kinetic characteristics and microbial community analysis by FISH in ANAMMOX lab-scale sequencing batch reactor

Directions for Elaboration:
Make the literature review on the topic of nitrogen removal by ANAMMOX process and ANAMMOX bacteria cultivation in lab-scale SBRs. Study the literature for analysis of nitrification bacteria by FISH.

In the practical part of your thesis use the samples from the ANAMMOX-lab-scale SBR, make the kinetic tests with aim to measure the rates of conversion of individual nitrogen forms. Process the samples for the FISH analysis and identify and quantify the presence of nitrifiers by this technique. Based on the results try to optimize the conditions in lab-scale SBRs and produce sufficient amount of ANAMMOX biomass for PVA immobilization.
Recommended Literature:

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Head of Department  Dean

Prague 17.02.2014
DECLARATION

This thesis/dissertation was written at the Department of Water Technology and Environmental Engineering of the Institute of Chemical Technology in Prague, February-August 2014.

I hereby declare that this thesis is my own work. Where other sources of information have been used, they have been acknowledged and referenced in the list of used literature and other sources.

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In Prague on ............................
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ABSTRACT

This diploma thesis based on part of the project which was financially supported by Government of Industry and Business Czech Republic by grant signed FR – TI4/254. Besides, this project is the cooperation of ICT Prague with several companies, including LentiKat’s a.s., Satturn Holešov spol. s r.o. and Asio spol. s r.o.

The objective of the project is developing the pilot-scale nitrogen removal technology for waste water treatment, especially for industrially waste water or digested sludge liquor. The innovative technique is the combination of partial nitrification and anammox process in 2 separated stages. Especially, all biomass (mainly nitrifying bacteria) will be immobilized in PVA biocarriers, the biocatalyst technology from LentiKat Company. To be more detailed, the first step converts half of ammonia nitrogen from influent wastewater to nitrite nitrogen by nitritation process. After that, second step will transform nitrite and ammonium nitrogen to nitrogen gas by anammox process. This is a long-term project from lab-scale, pilot-scale and possibly to a full-scale. However, this diploma thesis concentrated mainly on the smallest scale of the second stage. In this study, we operated a lab-scale SBR which showed the good retention of anammox biomass in both suspended solid and plastic carriers.

Briefly speaking, there are mainly 3 investigations in this diploma thesis:

- Investigating on the potential of anammox process for ammonium removal from raw digester liquor with lab-scale SBR
- Optimizing the operating conditions for enrichment of anammox bacteria by chemical kinetic test, including nitrogen forms, TSS, VSS, COD, DO and ORP.
- At the same time, sufficient anammox biomass was produced. By FISH analysis, the microbial community was investigated and quantified, mainly including AOB, NOB and anammox bacteria. Besides, identifying anammox species was part of the thesis work.

Because it was our first time running a lab-scale Anammox-SBR so in the first stage some mistakes happened which led to nitrite inhibition. However, we day by day improved the condition and maintained better working parameters for anammox process. Therefore, after several months, we could move to the second stage and start to enrich the anammox biomass. The nitrogen removal reached more than 80% and the ORP was around -50 mV which indicated the good condition for anammox process. Besides, after mostly 5 months of enrichment, we had sufficient amount of anammox bacteria in suspended solid and on the surface of plastic carriers to immobilize in PVA biocarriers. In details, FISH results showed that anammox bacteria accounted for 19% of total suspended biomass and there were 34% of them in biomass on surface of plastic carriers. Besides, the composition of anammox species included Candidatus *B. fulgida* (predominant), *S. wagneri*, and *A. propionicus*. 
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Anammox</td>
<td>Anaerobic Ammonia Oxidation</td>
</tr>
<tr>
<td>AMX</td>
<td>Anammox bacteria</td>
</tr>
<tr>
<td>AOB</td>
<td>Ammonia Oxidizing Bacteria</td>
</tr>
<tr>
<td>BNR</td>
<td>Biological Nitrogen Removal</td>
</tr>
<tr>
<td>CANON</td>
<td>Completely Autotrophic Nitrogen Removal over Nitrite</td>
</tr>
<tr>
<td>COD</td>
<td>Chemical Oxygen Demand</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DO</td>
<td>Dissolved Oxygen</td>
</tr>
<tr>
<td>DGGE</td>
<td>Denaturing Gradient Gel Electrophoresis</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent <em>in situ</em> Hybridization</td>
</tr>
<tr>
<td>Hh</td>
<td>Hydrazine Hydrolase</td>
</tr>
<tr>
<td>Hzo</td>
<td>Hydrazine Oxidoreductase</td>
</tr>
<tr>
<td>Nir</td>
<td>Nitrite Reductase</td>
</tr>
<tr>
<td>NirS</td>
<td>Nitrite Reductase variation S</td>
</tr>
<tr>
<td>NOB</td>
<td>Nitrite Oxidizing Bacteria</td>
</tr>
<tr>
<td>OLAND</td>
<td>Oxygen Limited Autotrophic Nitrification-Denitrification</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PNA</td>
<td>Peptide Nucleic Acid</td>
</tr>
<tr>
<td>PVA</td>
<td>Poly Vinyl Alcohol</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Real Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal Ribonucleic Acid</td>
</tr>
<tr>
<td>SBR</td>
<td>Sequencing Batch Reactor</td>
</tr>
<tr>
<td>SHARON</td>
<td>Single Reactor System for High Ammonium Removal over Nitrite</td>
</tr>
<tr>
<td>WWTP</td>
<td>Waste Water Treatment Plant</td>
</tr>
</tbody>
</table>
CHAPTER 1: LITERATURE REVIEW

1.1 The overview of nitrogen removal in wastewater treatment

The most common forms of nitrogen in municipal and industrial wastewater are ammonium (NH$_4^+$) in pH dependent equilibrium with ammonia (NH$_3$) and organic nitrogen compounds such as urea and amino acids of proteins (Seviour and Nielsen, 2010).

![Biogeochemical nitrogen cycle](image)

**Figure 1.1:** Schematic illustration of the biogeochemical nitrogen cycle

Ammonification is the process in which biologically catalyzed reactions proceed and release a portion of ammonium (NH$_3$/NH$_4^+$) from organic compounds. Further microbial process, namely Nitrification, give rise to aqueous oxidized inorganic nitrogen species including nitrite (NO$_2^-$), and nitrate (NO$_3^-$). However, Denitrification and Anammox processes further transform these into gaseous species, primarily di-nitrogen (N$_2$) or to a lesser extent nitric oxide (NO) and nitrous oxide (N$_2$O) which can release to the atmosphere particularly in oxygen limiting conditions (Seviour and Nielsen, 2010). Otherwise, in conditions of high aeration, for example, traditional biological reactors, some ammonia (NH$_3$) volatilization to the atmosphere will also occur.

Illegal disposal of wastewater containing high ammonium concentration can cause a huge damage to the environment. It is a key factor for the eutrophication in aquatic systems. Besides, dissolved ammonium is considered as a harmful agent for the aquatic life. Therefore, nitrogen removal from wastewater has become an important issue. Due to increasingly stringent environmental regulations, advanced and cost-effective techniques for the nitrogen removal from wastewater becomes a hot topic nowadays.

In conventional wastewater treatment plants (WWTPs), ammonium is removed by biological Nitrification-Denitrification over nitrate. New approach of nitrogen removal technique was based
on (partial) nitritation and the anaerobic ammonium oxidation (anammox) process. This innovation helps to remove the nitrogen in wastewater in more cost-effective, environmentally friendly, efficient and sustainable way. The combined partial nitritation and anammox process is a completely autotrophic process which can be performed either in one stage or in two stages (reactors).

There were a number of research groups who worked on autotrophic nitrogen removal processes, resulting in a variety of process configurations and various names. For example, as was the denitrification, the anammox process can be combined with Sustainable High rate Ammonium Removal Over Nitrite (SHARON) (van Dongen et al., 2001) to achieve nitrogen removal with reduced operational cost. In the combined SHARON-Anammox, AOB could convert mostly 50% of the ammonium in the influent to nitrite in the first aerated reactor, and then the mixture of ammonium and nitrite in the optimal ratio for anammox took place in the second anaerobic reactor. In contrast, Completely Autotrophic Nitrogen removal Over Nitrite (CANON) relied on a stable interaction between AOB and anammox bacteria to develop in one single reactor (Sliekers et al., 2002). In CANON reactor, NOB was inhibited by maintaining ammonium saturation and concomitant O₂ limitation. Similar to CANON, the Oxygen-Limited Autotrophic Nitrification/Denitrification (OLAND) system consisted of one reactor which worked under oxygen limiting condition (Kuai and Verstraete, 1998).

This next part will give further details of the principle pathways involved in biological nitrogen removal followed by an overview of the reactor conditions affecting the process. This knowledge is important to increase the effectiveness of biological nitrogen processes.

1.2 Nitrogen removal pathways

1.2.1 Conventional nitrification-denitrification over nitrate

The combined process of Nitrification/Denitrification is the most common method used for wastewater treatment nowadays. This biological treatment consists of 2 completely different microbial steps, namely nitrification and denitrification.

In nitrification, ammonia is oxidized to nitrite (nitritation) and further to nitrate (nitratation). Aerobic nitrifiers are the microbes contributing in nitrification. They form 2 distinct functional groups which are specialize in either ammonia or nitrite oxidization and namely Ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), respectively. They are both aerobic and chemolithoautotrophic because they fix CO₂ and gain energy for growth by oxidizing inorganic substances, including ammonia and nitrite, but some nitrifies also can work under anaerobic condition (Bock et al., 1995).

The pathways of conventional nitrification-denitrification over nitrate are illustrated via reactions:
Nitrification: \[ \text{NH}_4^+ + 1.5 \text{O}_2 \rightarrow \text{NO}_2^- + \text{H}_2\text{O} + \text{H}^+ \]
\[ \text{NO}_2^- + 0.5 \text{O}_2 \rightarrow \text{NO}_3^- \]
Overall: \[ \text{NH}_4^+ + 2 \text{O}_2 \rightarrow \text{NO}_3^- \]
Denitrification: \[ \text{NH}_4^+ + 2 \text{O}_2 \rightarrow \text{CO}_2 + 0.5 \text{N}_2 + \text{H}_2\text{O} \]

The second step of conventional biological nitrogen removal, *heterotrophic denitrification*, is an anaerobic process mediated mainly by facultative anaerobic heterotrophic bacteria. It comprises four step reduction processes in which nitrogen gas (N\textsubscript{2}) is formed from nitrate (NO\textsubscript{3}\textsuperscript{-}) over nitrite (NO\textsubscript{2}\textsuperscript{-}), nitric oxide (NO) and nitrous oxide (N\textsubscript{2}O). In details, the bacteria often use organic compounds as electron donors to reduce nitrate (NO\textsubscript{3}\textsuperscript{-}) and nitrite (NO\textsubscript{2}\textsuperscript{-}).

The main factors which might affect the efficiency of denitrification are:

- Dissolved oxygen (DO): increasing DO will decrease the denitrification rate.
- Presence of organic matter: the highest rate of denitrification can be achieved by adding an easily biodegradable and assimilated carbon source.
- Others factor, for example: pH, temperature, heavy metals and organic compounds.

Biological nitrification-denitrification over nitrate is considered as an efficient process with a relatively easy operation and moderate costs. It is generally used for the treatment of wastewater containing low nitrogen concentration. However, to some extend, it could be costly because of the aeration and input of organic carbon to maintain the denitrification. Moreover, this conventional technology could not treat wastewater with very high N-loading (low C/N ratio) and produces a big amount of sludge. Therefore, current interest focuses on the pathway to eliminate the aeration and the requirement for external carbon supply. In this thesis, we concentrated on anaerobic ammonium oxidation process (anammox). The overview of anammox, its application and the microbial community related to it will be revealed in the following parts.

### 1.2.2 ANAMMOX process

Conventionally, biological nitrogen removal is achieved by nitrification followed by denitrification process. However, the combination of partial nitrification and anammox are the innovative way for treatment of wastewater which contains high amount of nitrogen such as industrial wastewater and raw digested liquor.

ANAMMOX, an abbreviation for ANaerobic AMMonium OXidation, is a globally important microbial process of the nitrogen cycle. It can be applied as an alternative to the traditional nitrification-denitrification BNR pathway which was discussed previously. As the name suggests, Anammox, is an anaerobic microbially-catalyzed process in which ammonia serves as the electron donor, nitrite as the electron acceptor, and dinitrogen gas is the final product. The
process proceeds via the following reaction pathway (Jetten et al., 2009, Seviour and Nielsen, 2010):

Overall Reaction:

\[
\text{NH}_4^+ + \text{NO}_2^- \rightarrow \text{N}_2 + \text{H}_2\text{O}
\]

Metabolic Pathway:

\[
\begin{align*}
\text{NO}_2^- & \xrightarrow{\text{NirS}} \text{NO} \\
\text{NO} + \text{NH}_4^+ & \xrightarrow{\text{Hh}} \text{N}_2\text{H}_4 \xrightarrow{\text{Hzo}} \text{N}_2 + \text{H}_2\text{O}
\end{align*}
\]

Anammox Stoichiometry (Strous et al., 1998):

\[
\text{NH}_4^+ + 1.32\text{NO}_2^- + 0.066\text{HCO}_3^- + 0.13\text{H}^+ \rightarrow 1.02\text{N}_2 + 0.26\text{NO}_3^- + 0.066\text{CH}_2\text{O}_{0.5}\text{N}_{0.15} + 2.03\text{H}_2\text{O}
\]

Firstly, \(\text{NO}_2^-\) is reduced to \(\text{NO}\) by nitrite reductase (\(\text{NirS}\)). Then, hydrazine hydrolase (\(\text{Hh}\)), a novel enzyme, reduces nitric oxide while simultaneously oxidizing ammonium to form hydrazine (\(\text{N}_2\text{H}_4\)). Lastly, \(\text{N}_2\text{H}_4\) is oxidized by hydrazine oxidoreductase (\(\text{Hzo}\)) to produce dinitrogen gas, water, and a very small amount of byproduct nitrate (Jetten et al., 2009).

The anammox is a suitable process for N removal from wastewater with a high ammonium content and a low C:N ratio, such as liquor from sludge digester (Jetten et al., 2001). It poses some attractive advantages over the traditional nitrification-denitrification pathway. Firstly, there is no requirement for organic carbon sources which are often needed to boost the denitrification. Moreover, anammox microorganisms are obligate anaerobes, so it is unnecessary for aeration of anammox reactors. However, nitrite is required as substrate in this process and can be supplied by aerobic AOB (Seviour and Nielsen, 2010). Thus, the anammox process has the potential to reduce cost for N removal (Jetten et al., 2001, Schmidt et al., 2003). However, the main disadvantage of the process is the low growth rate of anammox bacteria. Thus, long start-up time is necessary. According to Schmidt et al. (2003), experience in anaerobic wastewater treatment has shown that this problem could be overcome once the first full-scale anammox plants were in operation (in Rotterdam, Netherlands) and seeding would become possible.

In order to remove ammonium successfully from wastewater using the anammox process, there should be a preceding aerobic partial nitrification which is the oxidation of wastewater ammonium to nitrite, but not to nitrate. To reach the partial nitrification, basically, the DO concentration needs to decrease. Besides, the temperature is adjusted higher than 25°C and free ammonia could be used to inhibit nitratation. The combination of partial nitrification and anammox has been studied by several authors at lab and pilot scale recent years with nitrogen removal efficiencies higher than 80%. In initial nitritation/anammox applications, the 2 reactions were separated in 2 compartments. Lately, it could be shown that AOB and anammox bacteria
can live together in one single reactor if DO concentration is low (below 0.5 g/m³). Moreover, nitrite concentration has to be low to prevent inhibition on anammox bacteria. Low oxygen and nitrite level will help to prevent nitration and NOB activity.

1.3 Microbial community in anammox reactor

This section addresses the community of microbes could be found in anammox reactor. Together with anammox bacteria, AOB and NOB could be identified by molecular techniques such as FISH. Besides, heterotrophic denitrifying microbes are also a part of the microbial community. The diversity of bacteria depends on the operating condition of the reactor.

1.3.1 Anammox bacteria

Anammox bacteria (AMX) are coccid and brown-reddish bacteria with an average diameter ranging from 800 to 1100 nm (van Niftrik et al., 2008). They are extremely slow-growing chemolithoautotrophs and obligate anaerobes with high sensitivity to oxygen. Growth of the microorganisms was irreversible inhibited even by oxygen concentration which was less than 0.5% air saturation (Strous et al., 1997a). They rely on CO₂ fixation and bicarbonate as their carbon sources, have doubling times between 10-20 days and have relatively low biomass yields (Seviour and Nielsen, 2010). Because of their low growth rate, they tend to thrive in natural environments that have low substrate concentrations.

Their extremely slow generation time is the main reason why it is not yet possible to grow anammox bacteria with standard microbial cultivation method. Until now, they cannot be grown in pure culture. When the biomass was studied with molecular techniques on the basis of its 16S rRNA, for example, PCR, clone libraries and FISH, it appeared that the anammox process is so far linked to one group of organisms (Strous et al., 1999a, Schmid et al., 2005). Although there is a relatively large phylogenetic distance among anammox genera, all of them belongs to the same order, namely Brocadiales, within the phylum Planctomycetes (Jetten et al., 2010). Until now, there are five anammox “Candidatus” genus have been described, including “Candidatus Kuenenia” (Schmid et al., 2000), “Candidatus Brocadia” (Kartal et al., 2004, Kartal et al., 2008), “Candidatus Anammoxglobus” (Kartal et al., 2007a), “Candidatus Jettenia” (Quan et al., 2008) and “Candidatus Scalindua” (Kuypers et al., 2003, Schmid et al., 2003a). They were given the status of Candidatus because of their non-cultivable characteristics. The first four genus have all been enriched from activated sludge while “Candidatus Scalindua” was found in natural habitats, especially from marine sediments and oxygen minimum zones. Anammox is estimated to be responsible for 33-50% of global nitrogen removal from marine ecosystems (Dalsgaard et al., 2005).
Figure 1.2: A 16s ribosomal RNA-gene-based phylogenetic tree of anammox bacteria (Kuenen, 2008) – illustrating the relationships of different families of anammox bacteria among the Planctomycetes. The sequence divergence of the Planctomycetes from other bacteria (indicated as outgroup) is high. The scale bar represents 10% sequence divergence.

Many studies in the last 2 decades showed that anammox bacteria are ubiquitous in natural ecosystems and the anammox process has been successfully applied for ammonium removal in both full and lab-scale wastewater treatment. The information of anammox species which was discovered until now is displayed in table 1-1.

Table 1-1: Microbial species of anammox bacteria discovered up to date

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brocadia</td>
<td>Candidatus Brocadia anammoxidans</td>
<td>Wastewater</td>
</tr>
<tr>
<td></td>
<td>Candidatus Brocadia fulgida</td>
<td>Wastewater</td>
</tr>
<tr>
<td>Kuenenia</td>
<td>Candidatus Kuenenia stuttgartiensis</td>
<td>Wastewater</td>
</tr>
<tr>
<td>Scalindua</td>
<td>Candidatus Scalindua brodae</td>
<td>Wastewater</td>
</tr>
<tr>
<td></td>
<td>Candidatus Scalindua wagneri</td>
<td>Wastewater</td>
</tr>
<tr>
<td></td>
<td>Candidatus Scalindua sorokinii</td>
<td>Seawater</td>
</tr>
<tr>
<td></td>
<td>Candidatus Scalindua arabica</td>
<td>Seawater</td>
</tr>
<tr>
<td>Jettenia</td>
<td>Candidatus Jettenia asiatica</td>
<td>Not reported</td>
</tr>
<tr>
<td>Anammoxoglobus</td>
<td>Candidatus A. propionicus</td>
<td>Wastewater</td>
</tr>
</tbody>
</table>
Back to the history, in 1999, *Brocadia anammoxidans* was first identified and described by physically purifying anammox cells from a laboratory enrichment culture (Strous et al., 1999a). The purified anammox cells converted ammonium and nitrite into nitrogen gas in the lack of oxygen and used fixed CO\(_2\) as carbon source only. After the first discovery, many wastewater treatment plants reported about the anammox reactions which explained for the nitrogen loss, especially in some landfill leachate treatment facilities. By the molecular tools, there was a clear presence of organisms affiliated with the anammox branch within the *Planctomycetes* in all these treatment plants.

In 2000, quantitative FISH analysis with a newly designed specific probe showed that a novel bacterium, classified as “*Candidatus Kuenenia stuttgartiensis*” constituted a dominant fraction in the anammox biofilm (Schmid et al., 2000).

In nature, anammox bacteria was first detected in the Black Sea, the world’s largest anoxic basin (Kuypers et al., 2003). The molecular tools showed the link between the anammox reaction and the presence of group of bacteria. As the result, anammox bacteria play a key role in the marine nitrogen cycle and belong to the “*Candidatus Scalindua*”. In the same year, the description of “*Candidatus Scalindua brodae*” and “*Candidatus Scalindua wagneri*” was published and it showed the average sequence similarity between them and “*Candidatus Brocadia*”, on the 16S rRNA level, was only 85% (Schmid et al., 2003a). Indeed, the phylogenetic depth of the anammox lineage is remarkable. It is deeper when comparing to lineage of aerobic beta-proteobacterial ammonia oxidizers which has sequence similarity between its most distant members is about 90%. Therefore, each species of anammox bacteria has some special characteristics and a specific but yet unknown niche.

In 2007, “*Candidatus Anammoxoglobus propionicus*” was described with a different niche, the co-oxidation of propionate and ammonium. This species could oxidize organic acid at high rate and then get an advantage to compete other genera for nitrite in the presence of propionate. Thus, it could be applied for treatment of nitrogen and/or COD containing wastewater. On the other hand, the enrichment of anammox bacteria in the presence of acetate showed that among the tested biomass, the biomass from “*Candidatus Brocadia fulgida*” enrichment culture oxidizes acetate at the highest rate. Moreover, continuous enrichment experiments displayed the outstanding competition of “*Candidatus Brocadia fulgida*” over other anammox bacteria (Kartal et al., 2008).

Members of all genera have been detected in wastewater treatment plants and have been enriched in many lab-scale bioreactors, but genome information is not yet available for all genera. In 2008, “*Candidatus Jettenia asiatica*” was identified as a new anammox bacteria which was dominant in a granular sludge anammox reactor (Quan et al., 2008). Its genome sequence was then analyzed and reported by sequencing and metagenomic analysis. Although it was impossible to distil a high quality draft genome of this organism from the metagenome, the scientists could identify all
essential genes for anammox metabolism based on the comparison with other anammox bacteria such as “Candidatus Kuenenia” (Hu et al., 2012).

Anammox bacteria used to be assumed that they are completely chemolithoautotrophic. However, recent researches show that next to ammonium, organic and inorganic compounds can be used as alternative electron donors. For example, methylamine was used by “Candidatus Brocadia fulgida” or ferrous ion by “Candidatus Kuenenia stuttgartiensis” (Kartal et al., 2008). Furthermore, it has been shown that, besides nitrite, iron and manganese oxides can also be used as alternative electron acceptors (Strous et al., 2006). Therefore, anammox bacteria do not all adhere strictly to chemolithoautotrophic lifestyle.

There are many factors which can affect the community composition in anammox reactors. It includes seeding sludge, the quality of influent wastewater and living condition for bacteria (temperature, pH and dissolved oxygen concentration). Besides, reactor condition (scale, type and age) could also influence the selectivity and the quantity of anammox bacteria. An insight should be carried out for optimizing the living condition to cultivate and enrich the high amount of anammox bacteria.

### 1.3.2 Ammonia oxidizing bacteria

Ammonia oxidizing bacteria (AOB) form two monophyletic groups, beta- and gamma-proteobacteria (Purkhold et al., 2000).

The beta-ammonia oxidizers comprise the genus *Nitrosomonas* and *Nitrosospira*. Most commonly found AOB in the nitrifying WWTPs belongs to genus *Nitrosomonas* (including *Nitrosococcus mobilis*), related to *N. europaea, N. eutropha, N. mobilis* and *N. oligotropha* (Nielsen et al., 2009). They are generally considered as aerobic chemolithoautotrophs, but recently organic compounds have been described to be carbon and energy source. The genome project of *Nitrosomonas europaea* nears completion. Although the relevance of this organism for wastewater treatment is disputable, it will still provide an invaluable source of information.

Genus *Nitrosospira* has occasionally been detected in WWTPs, but these AOB are generally popular in terrestrial habitats and seem to play only minor roles for wastewater treatment.

*Nitrosococcus* is the gamma-proteobacterial genus, which commonly includes 2 species: *Nitrosococcus halophilus* and *Nitrosococcus ocean*. This genus is mostly found in marine habitats. Therefore, by its high salt requirements, it is absent in most WWTPs.

They are generally considered as aerobic chemolithoautotrophs, but recently organic compounds have been described that can serve them as carbon and energy source (see below). The genome project of *Nitrosomonas europaea* nears completion. Although the relevance of this organism for wastewater treatment is disputable, it will still provide an invaluable source of information.
1.3.3 Nitrite-oxidizing bacteria

Nitrite-oxidizing bacteria (NOB) catalyze the second step of aerobic nitrification, the oxidation of nitrite (NO$_2^-$) to nitrate (NO$_3^-$). There are 4 different genera of NOB, including *Nitrobacter*, *Nitrococcus*, *Nitrospina* and *Nitrospira*. In most of WWTPs, the dominant NOB are members of the genus *Nitrospira* (Daims et al., 2001, Gieseke et al., 2003, Juretschko et al., 1998). This genus belongs to the distinct bacterial phylum *Nitrospirae*, and thus is not closely related to other known NOB, which are all *Proteobacteria*.

On the other hand, the genus *Nitrobacter* seems to play a minor role in wastewater treatment. It could be possible to obtained *Nitrobacter* cells in many bio-reactors, and then enrich or isolate them by incubation of activated sludge in artificial nitrite media, but FISH has showed that they do usually not reach the significant density in WWTPs (Wagner et al., 1996b). However, in temporarily-elevated-nitrite-containing reactors, such as SBRs treating highly concentrated sludge liquor, *Nitrobacter* can reach an exceptionally high abundance (Daims et al., 2001). It can be explained by the adaptation of these NOB to higher NO$_2^-$ concentration while *Nitrospira* are usually adapted to lower NO$_2^-$ concentration (Schramm et al., 1999).

In flocs and biofilm, the mutualistic symbiosis of AOB and NOB was reflected by the occurrence of NOB in the direct spatial neighborhood to AOB (Maixner et al., 2006).

Other NOB comprise the marine genera *Nitrococcus* and *Nitrospina* and the only recently discovered betaproteobacterial *Nitrotoga artica* (Alawi et al., 2007). Until now, none of these NOB has been shown by molecular tools to be functionally important for nitrification in wastewater treatment.

In this thesis, a SBR-Anammox reactor was operated and the community of microbes, including anammox bacteria, AOB and NOB, was identified and quantified by FISH analysis.

1.4 Sequencing batch reactor: an efficient way for anammox bacteria enrichment

1.4.1 Introduction of SBR

Sequencing Batch Reactor (SBR) is a special form of activated sludge treatment in which all of the treatment process takes place in the reactor tank and clarifiers is not required. This type of reactor treats the wastewater in batch mode and each batch is sequenced through a series of treatment stages.

There are five stages in the treatment process:

1. Fill: wastewater is filled in the tank and mixed with the biomass which is settled from the previous stage.
2. React: the process of reaction and biological treatment the targeted compounds. Mixing could be obtained by physical/mechanical methods.
3. Settle: mixing was stopped during this stage to allow the biomass and sludge to settle.
4. Decant: effluent is discharged.
5. Idle: idling time for the next part. If necessary, sludge removal occurs during this stage.

1.4.2 SBR is an advanced technique for anammox bacteria enrichment

The low specific growth rate and high doubling time of anammox bacteria (from 11 to 20 days) were considered as the main bottlenecks for the application of this process in wastewater treatment plants for nitrogen removal. Thus, the start-up period of anammox process is longer compared to other nitrogen removal techniques. However, after the recognition of its potential for treatment wastewater with high N-loading, there are high attempts to solve this problem. Many scientists tried to figure out which reactor system could enable the high and efficient sludge retention by both laboratory and full-scale reactor.

The first successful enrichment of anammox bacteria was the use of fluidized bed cultures fed with mineral medium containing only ammonium and nitrite only which growth the microbial community as biofilms on sand particles (Van de Graaf et al., 1996). However, this cultivation system was not satisfactory enough because it was difficult to operate fluidized bed reactor (FBR) in lab-scale and sometimes the biomass retention was not sufficient to maintain the anammox culture. Moreover, because of the long duration of enrichment (more than 1 year), it led to the failure of pH control and recycle pumps which could make the cultivation unsuccessful. The last problem was the inconstant condition of biofilms over the reactor by lack of bulk mixing in the FBR. For example, some parts of FBR could not receive substrate continuously and the biomass in this areas faced with starvation which caused the decrease of anammox activity and then difficulty for bacteria quantification (Strous et al., 1998).

In 1998, Strous et al. revealed that SBR was an appropriate system for growing anammox microorganisms (Strous et al., 1998). In this research, they tried to apply and optimize SBR for enrichment and quantitative study of very slowly growing anammox microbial community. As the result, SBR was considered to be a powerful experimental system because of following strengths:

- It has high and efficient biomass retention.
- It could distribute substrates, products and biomass aggregates in the reactor homogenously.
- It could be operated properly for more than 1 year.
- It is stable under substrate-limiting conditions.

SBR was concluded to be suitable for long-term enrichment, cultivation and quantitative analysis of a very slowly microbial community. Therefore, in this study, we use SBR for growing anammox bacteria under sludge digester media.
Besides, many researchers have studied various types of anammox reactors. They investigated on the performance and microbial community of the reactors. This knowledge helped to optimize the enrichment of anammox bacteria together with the better design for nitrogen removal efficiency by anammox process.

Table 1-2: Several studies of anammox process with different configurations

<table>
<thead>
<tr>
<th>Reactor types</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequencing batch reactor</td>
<td>(Strous et al., 1998)</td>
</tr>
<tr>
<td>Fluidized bed reactor</td>
<td>(Van de Graaf et al., 1996, Strous et al., 1997b)</td>
</tr>
<tr>
<td>Fixed bed biofilm reactor</td>
<td>(Strous et al., 1997b, Fux and Siegrist, 2004)</td>
</tr>
<tr>
<td>Up-flow anaerobic sludge blanket reactor</td>
<td>(Ahn et al., 2004)</td>
</tr>
<tr>
<td>Membrane SBR</td>
<td>(Trigo et al., 2006)</td>
</tr>
<tr>
<td>Rotating biological reactor</td>
<td>(Egli et al., 2001)</td>
</tr>
<tr>
<td>Gas-lift reactor</td>
<td>(Slickers et al., 2003)</td>
</tr>
</tbody>
</table>

1.5 Molecular tools: Fluorescence In Situ Hybridization (FISH)

Among cultivation-independent methods, FISH is a powerful tool to observe the morphology and to quantify numbers of bacteria or the equivalent biovolume in environmental samples from wastewater treatment plants (WWTP) such as activated sludge and biofilm. The principle of this method is that abundant rRNA molecules are probed with fluorescently labeled oligonucleotides which target specific site on the rRNA of organisms on interest. Under favorable and stringent conditions, the probes hybridize to their target cells, forming DNA/RNA duplexes which are possibly detected by fluorescent microscopy or flow cytometry (Seviour and Nielsen, 2010). FISH is useful in WWTP studies because it enables either morphological identification or quantification of microorganisms which present in aggregation down to the single cell level (Nielsen et al., 2009).

Although there exist other molecular methods, primarily PCR-based methods, such as quantitative PCR (q-PCR) which is more and more commonly applied in environmental samples, FISH is proved to have several key advantages. It is a faster and less expensive method. It also poses simplicity to observe the targeted cells under epifluorescent microscope. Comparing to those based on PCR, the result obtaining from FISH is the direct visual feedback during microscopic observance of the analyzed samples. It not only makes FISH more confident regarding to the accuracy of an experiment, but also converts FISH into a precise method to quantify the bacteria in the whole biovolume of samples. Because the quantitative FISH is based on the principle “count what you see”, it is less prone to biases than q-PCR approaches, which are quite sensitive to errors in method and contamination in samples (Nielsen et al., 2009). Moreover, until now, there is wide range of probes now used in FISH for targeted microbial
communities. We can recognize the existence of abundance and periodically examined databases, such as “probeBase” of the University of Vienna (Loy et al., 2003). Improvement and refinement of probes is also continually occurring when researchers identify sites of rRNA with highly limited access to oligonucleotide diffusion (Okten et al., 2012). Furthermore, PNA (peptide nucleic acid) probes was recently discovered and demonstrated greater specificity and thermal stability than some RNA and DNA alternatives (Lehtola et al., 2006).

However, there are still problems commonly encountered while using FISH including: mistakenly fluorescence of non-targeted cells and surrounding materials, such as inorganic matter. Besides, low signal intensity leads to the difficulty to get the proper image for quantification so the result cannot be precise. There are many reasons for the low or no signals in FISH analysis. Firstly, it can be explained by the inaccessibility of the probes but it can be overcome by the application of helper probes (Fuchs et al., 2000). Helper probes are unlabeled probes and believed to open secondary and tertiary structures. Thus, they can increase the accessibility of the FISH probes. Longer hybridization time is another solution (Yilmaz et al., 2006). Increasing hybridization time (up to 72 hours) can enhance probe diffusion into cells and decrease the kinetic barriers for targeted sites. Secondly, insufficient cell wall impermeability can also decrease the accessibility and cause no or low FISH signals. Different pre-treatment protocols can be used to solve the problems. Besides, the choice of fluorochrome is crucial. The larger the coefficient extinctions are, the stronger signals can be obtained (Nielsen et al., 2009). List of commonly used fluorescent dyes is shown in Table 1.3. Furthermore, the problem can be caused by other factors, for example, too long fixation time, too long exposure to light source or traces of ethanol left which can fade the fluorescent signals.

Table 1-3: Most commonly used fluorescent dyes to label oligonucleotides for FISH analysis (Nielsen et al., 2009)

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Color</th>
<th>Extinction coefficient (M⁻¹cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy3</td>
<td>Red</td>
<td>150,000</td>
</tr>
<tr>
<td>Cy5</td>
<td>Blue</td>
<td>250,000</td>
</tr>
<tr>
<td>DAPI</td>
<td>Blue</td>
<td>27,000</td>
</tr>
<tr>
<td>FLUOS</td>
<td>Green</td>
<td>74,000</td>
</tr>
<tr>
<td>TAMRA</td>
<td>Red</td>
<td>65,000</td>
</tr>
<tr>
<td>SYBR Green</td>
<td>Green</td>
<td>73,000</td>
</tr>
</tbody>
</table>

In this thesis, FISH analysis was applied to identify and quantify the microbial community in SBR-Anammox reactor, especially AOB, NOB and anammox bacteria (AMX). This result was then combined with chemical kinetic test for investigate the activity of bacteria and optimizing the enrichment condition of anammox bacteria.
2.1 Overview of experimental strategy

An overview of consequent stages undertaken within this thesis is displayed in figure 2.1.

![Experimental strategy overview](image)

**Figure 2.1:** Experimental strategy overview

The methodology for enrichment anammox organisms was conducted in 3 experimental stages:

1. **Start-up**
   - For anammox bacteria to adapt the environment and for lab-scale SBR (reactor E) to reach stable operating condition

2. **Enrichment**
   - Increase nitrogen loading and enrich anammox bacteria to obtain sufficient biomass for immobilization.

3. **Immobilization**
   - Immobilize anammox biomass in PVA biocarriers

**Stage 1: Start-up**

During the period May 15th, 2013 to January 13th, 2014, a laboratory scale reactor (indicated as E) was launched with the inoculation of anammox biomass from other reactor in ICT Prague (one plastic carrier). Because the previous reactor where the biomass taken from has a different operation and regime from our reactor E, so it took long time for bacteria to adapt the environment. Furthermore, reactor E was our first time running a lab-scale Anammox-SBR, hence in the first stage some mistakes happened (it will be discussed more in *Chapter 3*). It then led to the longer time for start-up period than we expected. However, we day by day improved the condition and maintained better working parameters for anammox process. Therefore, after several months, we could move to the second stage and start to enrich the anammox biomass.

**Stage 2: Enrichment**

During the period January 13th, 2014 to June 9th, 2014, we gained the stable operating condition for reactor E and a good environment for enrichment of anammox bacteria. In this stage, the nitrogen loading was increased slowly and the amount of anammox biomass grew up slowly. After 5 months, we obtained sufficient anammox biomass for immobilization in PVA biocarriers, and then move to the third stage.
Stage 3: Immobilization

As part of the project, anammox biomass needs to be immobilized in biocarriers (PVA pellets) from LentiKat’s company to improve the nitrogen removal efficiency and biomass stability. However, before mass producing of anammox PVA pellets, it is very important to investigate their lab-scale operation and feasibility for nitrogen removal in wastewater treatment.

However, because of the limited time, this thesis can only cover the initial one-month performance of the reactor with anammox PVA pellets (from June to July 2014).

In each stage, the activity of bacteria and nitrogen removal efficiency were determined throughout water chemistry analysis while the microbial community was determined with fluorescent in situ hybridization (FISH). The detailed protocols of methods are shown in section 2.3 and 2.4:

- Equipment, chemical and procedures used to monitor and follow the reactor operation by chemical kinetic test
- Facilities, supplies, reagents and probes applied in all stages of FISH: sample fixation, hybridization, imaging and image analysis

An introduction about SBR configuration and tested samples will be given in section 2.2.

2.2 Description of lab-scale SBR and collecting samples

2.2.1 About lab-scale SBR

2.2.1.1 Introduction of lab-scale Anammox-SBR

In this thesis, an enrichment culture of anammox bacteria was developed in a lab-scale SBR (namely reactor E) which had a maximum working volume of 6 L (figure 2.2). The system was maintained at 30°C (by temperature controller JULABO F250). pH value was not controlled but it was stable between 7 and 8. Reactor E was controlled at 6-7 days/batch for stage 1 and 3-4 days/batch for stage 2. The reactor contents were mixed uniformly using a stirrer.

During the first 5 minutes after filling, the anaerobic condition in the anammox reactor was maintained by the introduction of nitrogen gas and by activity of microorganisms. The oxygen concentration in the reactor was always checked and kept under inhibited level. The SBR was filled with feeding solutions which were prepared before by raw digested liquor and synthetic NaNO₂ solution.

For the decant period, before March 10th, in every batch we withdrew 3.7 L of effluent but after that, the we decreased the volume of output supernatant to only 2 L (in 6 L total working volume of reactor E).
2.2.1.2 Inoculum

The reactor E was firstly inoculated with one plastic carrier contained the anammox biomass from the previous experiment of Dr. Jan Bartacek in ICT Prague. In that experiment, he successfully operated a 5.5-litre SBR which combined partial nitrification and anammox in one stage. Furthermore, the origin of anammox biomass for Dr. Jan Bartacek's experiment is from another pilot-scale reactor which was situated in WWTP in Prague.

The inoculation of reactor E by the carrier with anammox biomass was on May 15th 2013. The carrier was initially inserted into reactor E on the surface of water and was later fixed by wire approximately 5 cm underwater.

2.2.1.3 Influent media

The source of ammonium nitrogen for this experiment is from the digested liquor of wastewater treatment plant in Prague. To prepare the feeding solution, we diluted the raw digested liquor to 600 mg/L of N-NH₄⁺ and stored it in 10-litre PE container. In table 2-1, some properties of this raw wastewater before and after dilution will be displayed in average value.
Table 2-1: Characteristics of raw digested liquor used for feeding solution

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Before dilution (mg/L)</th>
<th>After dilution (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-NH$_4^+$</td>
<td>1319</td>
<td>600</td>
</tr>
<tr>
<td>N-NO$_2^-$</td>
<td>0.6</td>
<td>0.2</td>
</tr>
<tr>
<td>N-NO$_3^-$</td>
<td>3.54</td>
<td>1.4</td>
</tr>
<tr>
<td>COD</td>
<td>2942</td>
<td>1163</td>
</tr>
</tbody>
</table>

For nitrite nitrogen input, we prepared the synthetic solution by dissolving 123 g NaNO$_2$ in 500mL flask of distilled water and then kept the solution in fridge.

According to the experimental strategy, the feeding solutions will be diluted to the required concentration of ammonium and nitrite.

**2.2.1.4 Operation of lab-scale Anammox-SBR**

The experiment of reactor E consists of 3 stages

**Stage 1: Start-up**

During the beginning period of this stage, the reactor was inoculated and nitrogen gas was continuously input to prevent the aerobic condition which was harmful to anammox bacteria. From May 15th 2013, during the first 9 weeks of the operation, reactor E worked with concentration of feeding solutions at 50 mg/L (both for ammonium and nitrite). From week 9, the load of nitrogen ammonium and nitrite increased from 50 to 100 mg/L. Besides, at this point, nitrogen gas input was stopped. On Oct 21st (end of week 23), we added 10 more plastic carriers (without biomass) to develop the attachment area for anammox bacteria.

In November 2013, we opened again the N$_2$ bubbling, and then tried to increase the nitrogen loading rate from 100 to 150 mg/L (week 26) and then to 200 mg/L (week 28). At the beginning of year 2014 (around week 30), we found out the inhibition of nitrite to anammox bacteria which led to the break-down of anammox microbial community (it will be discussed further in the next chapter). It could be explained by the sudden increase of nitrogen loading rate and 200 mgN-NO$_2^-$/L seemed too high for the anammox bacteria. Besides, in week 20, the removal of suspended biomass also assisted the inhibition. Therefore, we had to let the reactor E be recovered by pumping the nitrogen gas continuously and then decreased the ammonium and nitrite concentration in the influent to 30 mg/L. Fortunately, after this time, by checking the nitrogen removal efficiency everyday we could observe the proper operation of the reactor E and good signal for anammox bacteria enrichment.
**Stage 2: Enrichment**

At this stage, based on the experience from the first stage, we had to increase carefully and slowly the nitrogen loading rate. The concentration of ammonium and nitrite input is displayed by time in table 2-2.

**Table 2-2: Concentration of input N-NH$_4^+$ and N-NO$_2^-$ in stage 2**

<table>
<thead>
<tr>
<th>Date</th>
<th>Concentration of N-NH$_4^+$ (mg/L)</th>
<th>Concentration of N-NO$_2^-$ (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>From week 36</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>From week 42</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>From week 43</td>
<td>60</td>
<td>50</td>
</tr>
<tr>
<td>From week 44</td>
<td>70</td>
<td>60</td>
</tr>
<tr>
<td>From week 46</td>
<td>80</td>
<td>70</td>
</tr>
<tr>
<td>From week 48</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

The strategy was based on the concentration of nitrite and ammonium in the effluent. Once the concentration was closed to zero, an increase of nitrogen loading rate was applied. Otherwise, it was maintained at the existing level for several batches to make the operation of anammox reactor stable before being expanded. By using this strategy, it is possible to control and minimize the inhibitory effects of nitrite on anammox bacteria’s activities (which we experienced in the first stage when we increased suddenly the concentration of nitrite from 150 to 200 mg/L). Besides, the amount of biomass was a critical parameter to adjust the nitrogen loading.

On June 9th 2014 (week 57), we finished the second stage of the experiment which enriched the anammox bacteria in the suspended solid and sent all the biomass to the LentiKat’s for immobilizing them in PVA pellets.

**Stage 3: Immobilization**

On June 10th 2014, 1 kg PVA pellets which carry the biomass from the last stages inside was input in reactor E. After that, we operated the reactor with initial nitrogen loading rate of 50 mg/L for both ammonium and nitrite nitrogen concentration. Few weeks later, by investigating the performance of reactor, we decreased the influent concentration of both nitrogen forms to 30mg/L. In this stage, operation regime is similar to last stages but the effluent is removed during stirring and pellets are separated by a special filter as we would like to keep only biomass in PVA biocarriers.
2.2.2 About samples

2.2.2.1 Collecting samples for chemical analysis

We collected the influent and effluent samples. Besides, water samples were taken almost daily (for every batch) and were analyzed immediately or temporarily stored at -20°C in the freezer.

The chemical analysis of water samples was performed for (will be discussed more in section 2.3):

- Ammonium, nitrite and nitrate concentration
- pH, DO concentration, ORP, and COD

Besides, TSS and VSS were also investigated.

2.2.2.2 Collecting samples for FISH

The suspended solid was sampled from the reactor almost every month from May 2013 to May 2014. After that, it was fixed in the PFA and stored in the freezer at -20°C (see section 2.4.3).

In April and May 2014, beside suspended solid, we also took biomass samples from the plastic carriers to compare with the samples from suspended solid. The procedure of sampling, fixation and FISH analysis was similar to the previous samples.

2.3. Chemical Analysis

2.3.1 Kinetic test for inorganic nitrogen forms

The concentrations of inorganic nitrogen forms in reactor were analyzed on a regular basis. We measured them almost everyday in one working cycle. The parameters were N-NH₄⁺, N-NO₂⁻ and N-NO₃⁻ and they were determined using the following protocols.

2.3.1.1 Ammonium Nitrogen (N-NH₄⁺)

This protocol was adapted from Standard Methods in the Examination of Water and Wastewater 4500-NH₃.

Table 2-3: Nessler Reagent and Seignett Salt Solution preparation

<table>
<thead>
<tr>
<th>Nessler Reagent</th>
<th>Seignett Salt Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 g Hgl₂</td>
<td>2.5 g KNaC₄H₄O₆</td>
</tr>
<tr>
<td>17.5 g KI</td>
<td>50 mL dH₂O</td>
</tr>
<tr>
<td>Solution of NaOH (40 g NaOH in 125 mL dH₂O)</td>
<td></td>
</tr>
<tr>
<td>dH₂O fill to 250 mL</td>
<td></td>
</tr>
</tbody>
</table>
Step by step of \( \text{N-NH}_4^+ \) measurement:
- Load 5 mL of sample into a test tube
- Add 100 \( \mu \)L of Seignett salt solution and 100 \( \mu \)L of Nessler reagent
- Seal the test tube and mix thoroughly
- After 10 minutes measure the absorbance at 425 nm with a photoLab® 6100 VIS spectrophotometer (WTW GmbH, Weilheim, DE)
- Calculate the concentration of \( \text{N-NH}_4^+ \) using a calibration curve

2.3.1.2 Nitrite Nitrogen (\( \text{N-NO}_2^- \))

This protocol was adapted from Standard Methods in the Examination of Water and Wastewater 4500-\( \text{NO}_2^- \).

SANED Reagent preparation:
- 10 g Sulfanilamide
- 0.5 g N-(1-napthyl)-1,2-ethyendiamine-dichloride
- 25 mL concentrated \( \text{H}_3\text{PO}_4 \)
- \( \text{dH}_2\text{O} \) to 250 mL

Step by step of \( \text{N-NO}_2^- \) measurement:
- Load 5 mL of sample into a test tube
- Add 125 \( \mu \)L of SANED reagent and 1100 \( \mu \)L of \( \text{dH}_2\text{O} \)
- Seal the tube and mix thoroughly
- After 20 minutes measure the absorbance at 540 nm with a photoLab® 6100 VIS spectrophotometer (WTW GmbH, Weilheim, DE)
- Calculate the concentration of Ammonia using a calibration curve

2.3.1.3 Nitrate Nitrogen (\( \text{N-NO}_3^- \))

Nitrate analysis was performed by using Dr. Lange Küvetten-Test. Measurements are based on the reaction of nitrate ion with 2,6-dimethylphenol. This protocol was derived from ISO 7890-1:1986 (ISO, 1986).

Table 2-4: Preparation of solutions and reagents used in \( \text{N-NO}_3^- \) measurement

<table>
<thead>
<tr>
<th>Amide-Sulfuric Acid</th>
<th>Acid Mixture</th>
<th>DMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8 g amide-sulfuric acid</td>
<td>1 L concentrated ( \text{H}_2\text{SO}_4 )</td>
<td>0.24 g 2,6-dimethylphenol</td>
</tr>
<tr>
<td>( \text{dH}_2\text{O} ) to 100 mL</td>
<td>1 L concentrated ( \text{H}_3\text{PO}_4 )</td>
<td>200 mL glacial acetic acid</td>
</tr>
</tbody>
</table>

19
Four steps of N-NO₃⁻ measurement:

- Load 0.5 mL of sample into a test tube
- Add 50 µL of amide-sulfuric acid solution, 3.5 mL acid mixture, and 500 µL DMP solution
- Seal the tube and mix thoroughly
- After 10 minutes measure the absorbance at 360 nm with a photoLab® 6100 VIS spectrophotometer (WTW GmbH, Weilheim, DE)

2.3.2 Other parameters

Together with nitrogen species, other parameters were also measured: TSS, VSS, DO, ORP and COD. The procedure of measurement was adopted from Standard Methods.

2.3.2.1 Total suspended solid (TSS) and volatile suspended solid (VSS)

TSS is concentration of suspended solid in the water sample (unit: g/L).

It was measured step by step:

- Weight empty, dry filter (diameter of pores is 0.45 µm).
- Place the filter to vacuum filtration device and filtrate 50 mL of homogenized sample.
- After filtration, dry the filter with suspended solid at 105°C for 2 hours.
- After drying, cool down the filter paper and samples in desiccator.
- Weight of dry filter with solid material and calculate TSS.

VSS tells us about concentration of organic compounds in the sample.

It was measured step by step:

- Take dry filter with solid material from previous TSS measurement and place it to ceramic cup.
- Weight ceramic cup, add 4 drops of glycerin and put it in oven for 2 hours in 550°C.
- After heating and cooling, weight the cup and calculate the VSS.

2.3.2.2 Dissolved oxygen (DO), pH and oxidation-reduction potential (ORP)

ORP and pH are determined by InoLab® Multi 9420 IDS. The probes used for ORP and pH are from WTW Company which are SenTix 900 and SenTix 980, respectively.

For DO concentration, the instrument and probes are from Mettler Toledo Company with M200easy and probe EasySens O₂ 21.
2.3.2.3 COD and denitrification test

a. COD measurement

In this study, to investigate the activity of heterotrophs in reactor E, COD was checked with the procedure according to Standard Methods.

The procedure and reagents are different for high and low concentration of COD in samples.

*For high COD concentration*

Reagents:

- Oxidizing reagent – 20.4296 g K$_2$Cr$_2$O$_7$, 334 mL concentrated H$_2$SO$_4$ and 66.66 g HgSO$_4$ in 2 L volumetric flask fill by distilled water.
- Catalytic reagent – 10 g AgSO$_4$ in 1 L concentrated H$_2$SO$_4$.

The measurement was conducted step by step

- Test tube is filled with 2.5 mL of sample, 1.5 ml of oxidizing reagent and 3.5 mL catalytic reagent.
- Close and shake the test tube thoroughly. Test tube is then placed in mineralizator, for 2 hours in 150°C. After mineralization, let the temperature decrease to laboratory temperature.
- Test tube is then spectrophotometric evaluated. The wave length for evaluation is 600 nm.

*For low COD concentration*

Reagents:

- Oxidizing reagent – 1.022 g K$_2$Cr$_2$O$_7$, 167 mL concentrated H$_2$SO$_4$ and 33.33 g HgSO$_4$ in 1 L volumetric flask fill by distilled water.
- Catalytic reagent – 10 g AgSO$_4$ in 1 L concentrated H$_2$SO$_4$.

The procedure is the same as for high COD concentration but the test tube is spectrophotometric evaluated in 350 nm.

b. Denitrification test

Together with COD measurement, the matrices of reactor E was also put into denitrification test to investigate the potential for the growing of heterotrophic denitrifiers. The method was obtained from ICT Prague’s textbook, namely “Laboratory method in water technology” (Laboratorní metody v technologii vody) by Doc. Ing. Jana Zábranská et al.
In this experiment, the source of microorganisms (denitrifiers) was taken from the sludge from WWTP in Prague.

The feeding solutions for reactor E was tested as the substrate for denitrification (test 1). Besides, we also conducted the same procedure with acetate as organic substrates for denitrification (test 2). By comparing these 2 experiments, we can estimate the denitrifying activities in reactor E. The results of these tests are displayed in the amount of N-NO$_3^-$ removed per one gram of dry biomass in 1 hour (mg/(g.h)).

The procedure of denitrification test includes the preparation good living for conditions denitrifying bacteria.

- Add 1 liter of tested sludge to test cell (with dry biomass of 3 g/L),
- Add 3 mg of P-PO$_4^{3-}$, 15 mg of N-NH$_4^+$, 300 mg of COD (by the feeding solutions to reactor in test 1 and acetate in test 2) and 50 mg N-NO$_3^-$
- Take samples each 10 to 15 minutes for two hours. Keeping constant temperature (20°C) and pH value in range 7 and 7.3. Continuously input gas nitrogen for supporting anoxic condition in test cell.
- Samples are then evaluated after kinetic test, including N-NO$_2^-$, N-NO$_3^-$ and COD in each sample. Finally, make evaluation of results. Main parameter for evaluation is the drop of N-NO$_3^-$ concentration which is considered as the speed of denitrification.

2.4. Fluorescence in situ hybridization (FISH)

This internal protocol has been adapted from Amman (1995) in order to identify and quantify nitrifying microbes, including ammonia oxidizers (AOB), nitrite oxidizers (NOB) and anammox bacteria (AMX) by hybridization. The typical FISH protocol has four steps, including the fixation of the sample, hybridization of the targeted sequence to the probe, washing step to remove unbound probe, and the detection of labelled cells by microscopy.

The oligonucleotide probes were purchased from Generi Biotech (Hradec Králové, Czech Republic). The list of probes used in this study was displayed in table 2.7. Before using in FISH analysis, the stock probes need to be diluted with sterilized distilled water to 50 mM working solutions.

For observation the changing of the microbial community in 1 year of cultivation, the biomass was sampled from the reactor E almost every month from May 2013 to June 2014. After being taken from the reactor, all samples were fixed with paraformaldehyde (see section 2.4.3), and then stored in the freezer at -20°C.
2.4.1 Reagents and probes required in FISH

All the reagents and probes which were employed in the present study are introduced in this section. Below parts is information about reagents preparation for all steps of FISH.

To establish a baseline for kinetic test of microbial community, suspended solids was sampled from reactor E every month and subsequently fixed for hybridization. This result was then compared with inorganic nitrogen speciation from the previous chemical kinetic test. All samples were fixed with paraformaldehyde, stored at -20°C, and hybridized and observed via epifluorescent microscope.

<table>
<thead>
<tr>
<th>1 x Phosphate-Buffer-Saline (1xPBS)</th>
<th>3 x Phosphate-Buffer-Saline (3xPBS)</th>
<th>4% Paraformaldehyde (PFA)-PBS Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>• 8 g NaCl</td>
<td>• 24 g NaCl</td>
<td>• 66 mL ddH₂O (60°C)</td>
</tr>
<tr>
<td>• 0.2 g KCl</td>
<td>• 0.6 g KCl</td>
<td>• 4 g PFA</td>
</tr>
<tr>
<td>• 1.44 g Na₂HPO₄</td>
<td>• 4.32 g Na₂HPO₄</td>
<td>• 2-3 drops concentrated NaOH solution</td>
</tr>
<tr>
<td>• 0.2 g NaH₂PO₄</td>
<td>• 0.6 g NaH₂PO₄</td>
<td>• 34 mL 3 x PBS</td>
</tr>
<tr>
<td>• 1000 mL dH₂O</td>
<td>• 100 mL dH₂O</td>
<td>• pH 7-7.4 (HCl)</td>
</tr>
<tr>
<td>• pH 7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tris-HCl Buffer (1 M)</th>
<th>NaCl Stock (5 M)</th>
<th>SDS-solution (10%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>• 15.8 g Tris/HCl</td>
<td>• 29.2 g NaCl</td>
<td>• 10 g SDS</td>
</tr>
<tr>
<td>• 100 mL dH₂O</td>
<td>• 100 mL dH₂O</td>
<td>• 100 mL H₂Obidest</td>
</tr>
<tr>
<td>• pH 8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>EDTA (0.5 M)</th>
<th>Ethanol</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>• 18.6 g EDTA</td>
<td>• 50%</td>
<td></td>
</tr>
<tr>
<td>• 100 mL dH₂O</td>
<td>• 80%</td>
<td></td>
</tr>
<tr>
<td>• 96%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hybridization Buffer</th>
<th>Washing Buffer</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>• 360 mL NaCl (5M)</td>
<td>• 1000 µL Tris/HCl buffer</td>
<td></td>
</tr>
<tr>
<td>• 40 µL Tris/HCl buffer</td>
<td>• NaCl (5 M) (see table 2-6)</td>
<td></td>
</tr>
<tr>
<td>• PFA (see table 2-5)</td>
<td>• 500 µL EDTA (0.5 M)</td>
<td></td>
</tr>
<tr>
<td>• ddH₂O (see table 2-5)</td>
<td>• Dilute to 50 mL with dH₂O</td>
<td></td>
</tr>
<tr>
<td>• 2 µL SDS (10%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The amount of PFA and NaCl used for making hybridization and washing buffer in this thesis will be displayed in 2 below tables: table 2-5 and 2-6.

Table 2-5: Volume of PFA and ddH₂O by concentration of PFA in hybridization buffer

<table>
<thead>
<tr>
<th>Concentration of PFA in hybridization buffer[ % ]</th>
<th>PFA [μl]</th>
<th>ddH₂O [μl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>300</td>
<td>1300</td>
</tr>
<tr>
<td>20</td>
<td>400</td>
<td>1200</td>
</tr>
<tr>
<td>25</td>
<td>500</td>
<td>1100</td>
</tr>
<tr>
<td>30</td>
<td>600</td>
<td>1000</td>
</tr>
<tr>
<td>35</td>
<td>700</td>
<td>900</td>
</tr>
<tr>
<td>40</td>
<td>800</td>
<td>800</td>
</tr>
<tr>
<td>60</td>
<td>1200</td>
<td>400</td>
</tr>
</tbody>
</table>

Table 2-6: Volume of NaCl for washing buffer by concentration of PFA in hybridization buffer

<table>
<thead>
<tr>
<th>Concentration of PFA in hybridization buffer</th>
<th>Volume of NaCl in washing buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>15%</td>
<td>3180 μl</td>
</tr>
<tr>
<td>20%</td>
<td>2150 μl</td>
</tr>
<tr>
<td>25%</td>
<td>1490 μl</td>
</tr>
<tr>
<td>30%</td>
<td>1020 μl</td>
</tr>
<tr>
<td>35%</td>
<td>700 μl</td>
</tr>
<tr>
<td>40%</td>
<td>460 μl</td>
</tr>
<tr>
<td>60%</td>
<td>60 μl</td>
</tr>
</tbody>
</table>

List of probes employed in this thesis with their target bacteria, formamide concentrations and applied fluorophores will be shown in table 2-7. The total biomass is stained by DAPI (4',6-diamidino-2-phenylindole) right before the imaging process by epifluorescence microscope. It displays a blue color under the microscope.
Table 2-7: The oligonucleotide probes employed in this study (from Nielsen et al. 2009)

<table>
<thead>
<tr>
<th>Group</th>
<th>Probes</th>
<th>Target</th>
<th>Formamide</th>
<th>Florophore</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOB</td>
<td>Nso1225</td>
<td>Betaproteobacterial AOB</td>
<td>35%</td>
<td>Cy3</td>
<td>(Mobarry et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>Nso190</td>
<td>Betaproteobacterial AOB</td>
<td>35%</td>
<td>Cy3</td>
<td>(Mobarry et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>Ntspa712</td>
<td>Phylum Nitrospirae</td>
<td>35%</td>
<td>Cy3</td>
<td>(Daims et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>cNtspa712 (Competitor)</td>
<td>Ntspa712 non-target organisms</td>
<td>35%</td>
<td>None</td>
<td>(Daims et al., 2001)</td>
</tr>
<tr>
<td>NOB</td>
<td>Ntspa662</td>
<td>Genus Nitrospira</td>
<td>35%</td>
<td>Cy3</td>
<td>(Daims et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>cNtspa662 (Competitor)</td>
<td>Ntspa662 non-target organisms</td>
<td>35%</td>
<td>None</td>
<td>(Daims et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>NIT3</td>
<td>Genus Nitrobacter</td>
<td>40%</td>
<td>Cy3</td>
<td>(Wagner et al., 1996a)</td>
</tr>
<tr>
<td></td>
<td>cNIT3  (Competitor)</td>
<td>NIT3 non-target organisms</td>
<td>40%</td>
<td>none</td>
<td>(Wagner et al., 1996a)</td>
</tr>
<tr>
<td>AMX</td>
<td>Amx368</td>
<td>All anammox bacteria</td>
<td>15%</td>
<td>Cy3</td>
<td>(Schmid et al., 2003b)</td>
</tr>
<tr>
<td></td>
<td>Kst157</td>
<td>K. stuttgartiensis</td>
<td>25%</td>
<td>Cy3</td>
<td>(Schmid et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>Amx1015</td>
<td>B. anammoxidans</td>
<td>60%</td>
<td>Cy3</td>
<td>(Schmid et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>Bfu613</td>
<td>B. fulgida</td>
<td>30%</td>
<td>Cy3</td>
<td>(Kartal et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Apr820</td>
<td>A. propionicus</td>
<td>40%</td>
<td>Cy3</td>
<td>(Kartal et al., 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------</td>
<td>-------------</td>
<td>----------</td>
<td>----------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BS820</td>
<td><em>S. wagneri</em></td>
<td>40%</td>
<td>Cy3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scabr1114</td>
<td><em>S. brodae</em></td>
<td>20%</td>
<td>Cy3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.4.2 Equipment and supplies required for FISH

- Water bath (46°C)
- Incubator (48°C)
- Epoxide-coated glass slides separating 8 or 10 reaction fields (wells)
- Epifluorescent microscope equipped with the proper filters

2.4.3 Fixation with Paraformaldehyde

The fixation of samples were carried out step by step

- Collect samples randomly from the reactor and place in 2 mL Eppendorf tubes
- Centrifuge samples to separate activated sludge and supernatant, and then remove the supernatant. Repeat until getting enough biomass for investigation.
- Add 1.5 mL of 4% PFA-PBS solution
- Mix by shaking thoroughly, and then keep the sample in the refrigerator (at 4°C) for 4 hours.
- Remove and discard supernatant in PFA waste container.
- Add 0.5 mL of 1xPBS washing buffer and 0.5 mL of 96% Ethanol. Then, invert the tube several times to mix.
- Repeat the upper washing step 2 additional times.
- Add 0.5 mL of 1xPBS and 0.5 mL of 96% Ethanol (not denatured) into the tube
- Vortex to homogenize and the store the sample in the freezer at -20°C.
- After fixation, samples were ready for FISH analysis.

2.4.4 Hybridization

2.4.4.1 Immobilization

The samples need to be immobilized on the glass slides as preparing for FISH analysis:

- Place a 5-15 µl of the sample in each well in an epoxide-coated slide.
- Try to spread out the sample by using the pipette tip and then dry it in the incubator (48°C).
2.4.4.2 Dehydration

Dehydration of the immobilized sample can remove water from the sample in order to increase the resolution during microscopy. It can be done by serial increasing ethanol treatment:

- Firstly, dip the slide with sample in 50% Ethanol for 3 minutes
- Repeat step 2 using 80% Ethanol
- Repeat step 3 using 96% Ethanol
- Remove the supernatant and incubate the sample slides at 46°C for 20 minutes (or until it is dry)

2.4.4.3 Hybridization

The hybridization of the oligonucleotide to the ribosome in the target cells must be carried out under high stringency with proper formamide concentration:

- Add 8 µL of hybridization buffer into each well. The slide should be prepared one by one to avoid the evaporation of hybridization buffer.
- Add 1 µL of each gene probe and equal volume of complementary competitor probe (if needed) and mix gently by the pipette tip.
- Place the slide horizontally in to a 50 mL polyethylene tube with a piece of tissue wetted with 1-2 mL of hybridization buffer.
- Incubate the tube at 46°C for 3 hours.
- Remove the tube from incubator.

2.4.4.4 Washing

This step is for removing the unnecessary probes which remain after hybridization

- During the hybridization, prepare the washing buffer in a 50 mL polyethylene tube. Then, preheat the washing buffer in a 48°C water bath.
- After hybridization, rinse the slide in the washing tube gently 3 times. Put the slide into the washing buffer tube and place the tube in hot water bath at 48°C for 20 minutes.
- Remove the tube from hot water bath and then the slide from the washing buffer.
- Rinse the slide with cold distilled H₂O gently and wait until it dry
- Store the slide at 4°C for no more than 2 weeks to the imaging step

2.4.5 Imaging

Fluorescence detection was carried out with an Olympus BX51-RFAA microscope, images were captured with an Olympus MX10 CCD Camera using the fluorescence imaging software CELLF all products of Olympus Corporation (Tokyo, Japan). Image manipulations including: automatic white balance, manual brightness and contrast adjustment, and auto color sharpening were
carried out using GNU Image Manipulation Program (GIMP) version 2.8 (GNU Development Team, Berkeley, CA USA).

For quantifying the bio-volume of targeted microorganisms in next step, 30 random photos of each well in the slides was taken. The magnificence is 320 times for almost the analyzed pictures.

2.4.6 Image analysis

In this thesis, DAIME (acronym for Digital image Analysis In Microbial Ecology) was used for image analysis. This program has been written for analyzing the image obtained by FISH and other fluorescence techniques (Nielsen et al., 2009). In this program, by measuring the areas of the probe-stained cells, it helps counting and summing up the pixels that belong to these cells in each image. The process includes 2 steps:

- Segmenting the images to distinguish objective cells from the background.
- Counting the pixels belong to the objects and then quantifying the percentage of object in the biomass (with option biovolume fraction).
CHAPTER 3: RESULTS AND DISCUSSION

3.1 Performance of reactor E in stage 1

3.1.1 Reactor performance by chemical kinetic tests

3.1.1.1 Nitrogen forms

The start-up period (stage 1) of the studying reactor E was within 36 weeks from May 2013. In this stage, anammox bacteria needed time to adapt the new environment and they grew up very slowly. Another point to consider is possible diffusion of oxygen into the system. This is a critical factor especially during the initial period of enrichment since anammox activities are inhibited by even low DO concentrations (0.5 mg/L) and the anammox bacterial community could be destroyed by it. Therefore, the stabilization of reactor E for anaerobic condition was very important. Especially, anammox biomass was later protected by heterotrophic biomass which also developed and could consume most of DO in reactor. Besides, because of the changing of the environment of the biomass, from one-stage anammox process (partial nitritation and anammox in one reactor) of Dr. Jan Bartacek’s experiment to anammox process in 2-stage SBR (including the partial nitritation process before) in this study, the microbes needed time to adapt the new living conditions.

As we can see, in first 3 weeks only NH$_4^+$ was consumed, concentration of NO$_2^-$ and NO$_3^-$ increased slightly. However, in week 4, proper nitrogen removal was obtained. We can observe the consumption of both NH$_4^+$ and NO$_2^-$ (figure 3.1)

After 10 weeks of operation, an appreciable consumption of NH$_4^+$ and NO$_2^-$ was observed in the system (58 and 77 mg/L of NH$_4^+$ and NO$_2^-$, respectively) which can be considered as the indication for anammox activity. Together with anammox, aerobic nitrification was also indicated in this period because of the elevation of NO$_3^-$ concentration. By FISH, we can confirm all the nitrification activities (see details in section 3.1.2).

From week 4 to week 26, almost influent nitrogen compounds were removed from the wastewater with the removal efficiencies ranged from 85% to 98% for N–NH$_4^+$ and 73% to 100% for N–NO$_2^–$. As all NO$_2^–$ was consumed by the bacteria, nitrite became the limiting nutrient and anammox microorganisms could be at a growth rate lower than the maximum specific one. In order to supply efficient nutrients for biomass growth, their concentrations in the feed were increased stepwise by the experimental strategy (to 100 mg/L in week 11, to 150mg/L in week 26 and then to 200 mg/L in week 28). Compared to week 11, in week 26, nitrate rise became slighter, so we could experience the weakness of nitratation and then of NOB which catalyze this process (confirmed by FISH in section 3.1.2).
However, in week 28, after increasing the nitrogen concentration to 200 mg/L directly from 150 mg/L (in week 26), the disturbance happened in the reactor. Another mistake in this stage is the removal of suspended solids in week 20 which could later assist the nitrite inhibition effect. The fluctuation of nitrogen removal indicated that the instant rise of nitrite was not suitable for running lab-scale anammox reactors, which could result the drop in anammox activity. Learning from this experience, the nitrite concentration was then increased gradually in stage 2 (Enrichment). This information is valuable for the future operation of full-scale reactors in
wastewater treatment plant, in which the operational problem by nitrite concentration is one of the obstacles. The nitrite inhibition on anammox activity will be discussed in section 3.1.4.

After the nitrite inhibition, we had to decrease the nitrogen loading and during last 8 weeks of the first stage, reactor E worked inappropriately and it led the low efficiency of nitrogen removal (below 50%). Fortunately, in this period, reactor also took time for recovery and we turned to stage 2 with better experience for dealing with lab-scale Anammox-SBR. Therefore, in stage 2, the suspended solids would not be removed and the nitrogen load to reactor E was increased gradually with better investigation.

3.1.1.2 TSS and VSS

In stage 1, TSS and VSS were measure for investigation of concentration of activated sludge (TSS) and its organic fraction (VSS).

Figure 3.3 shows the correlation between TSS and anammox bacteria development. Therefore, it revealed the support of TSS for anammox biomass growing. In week 20, an amount of suspended solid was removed which led to the decrease of TSS. Besides, it later assisted the nitrite inhibition so a sharp declination of anammox bacteria biovolume occurred in reactor E. This problem will be discussed in details in section 1.4 (nitrite inhibition on anammox activity).

![Figure 3.3: Relationship between TSS and AMX quantification in stage 1 by FISH](image)

About VSS, the results exhibited a significant rise of organic proportion of sludge, from 24.6% (in week 1) to 74 % (in week 20). After removal of suspended solid, the percentage of organic content rocketed to 95%.

3.1.2 Quantification of AOB, NOB and anammox bacteria by FISH

As mentioned in the previous section, the quantitative FISH results could be applied to validate the kinetic test. To detect and quantify most of the AOB in the samples at once, the probe mixture consisting Nso1225 and Nso190 was used. Besides, for NOB quantification, we used the
combination of 3 probes (Ntspa712, Ntspa662 and NIT3) and their competitor oligonucleotides. Finally, probe Amx368 was used to target all anammox bacteria. The details of probes can be checked in table 2.17. For verifying our assumption taken from kinetic tests, 30 FISH images of each targeted microbes were taken and analyzed to determine the percentage of AOB, NOB and AMX to the total bacteria in the biomass. The quantification results of FISH analysis (by DAIME) will be summarize in figure 3.4.

<table>
<thead>
<tr>
<th>Week</th>
<th>AOB (%)</th>
<th>NOB (%)</th>
<th>AMX (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Free</td>
<td>Free</td>
<td>5.5</td>
</tr>
<tr>
<td>8</td>
<td>8.25</td>
<td>9.5</td>
<td>0.7</td>
</tr>
<tr>
<td>14</td>
<td>6.3</td>
<td>15.3</td>
<td>2.6</td>
</tr>
<tr>
<td>20</td>
<td>8.75</td>
<td>8.3</td>
<td>4.9</td>
</tr>
<tr>
<td>23</td>
<td>6.2</td>
<td>7.6</td>
<td>4.0</td>
</tr>
<tr>
<td>29</td>
<td>9.6</td>
<td>5.53</td>
<td>6.2</td>
</tr>
<tr>
<td>36</td>
<td>17</td>
<td>4.5</td>
<td>Free</td>
</tr>
</tbody>
</table>

(*Free: FISH analysis showed the free cells of targeted organisms. However, it was impossible to quantify the amount of free cells properly but we could estimate the amount is less than 2%)

**Figure 3.4:** Biovolume fraction of nitrifiers in stage 1

By the chemical kinetic tests, we assumed the co-operation of both aerobic and anaerobic nitrification in the first stage, with the efficient removal of N–NO$_2^-$ and N–NH$_4^+$ (proof for anammox process) and the increase of N–NO$_3^-$ (proof of nitrification, nitratation and part of anammox process). Then, FISH results, which showed the presence of AOB, NOB and AMX, verified the hypothesis we delivered before. The details of FISH results will be discussed more in next paragraphs.

From week 1 to week 8, according to chemical kinetic test result of nitrogen, we could see the transformation of ammonium and nitrite to nitrate. It brings a possibility that due to the aerobic nitrification, AOB and NOB worked strongly for catalyzing the nitritation and nitratation, respectively. From the table of FISH analysis result (**figure 3.4**) before the reactor operation, the biomass in reactor E contained 5.5% of AMX, while AOB and NOB were only free cells in the microbes community which accounted for less than 2% (estimation). However, after 8 weeks of operation, the amount of AMX decreased significantly (from 5.5% to less than 1%) while AOB and NOB increased sharply (to 8.25% and 9.5%, respectively). By this way, FISH analysis confirmed the information which was assumed before from kinetic tests. In **figure 3.5**, we can observe the alteration of nitrifiers between week 1 and week 8 of reactor operation.
Week 1, AOB (Cy3, red), 320x, infrequent free cells
Week 1, NOB (Cy3, red), 320x, very few free cells
Week 1, AMX (Cy3, red), 320x, good signals, small and medium clusters

Week 8, AOB (Cy3, red), 320x, strong signals, high-density small clusters
Week 8, NOB (Cy3, red), 320x, strong signals, high-density small clusters
Week 8, AMX (Cy3, red), 320x, infrequent free cells, weak signal

*Note: Representative images used for the illustration, targeted bacteria are shown in red (Cy3) and the total biomass (DAPI) in blue.

Figure 3.5: FISH images to illustrate the changing of nitrifiers from week 1 to week 8

The break-down of anammox bacteria could be explained by the presence of the dissolved oxygen and the inhibition of nitrite in the reactor E (read section 3.1.4). Besides, the initial condition of reactor E was proper for aerobic nitrifying bacteria (including AOB and NOB) to grow up. Furthermore, low activity of heterotrophs (indicated by low amount of biomass) could not help to decrease DO concentration as the expectation. In week 14, AOB and NOB occupied nearly 25% of the bacterial community, in which, there was 15.3% of NOB. Thus, it can explain the high concentration of nitrate in the effluent. On the other hand, anammox bacteria accounted for less than 3% which played a minor role in this period.

After week 14, we noticed the decline of NOB, stability of AOB and extension of AMX (from week 20 to week 29). It explained the presence of anammox activity and weakness of nitrification process in this period (as mentioned before in kinetic tests). As the result, we can say that anaerobic condition for anammox was obtained. The collapse of NOB could be explained by low DO concentration. Besides, the increasing activity of heterotrophs could lead to reduction of DO and then NOB biovolume. However, long non-aeration periods have a stronger effect on NOB than AOB so biomass of AOB was stable (Mota et al., 2005). Besides, many studies found the
possibility for AOB and AMX to grow concomitantly in a single SBR if oxygen levels are low (below 0.5mg/L of DO concentration).

However, after week 28, because of the nitrite inhibition (read section 3.1.4), anammox activity and nitrogen removal was badly affected. To prove what we experienced from kinetic test, FISH results display a significant reduction of AMX but sharp increase of AOB (to 17%) (See more in figure 3.4). Combination of kinetic test and FISH concludes the failure of anammox process and strength of nitritation in the end of stage 1. This conclusion could be generally explained by 3 points:

1. Nitrite inhibition on anammox activities after sudden rise of nitrite nitrogen from 150 to 200 mg/L in week 28.
2. The removal of suspended solids in week 20 could make the bacteria be more sensitive to the inhibition.
3. Anammox bacteria are more sensitive than AOB to the change of the living conditions.

![Figure 3.6: FISH images to illustrate the nitrite inhibition on anammox bacteria from week 20](image)

**Figure 3.6**: FISH images to illustrate the nitrite inhibition on anammox bacteria from week 20

### 3.1.3 Anammox bacteria species identification by FISH

Together with quantification of AOB, NOB, and total anammox bacteria, other probes were also used in FISH to identify the presence of anammox species. Information about name of anammox species with their targeted probes and description of their population in reactor E are displayed in table 3-1.
Table 3-1: Identification of AMX species by FISH in stage 1

<table>
<thead>
<tr>
<th>Week</th>
<th>B. anammoxidans</th>
<th>B. fulgida</th>
<th>K. stuttgartiensis</th>
<th>S. brodae</th>
<th>S. wagneri</th>
<th>A. propionicus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Amx1015</td>
<td>Bfu613</td>
<td>Kst157</td>
<td>Scabr1114</td>
<td>BS820</td>
<td>Apr820</td>
</tr>
<tr>
<td>8</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>Free cells</td>
<td>Free cells</td>
<td>−</td>
</tr>
<tr>
<td>14</td>
<td>−</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>20</td>
<td>−</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>23</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>29</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>Free cells</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>36</td>
<td>Free cells</td>
<td>+</td>
<td>Free cells</td>
<td>Free cells</td>
<td>+</td>
<td>Free cells</td>
</tr>
</tbody>
</table>

*Scale of amount: −: no detection; +: few; ++: medium; +++: dominant
*Free cells: FISH analysis showed the scattered distribution of targeted organisms, not in clusters

In the beginning, FISH images displayed the presence of 3 species in anammox bacteria community, including *B. fulgida, S. wagneri,* and *A. propionicus.* However, from week 1 to week 8, parallel to the reduction of total anammox bacteria, there was the disappearance of *A. propionicus* and the number of other species also declined. Especially, *Candidatus S. wagneri* was broken down from clusters to free cells. It was the signal of the negatively changing in condition of living environment for bacteria.

After week 8, anammox bacteria adapted the condition and then grew up slowly. We could experience the great development of *Candidatus B. fulgida* and *Candidatus A. propionicus.* In week 20, *Candidatus B. fulgida* which stained with Bfu613 probe formed high-density clusters became the dominant species among anammox bacteria community in reactor E. Besides, the good signal of *S. wagneri* indicated its presence in the community.

As mentioned in chapter 1 (this thesis), anammox bacteria used to be considered as absolute chemolithoautotrophs. However, recent studies have shown that they have the versatile metabolism. In addition to the anaerobic oxidation of ammonium, they can also use organic acids (such as acetate and propionate) as electron donors. In the study of Kartal (2008), some insights about *B. fulgida* was explored. The enrichment of anammox bacteria in the presence of acetate showed that among the tested biomass, the biomass from “*Candidatus Brocadia fulgida*” enrichment culture oxidized acetate at the highest rate. This result showed that “*Candidatus
Brocadia fulgida” out-competed other anammox bacteria in the existence of acetate, ammonium, nitrite and nitrate. Besides, “Candidatus Anammoxoglobus propionicus”, was shown to out-compete other anammox bacteria as well as heterotrophic denitrifiers for propionate as the supplementary electron donor in the presence of ammonium. This species could oxidize organic acid at high rate and then get an advantage to compete other genera for nitrite in the presence of propionate. Thus, it could be applied for treatment of nitrogen and/or COD containing wastewater.

These propositions are appropriate with reactor E’s condition which contains all 3 nitrogen forms and medium COD. However, a further investigation on acetate and propionate (organic acids) might be useful to draw a firmer conclusion.

On the other hand, from week 23, because of the nitrite inhibition which was mentioned in the kinetic test, the disintegration of anammox bacteria community underwent in the reactor E. Therefore, until week 36, anammox bacteria were too weak to shape the clusters and the signals of targeted anammox species were only infrequent free bacteria and weak small clusters.

### 3.1.4 Nitrite inhibition on anammox activities

As mentioned in the results of kinetic tests, in this section, more information about nitrite inhibition and recommendation for the 2\(^{nd}\) stage of reactor E operation is presented.

During the beginning of the experiment, the input N-NO\(_2^-\) concentration was raised by a step of 50 mg/L. At low concentrations (50-100 mg/L), this strategy did not cause the fluctuation. On week 28, when nitrite concentration increased to 200 mg N/L directly from 150 mg/L, the disturbance happened in the reactor. The fluctuation of nitrogen removal indicated that the instant increase of nitrite was not suitable for running lab-scale anammox reactors, which could result the drop in anammox activity. Since then, the nitrite concentration was increased gradually in stage 2 (Enrichment). This information is valuable for the future operation of full-scale reactors in wastewater treatment plant, in which the operational problem is one of the obstacles. Another important point is the biomass should be kept stable in the reactor because the removal of suspended biomass could change the composition of microorganisms which already adapted to reactor conditions after a period of operation.

The fluctuation of anammox bacteria affected nitrite removal more significantly than ammonium removal, resulting in more decreasing in nitrite removal. To explain for this result, we need to look back the FISH results. Bacteria quantification by FISH indicated that heterotrophic denitrifiers, autotrophic aerobic nitrifiers (AOB and NOB) and anammox microorganisms coexisted in the community (more details in section 3.1.3). Especially, AOB rocketed to 17% in week 36 which made them become the dominant species in reactor E after the disturbance. During the fluctuation period, AOB could oxidize ammonium to nitrite and compensate for the loss of ammonium removal from anammox activity. This could be responsible for the above phenomena. Besides, anammox bacteria were more sensitive to the inhibition than AOB and the
removal of suspended biomass before could assist nitrite inhibition as the biomass was overloaded by nitrogen substrate, especially nitrite nitrogen.

In summary, reactor condition was more preferable for AOB than anammox bacteria in this period. In figure 3.7, the failure of nitrogen removal after nitrite inhibition (in week 28) is clearly showed.

![Figure 3.7: Nitrogen removal efficiency in stage 1](image)

Nitrogen removal efficiency declined when input NO$_2^-$ concentration was extended to 200 mg/L on week 28. Similar phenomena were observed in several studies (table 3-2). In this thesis, the fluctuation of nitrogen removal efficiency could be the result from a sudden shock of nitrite concentration. Besides, this may be caused by the toxic effects of ammonium concentration. However, usually nitrite concentrations above 100 mg/L would inhibit anammox activity, while the value was much higher for ammonium, i.e. 50 mM (Dapena-Mora et al., 2007). In this thesis, the disturbance was marked when influent N–NH$_4^+$ concentration increased to only 200mg/L. Hence, ammonium was not the potential inhibitor, because the concentration of ammonium on week 28 was far lower than the inhibition values which were found in other study. According to previous researches, nitrite is absolutely the inhibitor, since its toxicity to anammox bacteria was well accepted.

After the nitrite inhibition, anammox activities could be recovered by hydrazine and hydroxylamine addition (Strous et al., 1999b). Since anammox process produced hydrazine/hydroxylamine continuously, without addition of any chemicals, the lab-scale reactor in this study was recovered by stopping increasing influent loading rates and changing the feed strategy (decreasing sharply and then increasing slowly in stage 2). This strategy was proved to be successful although it took long time for the disturbance to stop (in 2 months). Furthermore, no fluctuation happened again after the recovery (in stage 2), even later the influent ammonium and nitrite concentrations increased to above 100 mg/L. Furthermore, in stage 2, the suspended biomass would never be removed.
Table 3-2: Comparison between literature data of nitrite inhibition on the anammox bacteria and the experiment results of this study

<table>
<thead>
<tr>
<th>Reference</th>
<th>Nitrite concentration</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Strous et al., 1999b)</td>
<td>100 mg/L</td>
<td>Completely inhibition</td>
</tr>
<tr>
<td>(Jung et al., 2007)</td>
<td>70 mg/L</td>
<td>Activity decrease</td>
</tr>
<tr>
<td>(López et al., 2008)</td>
<td>100 mg/L</td>
<td>Completely inhibition</td>
</tr>
<tr>
<td>(Bettazzi et al., 2010)</td>
<td>60 mg/L</td>
<td>Activity decrease</td>
</tr>
<tr>
<td>(Cho et al., 2010)</td>
<td>30 mg/L (long exposure)</td>
<td>Activity decrease</td>
</tr>
<tr>
<td>(Bettazzi et al., 2010)</td>
<td>250 mg/L</td>
<td>Completely inhibited</td>
</tr>
<tr>
<td>This thesis</td>
<td>200 mg/L</td>
<td>Inhibited</td>
</tr>
</tbody>
</table>

3.2. Performance of reactor E in stage 2

3.2.1 Reactor performance by chemical kinetic tests

3.2.1.1 Nitrogen forms

The second stage (Enrichment) of reactor E lasted for 16 weeks from the middle of January 2014 (from week 36 to week 52 of the whole experiment time). The figure 3.8 generalizes the performance of reactor E during this stage. In this stage, by experiences getting from the last stage, the nitrogen loading rate was increased gradually.

![Figure 3.8](image-url)

Figure 3.8: Performance of reactor E during the second stage of this study
In first 5 weeks (from week 36 to week 40), the concentration of N-NH$_4^+$ and N-NO$_2^-$ ranged from 30 to 50 mg/L. In week 40, all the ammonium and nitrite was removed from the feed. Therefore, we started to increase stepwise the concentration of the input N-NH$_4^+$ and N-NO$_2^-$. In the end of stage 2, the nitrogen feeding is 100 mgN-NH$_4^+$/L and 100 mgN-NO$_2^-$/L and. After this time, the efficient nitrogen treatment was obtained. In details, the efficiency was from 82% to 98% for ammonium removal and from 90% to 100% for nitrite removal (figure 3.9).

![Figure 3.9](image)

**Figure 3.9:** Nitrogen removal efficiency in stage 2

About nitrate compounds, its concentration was relatively stable in stage 2. It indicates the low potential of nitratation which was then confirmed by low amount of NOB in the total biomass by FISH analysis. It showed that there was low concentration of DO which did not allow NOB to develop in reactor E.

### 3.2.1.2 TSS and VSS

In stage 2, the TSS concentration increased from 0.067 g/L at week 36 to 0.838 mg/L at week 52. It increased by the rise of influent N-NH$_4^+$ and N-NO$_2^-$ concentration. The TSS concentration which obtained from reactor E poses quite similarity to other anammox SBRs in regards with experiment duration (*table 3-3*).

<table>
<thead>
<tr>
<th>Duration of experiment</th>
<th>TSS (g/L)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 days</td>
<td>0.354</td>
<td>(Fux et al., 2002)</td>
</tr>
<tr>
<td>365 days</td>
<td>2.1</td>
<td>(López et al., 2008)</td>
</tr>
<tr>
<td>112 days</td>
<td>0.067–0.838</td>
<td>This thesis</td>
</tr>
</tbody>
</table>
The growth of the anammox biomass can hardly be determined by the TSS concentration in the reactor because of the possibility of solids and biomass accumulation from the raw digested liquor which was used for feeding solution. Besides, heterotrophs and organic compounds accounted for a large portion in the biomass. However, we can see the correlation of anammox bacteria amount with TSS (figure 3.10). It clearly shows that the growing of anammox bacteria will lead to the elevation of TSS concentration.

Furthermore, together with TSS, we also measured VSS in reactor E. The result shows the percentage of organic compounds in suspended solids was relatively high (the average value is around 80%).

![Figure 3.10: Relationship between TSS and AMX biovolume fraction in stage 2](image)

**3.2.1.3 ORP and DO concentration**

Oxidation Reduction Potential (ORP) or Redox is an electrochemical parameter that shows the activity of oxidizers and reducers in respect to their concentration. Positive values of ORP show that oxidizing substances (the ones that attract electrons or so-called electron acceptors) are dominant in the analyzed environment and negative values shows that reducing conditions (so-called electron donors) prevail. In the section of wastewater treatment, ORP can be used in investigating biological nutrient removal, such as nitrogen removal.

Classical biological nutrient removal systems based on activated sludge process consists of anaerobic, anoxic and aerobic stages. When oxidation conditions of aerobic stage can be controlled using DO parameter, its application for control of anoxic and anaerobic stages is not possible because of low levels of DO. In this case ORP can be more efficient because it has both positive and negative values. Therefore, in anaerobic environments where DO shows very little or no change, ORP may significantly change its value to response of alternative biochemical conditions (Holman and Wareham, 2003). In this study, application of ORP could be used as a control tool for anammox process when dissolved oxygen (DO) in reactor was close to zero and the results are discussed in more detail further.
ORP sharply decreases during the experiment of stage 2. This can be explained by the facts that DO has strong influence on ORP, decreasing DO concentration could lead to decreasing ORP. However, after addition of nitrite in the influent, ORP could increase because of increasing DO which came together with added nitrite solution. However, together with nitrite, nitrogen substrates also contain ammonium nitrogen which can decrease ORP. In our study, In the beginning of each batch (after feeding the reactor E with nitrite and ammonium solutions), the ORP value tended to be the lowest and then elevated during the reaction.

Measuring ORP can serve as the signal of anammox reaction only when DO is very close to zero, which could not make an observable changing. In our experiment, although DO concentration did not change so much during the time (always between 0 to 0.5 mg/L), ORP had the alteration during the time.

![Figure 3.11: Relationship between ORP value and AMX biovolume fraction](image)

The figure 3.11 shows the relationship of ORP and AMX biovolume fraction (obtained from FISH) which clearly points out that anammox bacteria developed strongly by the decreasing of ORP value. From previous study, ORP value of smaller than -50 mV is found appropriate for stable nitrogen removal in the anammox process (Viet and Kim, 2008). By the end of stage 2, in week 54, ORP value in reactor E increased from -90 mV to -52 mV which showed that, the condition is appropriate for the growing of anammox bacteria.

### 3.2.1.4 COD removal and denitrification test

In week 52 and week 54, together with other parameters, COD was also measured daily in one batch. The result of COD in these 2 weeks is displayed in table 3-4. As the result, we can see the decrease of COD during one batch. The COD removal was obtained by the activity of heterotrophs in reactor E which accounted for almost 50% of total biomass (in week 52). Besides, the activites of heterotrophs was also useful for anammox bacteria as they could consume DO and decrease ORP value.
Table 3-4: COD in reactor E in week 52 and 54

<table>
<thead>
<tr>
<th>Time</th>
<th>COD (mg/L)</th>
<th>Week 52</th>
<th>Week 54</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influent</td>
<td>352</td>
<td></td>
<td>490</td>
</tr>
<tr>
<td>Day 1</td>
<td>245</td>
<td></td>
<td>316</td>
</tr>
<tr>
<td>Day 2</td>
<td>182</td>
<td></td>
<td>234</td>
</tr>
<tr>
<td>Day 3 (effluent)</td>
<td>122</td>
<td></td>
<td>181</td>
</tr>
</tbody>
</table>

Besides, because the denitrification and denitrifiers depend on organic substrates we also conducted denitrification test to evaluate the potential activity of heterotrophic denitrifiers. By conducting 2 parallel test, with the matrices (feeding solution) of reactor E (test 1) and acetate (test 2) as the substrates, we had the benchmark to estimate the possibility of denitrification of reactor T supported by input solutions since acetate is easy for denitrifying heterotrophs to consume (see table 3-5). In other way, it was the experiment to check if the substrate in reactor E was good for the living of denitrifiers.

Table 3-5: Denitrification test for investigating the potential activities of denitrifiers supported by organic substrates in reactor E

<table>
<thead>
<tr>
<th>Time</th>
<th>Denitrification speed by acetate (mg N-NO₃₋/(g.h))</th>
<th>Denitrification speed by reactor E’s matrices (mg N-NO₃₋/(g.h))</th>
</tr>
</thead>
<tbody>
<tr>
<td>March 26th 2014</td>
<td>8.51</td>
<td>2.36</td>
</tr>
<tr>
<td>April 29th 2014</td>
<td>8.86</td>
<td>1.73</td>
</tr>
<tr>
<td>Average</td>
<td>8.685</td>
<td>2.045</td>
</tr>
</tbody>
</table>

As the result, we can approximate that the matrices of reactor E was quite appropriate for denitrifying activity. It was proved by the reduction of COD concentration during a batch and from FISH analysis, the heterotrophs constituted almost 50%.

3.2.2 Quantification of AOB, NOB and anammox bacteria by FISH

Same as stage 1, in stage 2, suspended biomass samples were taken for FISH analysis to confirm nitrifying activities which were mentioned from previous chemical kinetic tests. The figure 3.12 displays the development of nitrifiers (indicated by biovolume fraction in total biomass), including AOB, NOB and anammox bacteria during stage 2.
(*Free: FISH analysis showed the free cells of targeted organisms. However, it was impossible to quantify the amount of free cells properly but we could estimate the amount is less than 2%)

**Figure 3.12:** Biovolume fraction of nitrifiers in stage 2

In stage 2, because anammox bacteria already adapted the condition in the reactor so their biovolume increased slowly by the time. NOB decreased because of the anaerobic condition, and so did AOB. In this stage, because the DO concentration was almost zero, AOB cannot survive in this stage like the previous one which DO concentration is very low but high enough for the survival of AOB. Therefore, ORP should be measured for investigation of the reactor condition (see section 3.2.1.3).

**Figure 3.13** shows the increase in influent ammonium and nitrite concentration and the resulting elevation of AMX biovolume fraction in total biomass. From week 48, when input NH$_4^+$ and NO$_2^-$ concentration rose to about 100 mg/L, anammox bacteria reached 14% and became the dominant species in this period. It showed the good correlation between nitrogen load and the anammox biomass growth. On the other hand, AOB and NOB were only free cells among the community and weakly active.

**Figure 3.13:** Relationship of AMX development with nitrogen loading concentration
However, in week 52, AOB suddenly became dominant in reactor E which accounted for 33%. Beside, anammox bacteria were also abundant with constitution of almost 19% in the total biomass. In figure 3.14, the FISH images of AOB, NOB and anammox bacteria is displayed for a better view of microbial community in reactor E in week 52.

When comparing the anammox growth in stage 2 with stage 1, we could experience the more gradual development of them after the reactor E reaching the stable condition for AMX enrichment. The better result confirmed the experiences we got from stage 1, including gradually changing of nitrogen load and avoidance of removing suspended biomass.

![FISH images of nitrifying bacteria community in week 52](image)

**Figure 3.14:** FISH images of nitrifying bacteria community in week 52

In reactor E, 10 plastic carriers were used to enhance the biomass retention. In last weeks of stage 2, together with suspended solids, biomass which was on the surface of plastic carriers was also analyzed by FISH. The result is displayed in [table 3-6].

Anammox biomass on the surface of plastic carrier tended to aggregate and form a small granule. By this way, they were hardly washed out of the reactor and the biomass retention will be improved. Besides, the anammox granules spread from the surface of carriers to suspended biomass and further grew up there (figure 3.16).

**Table 3-6:** Quantification of AOB, NOB and anammox bacteria in total biomass on the surface of carrier by FISH

<table>
<thead>
<tr>
<th>Biovolume fraction in total biomass (%)</th>
<th>AOB</th>
<th>NOB</th>
<th>AMX</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Week 48</strong></td>
<td>No signal</td>
<td>No signal</td>
<td>33%</td>
</tr>
<tr>
<td><strong>Week 52</strong></td>
<td>No signal</td>
<td>No signal</td>
<td>34%</td>
</tr>
</tbody>
</table>
Week 48, AMX (Cy3, red), 320x, biomass on surface of plastic carriers, strong signals, high-density small and medium clusters (33%)

Week 52, AMX (Cy3, red), 320x, biomass on surface of plastic carriers, strong signals, abundant, medium-density big clusters (34%)

Figure 3.15: FISH images of AMX on the surface of plastic carriers

Figure 3.16: (a) Anammox biomass on the surface of plastic carriers; (b) Anammox biomass granules in suspended biomass in reactor E
3.2.3 Identification of anammox bacteria species by FISH

Similar to stage 1, together with AOB, NOB, and total anammox bacteria, other probes were also used in FISH to identify the presence of AMX species in stage 2. Information about name of anammox species with their targeted probes and description of their population are displayed in below table 3-7.

Table 3-7: Identification of AMX species by FISH in stage 2

<table>
<thead>
<tr>
<th>Week</th>
<th><strong>B. anammostdans</strong></th>
<th><strong>B. fulgida</strong></th>
<th><strong>K. stuttgartiensis</strong></th>
<th><strong>S. brodae</strong></th>
<th><strong>S. wagneri</strong></th>
<th><strong>A. propionicus</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>36</td>
<td>Free cells</td>
<td>+</td>
<td>Free cells</td>
<td>Free cells</td>
<td>+</td>
<td>Free cells</td>
</tr>
<tr>
<td>40</td>
<td>Free cells</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>44</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>48</td>
<td>–</td>
<td>+++</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>52</td>
<td>–</td>
<td>++++</td>
<td>–</td>
<td>–</td>
<td>+++</td>
<td>++</td>
</tr>
</tbody>
</table>

*Scale of amount: −: no detection; +: few; ++: medium; +++: medium high; ++++: dominant
*Free cells: FISH analysis showed the scattered distribution of targeted organisms, not in clusters

From week 52, microbial analysis (FISH) confirmed the coexistence of aerobic nitrifiers (AOB) and the anammox bacteria, including Candidatus *B. fulgida*, Candidatus *S. wagneri*, and Candidatus *A. propionicus* in the reactor E. These data confirmed the possibility of the simultaneous partial nitrification and anammox process in the nitrogen removal (see figure 3.17)

Especially, image of epifluorescence microscopy presented a high density of Candidatus *B. fulgida* which stained with probe Bfu613 and aggregated in medium and big clusters. Besides, they were the dominant *Candidatus* among anammox bacteria community.

Besides, we experience the great development of *B. fulgida* from week 40, not only the amount but also the strength of living. They formed in the high-density big clusters which were considered as a good signal. By combining to each other, bacteria could obtain a better and more stable living condition.
We also identified AMX species on plastic carriers by FISH, the procedure was the same as with suspended solid. The summary of result is displayed in below table.

Table 3-8: Identification of AMX species (on the surface of plastic carriers) by FISH

<table>
<thead>
<tr>
<th></th>
<th>B. anammoxidans</th>
<th>B. fulgida</th>
<th>K. stuttartiensis</th>
<th>S. brodae</th>
<th>S. wagneri</th>
<th>A. propionicus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week</td>
<td>Amx1015</td>
<td>Bfu613</td>
<td>Kst157</td>
<td>Scabr1114</td>
<td>BS820</td>
<td>Apr820</td>
</tr>
<tr>
<td>48</td>
<td>−</td>
<td>+++</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>52</td>
<td>−</td>
<td>++++</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

*Scale of amount: −: no detection; +: few; ++: medium; +++: medium high; ++++: dominant*

From the table, we can see that, the community of anammox bacteria on the surface of plastic carriers was totally similar to suspended solid. It was also the presence Candidatus B. fulgida, Candidatus S. wagneri, and Candidatus A. propionicus. Among the Candidatus, B. fulgida was the most dominant with high and strong fluorescent signal in FISH images (see figure 3.18).
3.3 Initial performance of reactor E in stage 3

After more than a year of anammox bacteria enrichment, at the end of stage 2, we obtained sufficient biomass for immobilization in PVA biocarriers. The result of TSS and VSS measurement showed that weight of dry suspended solid for immobilization was 3.37 g, containing 64%, of organic biomass (2.16 g). Figure 3.19 illustrates reactor E when it was filled with PVA carriers and a schematic for LentiKat biocatalyst.
In the beginning of stage 3 (week 57 of experiment), reactor E operated with 1 kg of wet pellets with immobilized biomass of approximately 1.27 g. Nitrogen gas was bubbled continuously to ensure the anaerobic condition for anammox process. At the same time, 700 g of similar wet pellets is used for another reactor in LentiKat’s Company. Because of the limited time, this thesis could not cover the result of stage 3. Therefore, only short description of reactor E’s initial performance is introduced in next paragraphs.

At first, the influent concentration of N-NH$_4^+$ and N-NO$_2^-$ was around 60 and 50 mg/L, respectively. However, the nitrogen removal occurred slowly, so their concentration was then decreased to about 30 mg/L. About the nitrogen removal, after 1 month of stage 3, the removal efficiency is 50% for N-NH$_4^+$ and 60% for N-NO$_2^-$. Besides, the removal rate is elevating batch by batch. Furthermore, we can observe the declination of COD which is considered as signal for activities of heterotrophs. Finally, ORP is continuously measured in stage 3 and it decreased gradually during one month of operation (about -40 mV in week 60) which could indicate the proper anaerobic conditions in reactor until now.

About molecular techniques, the biocatalysts (PVA pellets) were sampled for FISH in week 57 and 59. However, we could not observe any signal of nitrifying bacteria, including AOB, NOB and AMX. The experience is still carried on for investigating the potential for immobilization of anammox biomass in PVA biocarriers.
CHAPTER 4: CONCLUSION AND RECOMMENDATION

4.1 Conclusion

In this thesis, we operated an anammox lab-scale SBR (reactor E) for more than one year, and attained a good result for nitrogen removal and sufficient biomass to immobilize in PVA biocarriers. The start-up of anammox process in SBR required a long period of time (almost 9 months). However, after the stabilization, the maintenance of this process was not so difficult and we could enrich sufficient anammox biomass for PVA immobilization after next 5 months. Besides, the SBR is a suitable system for the enrichment of microorganisms which have an extremely slow growth rate, such anammox bacteria. SBR supports a homogeneous distribution of substrates, products and biomass. The operation of reactor E was relatively stable, especially in stage 2, and high nitrogen removal efficiency was reached.

The most important remark is the DO concentration for the reactor’s operation which needs to be kept below 0.5 mg/L. The anammox bacteria are very sensitive and easily suppressed by even low oxygen amount. Besides, the nitrite inhibition on anammox activities needs to be taken into consideration. In the first stage of the study, we experienced the trouble by nitrite inhibition after a sudden rise of influent N-NO\(_2^-\) concentration (from 150 mg/L to 200 mg/L) and it took more than 2 months for reactor to re-stabilize. The key is that nitrite concentration for feeding the reactor should be adjusted and increased slowly in one batch, following the reactor performance in the previous batch by chemical kinetic tests. Therefore, in stage 2, the input concentrations of nitrogen solutions was gradually elevated from 30 mg/L to 100 mg/L and they were connected with the development of suspended biomass. Furthermore, suspended solids should not be removed during the period of enrichment, because the removal might badly affect the adapted biomass and assist the nitrite inhibition on anammox activity. During enrichment stage, TSS increased from 0.067 to 0.838 g/L and higher concentration and activity of biomass also led to the development of anammox bacteria.

FISH analysis revealed that the nitrifying biomass which was obtained from reactor E finally mainly consists of AOB and 3 species of anammox bacteria, including *B. fulgida*, *S. wagneri*, and *A. propionicus*. Among anammox bacteria, *B. fulgida* was the predominant species. After 1 year of enrichment, the anammox bacteria constituted 19% of the total bacteria in suspended biomass. Besides, AOB was present in the reactor with the abundance of 33% of total biomass and the nitrogen removal efficiencies were higher than 80%. The results of both chemical kinetic tests for nitrogen form speciation and FISH analysis for nitrifiers identification showed the incorporation of anammox and nitritation process in the reactor E (table 4-1). It led to the elevation of nitrogen removal efficiency and anammox biomass in stage 2. Furthermore, biomass on the surface of plastic carriers was also investigated by FISH and the result displayed a higher abundance of anammox bacteria. Anammox bacteria tended to aggregate on the surface of carriers and accounted for 34% of total biomass while there was no signal of AOB and NOB.
The composition of anammox species on the surface of plastic carriers was similar to suspended solid, which also included *B. fulgida* (predominant), *S. wagneri*, and *A. propionicus*.

Together with kinetic test for nitrogen forms, other parameters were also investigated in this study. Firstly, the DO and ORP value exhibited a good performance of reactor E with an appropriate anaerobic condition for anammox process. Secondly, COD measurement which showed the decrease of COD during a batch. Together with denitrification test, they revealed the potential activities of heterotrophic denitrifying bacteria in reactor E. In the end of stage 2, the ORP value in reactor was around -50 mV which indicate the stable condition for anammox activities. From the FISH analysis of autotrophic nitrifiers (including AOB, NOB and AMX), heterotrophs which were assumed to be the rest of biomass accounted about 50% of total biomass. The activity of heterotrophs also supported the decrease of DO and ORP value.

**Table 4-1:** The performance of reactor E during one-year operation is summarized in table

<table>
<thead>
<tr>
<th>Stage</th>
<th>STAGE 1: START-UP</th>
<th>STAGE 2: ENRICHMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overview</td>
<td>Anammox and nitritation process</td>
<td>Nitrite inhibition on anammox activity</td>
</tr>
<tr>
<td>N–NH₄⁺ removal</td>
<td>85% – 98%</td>
<td>52% – 70%</td>
</tr>
<tr>
<td>N–NO₂⁻ removal</td>
<td>73% – 100%</td>
<td>25% – 50%</td>
</tr>
<tr>
<td>AOB</td>
<td>6.3% – 9.6%</td>
<td>17%</td>
</tr>
<tr>
<td>NOB</td>
<td>5.5% – 15%</td>
<td>4.5%</td>
</tr>
<tr>
<td>AMX</td>
<td>1% – 6.2%</td>
<td>Free cells only</td>
</tr>
<tr>
<td>AMX species</td>
<td><em>B. fulgida</em> (dominant), <em>S. wagneri</em>, and <em>A. propionicus</em></td>
<td>Free cells only, very low signal for all species</td>
</tr>
</tbody>
</table>

*Note: stage 3 (immobilization) is not included in this table*

During this present study, there were several difficulties. At first, anammox bacteria have slow growth rate and are sensitive to negative conditions, such as DO and nitrite inhibition. Therefore, it was not easy to keep the constant reactor performance and effective anammox bacteria enrichment. Especially in stage 1, many mistakes happened which led to the temporary collapse of anammox bacteria.
Secondly, due to the low amount of anammox bacteria in the suspended biomass in some samples, it was not easy to quantify the biovolume fraction of them by FISH. After hybridization, the FISH samples were observed under fluorescence microscope, but in some cases, the signals were very low. It was then improved by adjusting the FISH protocol, for example, longer hybridization time and lesser amount of samples in each well. In details, we increased the hybridization time from 3 hours to 4 hours and place only 5 µl of sample in stead of 8 µl as usual. By this way, we could obtain stronger signals of samples and take 30 pictures which quality was possible to be quantified by DAIME. Furthermore, the mix of probes which target each anammox species was also applied and compared with the efficiency of Amx368 probe (targeting all anammox bacteria). The results showed no remarkable difference between 2 experiments.

4.2 Recommendation

In fact, the percentage of anammox bacteria in total suspended biomass after 18 weeks of enrichment in stage 2 (19%) was not as high as other studies. The reasons could be that the enrichment time was shorter and there were plastic carriers in reactor E where anammox bacteria preferred to attach on. Besides, because of using the raw digested liquor for ammonium feeding, the cultivated biomass contained not only nitrifiers but also high amount of heterotrophs. However, it brought the real condition for investigating anammox activity as the final purpose of the enriched biomass would be used for waste water treatment.

From this study, I have some recommendation for the future works:

- For molecular technique, beside FISH, consider PCR-DGGE to identify the microbial diversity in the biomass samples.
- Combine partial nitritation and anammox process in a single reactor. In reactor E, we can observe the co-existence of AOB and anammox bacteria.
- Combine anammox and denitrification in a single reactor. It is technically feasibly and economically favorable, especially when the waste water contains both ammonium and organic carbon.
- Apply anammox granular systems which have good biomass retention and more stable conditions.
REFERENCES


