Evaluation of compounds generated in the thermal hydrolysis process on microbial activities in partial nitritation/anammox process

By

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Abstract

Anaerobic digestion (AD) is a traditional technique to treat sewage sludge and produce biogas. Thermal hydrolysis process (THP) is a feasible pretreatment for AD by improving the biodegrdability of organic compounds. Partial nitritation/anammox (PN/A) process could be applied in side-stream for treating the reject water from AD since the reject water has a low ratio of COD/N. The prime microorganisms involved in PN/A process are anammox biomass, denitrifiers, ammonium oxidizing organisms (AOO) and nitrite oxidizing organisms (NOO). However, the compounds generated in THP may cause negative effect on microbial activities. In this research, characteristics of the reject water was clarified, and manometric method and respirometry method were used to determine the microbial activities by measuring the conversion rates of the substrates. A higher concentration of soluble COD, total ammonium as nitrogen (TAN) and refractory compounds were in the wastewater treatment plants (WWTPs) with THP installation (*i.e.* Tilburg, Hengelo, Apeldoorn, and Amersfoort) than the ones in the WWTPs without THP pretreatment (*i.e.* Sluisjesdijk and Olburgen). Sluisjesdijk has one step PN/A process, while Olburgen has two steps PN/A process. Maximum specific anammox activity(SAA) among the studied WWTPs was found in Sluisjesdijk as 1.32±0.02 gN/gVS/day. THP installation could suppress the SAA value. The presence of refractory compounds may lead to the decrease of SAA. The denitrification activity was enhanced by the high concentration of COD generated in THP. The ratio of AOO activity to anammox activity were similar to the stoichiometry value of 1.32 in Sluisjesdijk Sharon reactor, Tilburg, Hengelo and Amersfoort. Molecular methods may recommended for further study in the area of this research.

Keywords: Thermal hydrolysis process (THP); PN/A process; melanoidins; specific anammox activity (SAA); specific denitrification activity (SDA)

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Abbreviations

AD	Anaerobic digestion
Anammox	Anaerobic ammonium oxidation
AOO	Ammonium oxidizing organisms
COD	Chemical oxygen demand
DNRA	Dissimilatory nitrate reduction to ammonium
DO	Dissolved oxygen
FA	Free ammonia
FNA	Free nitrous acid
HMF	5-hydroxymethyl-furfural
HS	Humic substance
MW	Molecular weight
NOO	Nitrite oxidizing organisms
PN/A	Partial nitritation/anammox
SAA	Specific anammox activity
SDA	Specific denitrification activity
SUVA	Specific ultraviolet absorbance
TAN	Total ammonium as nitrogen
THP	Thermal hydrolysis process
TOC	Total organic carbon
VFA	Volatile fatty acid
VS	Volatile solid
VSS	Volatile suspended solid
WAS	Waste activated sludge
WWTP	Wastewater treatment plant

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1.Introduction

1.1 Background knowledge

The effects on the microbial activity of the distinct compounds generated in the reject water after sludge anaerobic digestion should be studied for the purpose of evaluating the applicability at industrial-scale of the partial nitritation/anammox (PN/A) process. In this chapter, the background of thermal hydrolysis process and the studied microorganism were introduced briefly.

1.1.1 Thermal hydrolysis process (THP)

Anaerobic digestion(AD) is a technique applied all over the world to treat the municipal sludge so as to degrade the organic matter and at the same time produce methane as a renewable energy source (Chen *et al*., 2008; Van Lier *et al*., 2001). After AD, the digested sludge goes into the dewatering process to diminish the water content. The dewatered sludge is disposed, incinerated or used as soil conditioner (Fytili & Zabaniotou, 2008) and the reject water is treated to remove nutrients and reflowed to the mainstream of the treatment. In order to increase the efficiency of AD and improve the dewaterability of digested sludge, multiple pretreatment methods have been proposed, for example, mechanical disintegration methods, chemical methods and THP among others (Ariunbaatar *et al*., 2014; Carrere *et al*., 2010). THP is a reliable pretreatment method used in anaerobic digestion, and the biodegradability and dewaterability of secondary sludge produced in the WWTPs could be improved by lysing cells under the high temperature (Barber, 2016; Xue *et al*., 2015). The optimum conditions for THP operation, is reported at range of 160-180°C for 30-60 min (Barber, 2016; Sapkaite *et al*., 2017). It has been documented that high solubility of COD and high solubility of protein was observed in the reject water after THP (Elliott & Mahmood, 2012; Phothilangka *et al*., 2008), leading to high concentration of soluble COD and high concentration of ammonium detected in the reject water. Besides, due to the application of THP, an increase of refractory materials especially melanoidins production is manifested (Penaud *et al*., 2000; Stuckey & McCarty, 1984). The amount of melanoidins is temperature dependent over time, which means more melanoidins are produced with higher operating temperature (Dwyer *et al*., 2008). The refractory compounds (melanoidions) might influence the efficiency of UV disinfection and increase the concentration of organically bounded nitrogen in the effluent (Martins *et al*., 2000). Moreover, a negative effect of the reject water after THP on the microbial activity was reported by Zhang *et al*. (2018).

1.1.2 Microorganisms involved in the PN/A process

PN/A process (also known as deammonification) is used abroad for the treatment of the reject water and it could also be used for the reject water produced after THP (Ganigue *et al*., 2008; Zhang *et al*., 2016). A sludge treatment scheme is shown in Fig 1.1, and the steps of the sludge treatment process and the production of reject water were represented in this scheme. PN/A process is a completely autotrophic process which contains two steps, which are partial nitritation and anammox. In the first step, ammonia is partially oxidized to nitrite by aerobic organisms called ammonium oxidizing orgamisms (AOO). In the subsequent step, anammox biomass produces molecular nitrogen by the remaining ammonium and the nitrite produced (Sliekers *et al*., 2002; Van Dongen *et al*., 2001). Anammox bacteria cannot use COD in their metabolism, therefore COD that is present in reject water will lead to the growth of denitrifiers and other heterophic organisms. Nitrite-oxidizing organisms(NOO) exists in the process due to nitrite produced in the first step by AOO. However, the limited oxygen concentration and high temperature (>25℃) cause the suppression of NOO in the PN/A process (Laureni *et al*., 2016).

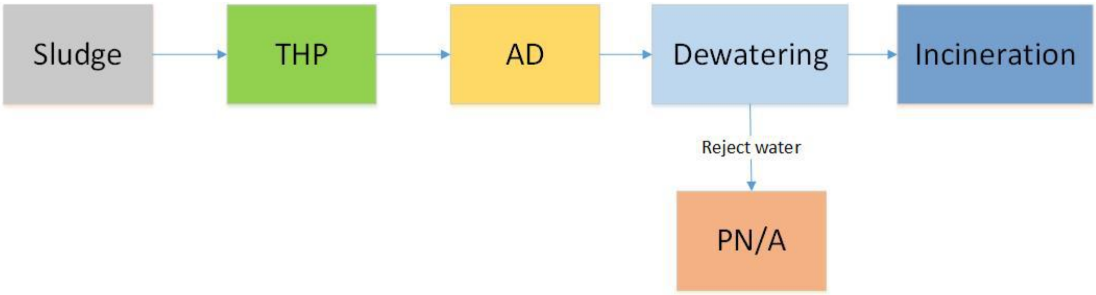

Fig 1.1 The sludge treatment scheme.

1.1.3 Research focus

Previous research has shown that with THP, a higher concentration of organic compounds compared to conventional AD are present in reject water, which may inhibit the activity of anammox and ammonium oxidizing organisms (AOO) (Figdore *et al*., 2011; Zhang *et al*., 2018). Denitrifiers may also exist in the PN/A process if there is COD remained in the PN/A reactor and the presence of denitrifiers might lead to the wash-out of anammox (Gu *et al*., 2018) or cause imbalance in the populations due to the competition of the substrates among different biomass in the PN/A reactor. The suppression of anammox biomass in the PN/A process could be explained by the inhibition of the compounds generated in the THP and/or mass transport limitation due to the granular sludge in the PN/A process (Zhang *et al*., 2016; Zhang *et al.,* 2018). Figdore *et al*.(2011) proposed that the soluble COD fraction may have negative effect on the biomass activity, however, no specific compounds were reported in that study. Reject water treatment design could be improved if the effect of particular compounds on the microbial activity measured as conversion rate would be known. Besides, the researches on the microbial inhibition mainly focus on the lab-scale system. For full-scale situation, mainly 16S rRNA and qFISH techniques instead of conversion rates were studied. For WWTPs, conversion rate could represent the conversion capacity of the full-scale reactor and demonstrate the operation of the reactor directly. And the effect of the reject water on the balance of different biomass in the full-scale PN/A reactor was not clear yet. Thus, the objective of this research is to identify the difference on the conversion rates from diverse wastewater treatment plants (WWTPs) with/without THP. Hereto, batch experiments were performed to assess the effects of THP on the PN/A process analyzing the conversion rates of these. This research can help to evaluate the specific conversion rates in the PN/A process caused by the presence of humic-like substances produces in THP. And feasibility of the combination of THP and the PN/A process will be evaluated.

1.2 Research question and objectives

According to the background aforementioned, the specific questions of this study are:

- What are the characteristics of reject water from AD after THP?
- Are there differences in the microbial activity among WWTPs with and without THP?
- Is there a link between the composition of reject water and the microbial activities in a subsequent PN/A system?
- What is the oxygen demand in the PN/A process?

Specifically, the research questions could be answered by the specific objectives of this study:

1) Identification of the differences in the conversion processes in full-scale PN/A systems with/without THP.
2) Determination of the biochemical charaterisation of the reject water with/without THP installation.
3) Identification of the oxygen demand in the PN/A process.

The hypothesis of this study is that the installation of THP could influence the characteristics of the reject water and the compounds generated in THP may cause suppression on the biomass activity. Besides, the application of PN/A system could limit the oxygen demand for nitrogen removal. Objective 1) is accomplished in the Literature Review (Chapter 2), and involve the research of areas such as inhibition and limitation of anammox biomass, AOO and denitrifiers. The background of objective 2) is illustrated in the Literature Review (Chapter 2) and the results of lab measurement is provided in Chapter 4. Objective 3) is analyzed in Chapter 4.

1.3 Thesis outline structure

This master thesis includes 5 chapters.

Chapter 1 introduces the background of the research. The research objective, and research questions are introduced.

Chapter 2 discusses the literature review related to the research questions. The literauture review of thermal hydrolysis of sewage sludge and PN/A process are provided in this chapter.

Chapter 3 describes the research method and analysis techniques applied in this study.

Chapter 4 discusses characteristics of WWTPs, and the experiments with PN/A sludge on the conversion rates with the PN/A biomass.

Chapter 5 presents the conclusion and recommendations for further study.

2.Literature review

In this chapter, the previous researches regarding the compounds generated in THP and microorganisms involved in the PN/A were introduced.

2.1 Thermal hydrolysis of sewage sludge

2.1.1 Thermal Hydrolysis Process (THP)

Anaerobic digestion is applied worldwide as a reliable technology to treat sludge before disposal (Gavala *et al*., 2003; Xue *et al*., 2015). Hydrolysis, acidogenesis, acetogenesis and methanogenesis are the four major steps contained in the degradation of complex compounds in AD process. Hydrolysis is the rate-limiting stage for the sludge (Choi *et al*., 2018; Xue *et al*., 2015). Pretreatments that can improve the biodegradability of sludge were proposed in AD. THP is a reliable and commercial pretreatment to promote the biodegradability and dewaterability of the sludge produced in the WWTPs and is able to achieve biosolid A quality of sludge (Barber, 2016; Xue *et al*., 2015). Through THP, sludge is hydrolyzed by two mechanisms: i)the elevated temperature could cause thermal influence and ii)steam explosion generated by a rapid decompression (Pérez-Elvira *et al*., 2008). A sequence of reactions happen at high temperature (>100℃), and these reactions are shown in Fig 2.1. As a result, operating temperature and reaction time are two main factors of the design of THP installations. The optimum conditions for THP operation to achieve the highest methane production are reported by the researchers at the range of 160-180℃ and 30-60 min (Barber, 2016; Sapkaite *et al*., 2017). The differences in the optimum temperature and reaction time in different studies are probably related to the different characteristics of the sludge (Barber, 2016).

The influence of THP on sludge have been studied for years and is reviewed in Table 2.1. Heating and sudden decompression can break down the organic matter which can be verified by the increase of COD solubilization leading to a reduction in the particle size and viscosity of the sludge. Xue *et al*. (2015) found that the solubilization of COD increased from 4.5% to 42.5% at 160℃ holding for 180 min and the viscosity decreased from 4500 mPa*s to 5.8 mPa*s at the same condition. Since the solubility of carbohydrates and proteins are increased with the temperature, their presence in the solution results in the occurrence of Maillard reaction ending up in the formation of melanoidins (end products of Maillard reaction). A concentration of 5.9 g eqSAB/L (where SAB was SH3 domain-bingding protein) of soluble protein measured in the sludge after THP which was nearly 20 times than it in the untreated sludge was reported by Bougrier *et al*. (2008) at 170℃. Bougrier *et al*. (2008) also found more carbohydrates under 170℃ treatment (0.83 g eqCOD/L). Due to the improvement in the biodegradability of the sludge and viscosity decrease, more biogas production can be expected. Choi *et al*. (2018) proposed that methane production of the sludge after THP was 1.6 times higher than the conventional AD process which was similar to the 1.3 times suggested by Pérez-Elvira and Fdz-Polanco (2012).

There are diverse motivations to apply THP to WAS. It can be used as a sanitation technique to achieve Class A standard defined by EPA (USEPA, 1999). Also, less energy is needed for the dewatering process due to increased dewaterability of the sludge.

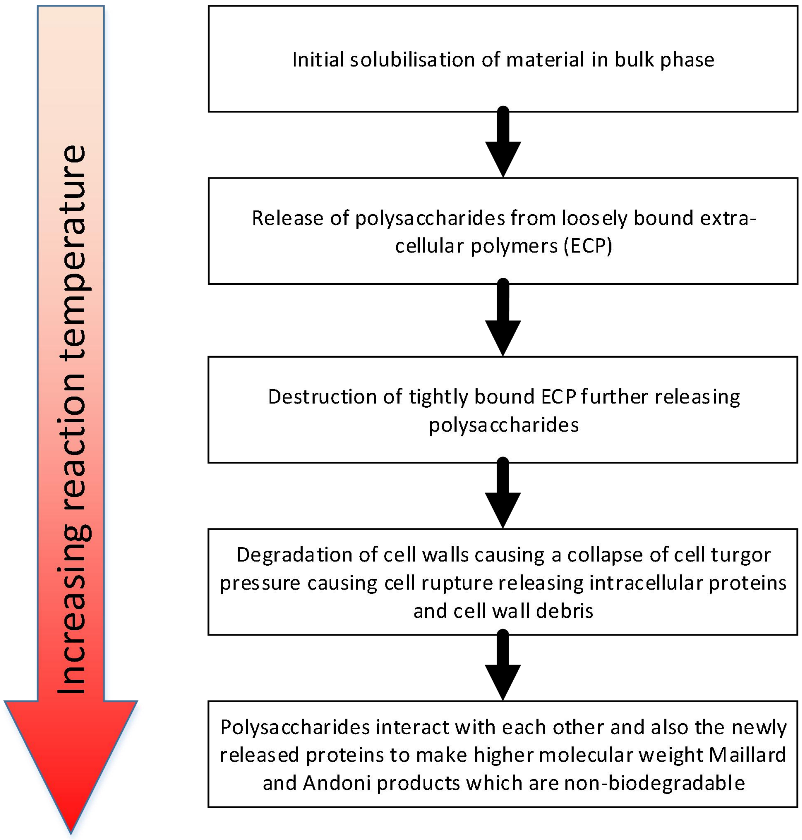

Fig 2.1 Sequence of reactions in the THP system (Barber, 2016).

Table 2.1 Summary of influence of thermal hydrolysis pretreatment on sludge

Observation	Operating temperature	Reaction time	References
COD solubilisation increased with higher operating temperature	<200°C	10-60 min	(Bougrier *et al.*, 2008; Xue *et al.*, 2015)
Protein solubilisation increased	<130°C	60 min	(Li & Noike, 1992; Liu *et al.*, 2012)
Carbohydrates solubilisation	130-170°C	10-30 min	(Bougrier *et al.*, 2008; Liu *et al.*, 2012)

increased			
Viscosity decreased	170℃	30-60 min	(Bougrier *et al.*, 2006; Liu *et al.*, 2012)
Dewaterability increased	180℃	-	(Phothilangka *et al.*, 2008)
Gas production increased with higher reaction time	150-170℃	-	(Phothilangka *et al.*, 2008; Wilson *et al.*, 2011)
Production of refractory compounds	165℃	20 min	(Liu *et al.*, 2012; Oosterhuis *et al.*, 2014)

2.1.2 Full scale installations

As mentioned before, THP has multiple advantages, for instance, safe for disposal. As a result, THP has been widely implemented in industrial plants. Four different technologies were discussed here in accordance with the technologies installed in the WWTPs in this study.

After the first full-scale Cambi installed in Hamar, Norway (Kepp *et al.*, 2000), more than 50 full-scale projects of this company have been built worldwide (Sargalski, Solheim & Fjordside, 2007). The scheme of Cambi system is shown in Fig 2.2Treating organic waste with Cambi® THP (Williams & Burrowes, 2016). The operation temperature for Cambi system is 165-180℃ and reaction time is 10-30 min (Kepp *et al.*, 2000). Three reactors are contained in Cambi system which are pulper, main reactor and flash tank. Sludge is heating to circa 90℃ in pulper by the recycled steam from the flash tank and then flows to the main reactor with the pressure between 2.6 to 6 bar, and the temperature in the reactor is 170℃ holding for 30 min. The temperature in the reactor is achieved by adding extra steam. Finally, the sludge is flashed to circa 0.5 bar in the flash tank where the rapid expansion happened (Kepp *et al.*, 2000). Besides, cooling device is needed to cool the sludge at circa 37℃ before the sludge enters to AD device.

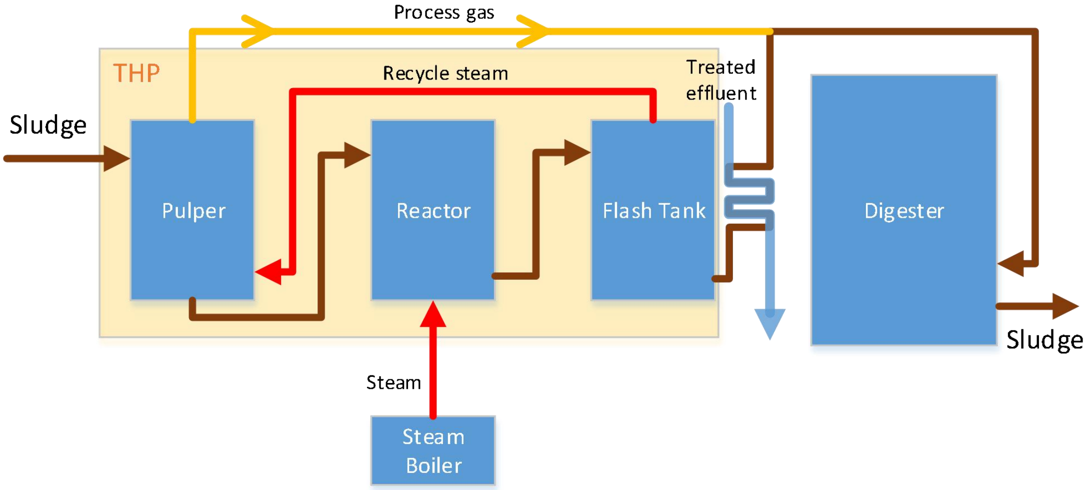

Fig 2.2 - Cambi™ Thermal Hydrolysis Process Schematic, Mark II System Configuration

(Williams & Burrowes, 2016).

Sustec TurboTec THP system is another commercial process THP system which was applied in two full-scale plants in Europe (Williams & Burrowes, 2016). The scheme of Sustc system is shown in Fig 2.3 (Williams & Burrowes, 2016). Raw secondary sludge mixes with the hydrolysed sludge from the hydrolysis reactor to circa 65℃. Subsequently, the mixed sludge is separated in the separator. The thin sludge goes into the digester directly while the thicker sludge is hydrolysed in the reactor at 140℃ (Williams & Burrowes, 2016). The advantage of Sustec system is that it needs less steam for heating.

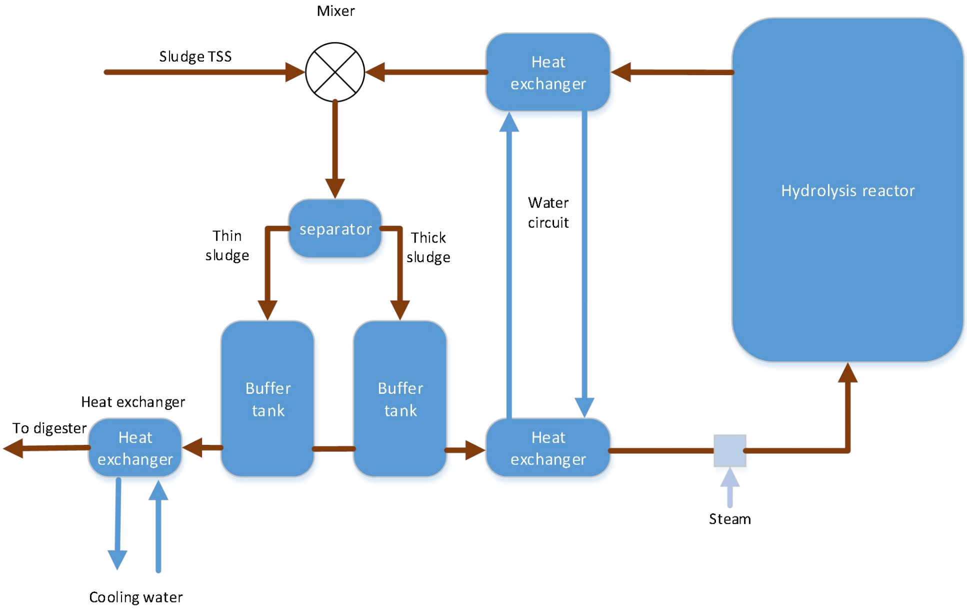

Fig 2.3 Sustec TurboTec Thermal Hydrolysis Process Schematic (Williams & Burrowes, 2016). The brown line is the sludge flow and blue line is water flow.

Lysotherm is a continuous tubular operation THP system applied by Eliquo Water Group. Four zones are contained in Lysotherm system: pre-heating zone, top-heating zone, reactor zone and cooling zone. The difference between Lysotherm system and other THP system is that heating circuits (thermal oil heating circuit and water heating circuit) instead of steam are supplied to heat the sludge. The sludge is preheated by the recycled heat from cooling system. And then the sludge is heated to the operational temperature by thermal oil. In the reactor zone, the temperature is maintained at 140-170℃ for 15-30 min (Williams & Burrowes, 2016). Finally, the sludge is cooled before feeding into the digester.

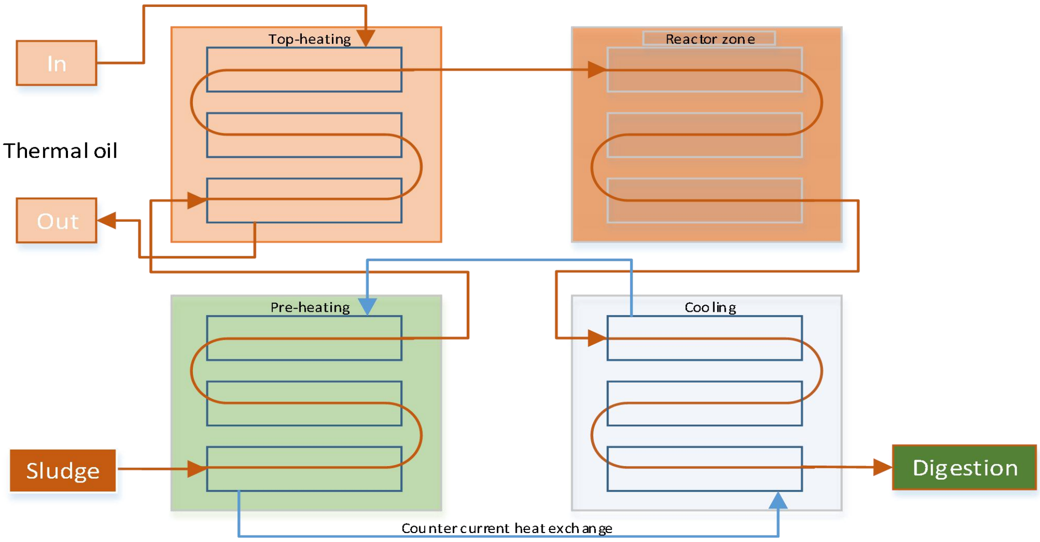

Fig 2.4 Lysotherm Thermal Hydrolysis Process Schematic (Williams & Burrowes, 2016).

2.1.3 Production of refractory compounds in THP.

According to the previous section, THP operation has shown an increase of refractory materials production. Maillard and Amadori products are the major compositions of the refractory materials produced in THP (Barber, 2016; Barker & Stuckey, 1999). These compounds are well known as nonenzymic browning-causing nitrogenous polymers, with high molecular weight(>10 kDa) (Barker & Stuckey, 1999). The refractory compounds might influence the efficiency of UV disinfection and increase the concentration of organically bounded nitrogen in the effluent (Martins *et al.*, 2000). Besides, the exist refractory compounds in the effluent from AD may cause inhibition by competing and/or limitation of substrate penetration on the microbial population activities in the PN/A process because of its nonbiodegradable characteristic.

Maillard Reaction

The Maillard Reaction is named after Louis Maillard who first described it in 1912 and is widely spread in the nature, especially in the food field, this process takes place as the proteins and reducer sugars react to form products called melanoidins.

The formation of melanoidins is a complex process and it is open to debate, among all the definitions, Hodge's definition in 1953 is well approved (Nursten, 2005). According to this hypothesis, the process is divided in three stages as follows:

I Initial stage:

Reaction A:Sugar-amine condensation

Reaction B:Amadori rearrangement

II Intermediate stage:

Reaction C:Sugar dehydration (formation of furfural and hydroxymethylfurfural)

Reaction D:Sugar fragmentation

Reaction E:Amino acid degradation

III Final stage:

Reaction F:Aldol condensation

Reaction G:Aldehyde-amine condensation and formation of heterocyclic nitrogen compounds

The rate and extent of Maillard reaction increases with the temperature , therefore in the range of 140-165℃ it is likely to occur which is the range of THP (Barber, 2016). As a result, it is prospective to detect melanoidins and its intermediates in the THP reactor, anaerobic digester and subsequent steps of the treatment. The scheme of melanoidins formation is shown in Fig 2.2 (Barber, 2016). The figure shows that the reactions happens between amino acids and sugars which can be provided by WAS and enhanced due to THP. Liu *et al*. (2012) reported that the UV absorbance at 254 nm of kitchen waste was around 8 times higher than that of waste activated sludge which showed the enrichment of proteins and sugars could enhance the production of melanoidins. From the Fig 2.2, it can also be seen than pH conditions drives different pathways and divergent intermediates can be produced. According to the work of Bougrier *et al*. (2006), pH decrease in THP to 5.8 because of the formation of acidic compounds. As a result, furfural and HMF may exist in the effluent as intermediate products.

The production of melanoidins in THP have been documented by many researchers (Penaud *et al*., 2000; Stuckey & McCarty, 1984). The presence of melanoidins in THP was hypothesized by Stuckey and McCarty (1984) due to decreased bioconvertibility of nitrogen compounds. Liu *et al*. (2012) indicated that low MW(<10kDa) may react to form refractory compounds resulting in the increase of high MW(>10kDa) and higher UV 254nm absorbance was detected after THP (Liu *et al*., 2012). Lower biogas production was observed by Pinnekamp (1988) in AD, production of melanoidins was considered as the responsible reason. However, at the condition temperature of 180℃ and holding for 76 min, Choi *et al*. (2018) reported that no melanoidins generated in THP for sewage sludge.

Melanodins formation is temperature dependent, Dwyer *et al*. (2008) found that melanodins have a positive relationship with increasing temperature and the color of the THP effluent in the full-scale plant in Oxley Creek, Australia. A decrease from 12,677 mg-PtCo/L to 3837 mg-PtCo/L was also observed when the operation temperature was reduced from 165℃ to 140℃ (Dwyer *et al*., 2008).

Caramelisation reactions

Besides Maillard reaction, caramelisation is another non-enzymatic reaction leading to the brownish of the water that might happen at high temperature during THP(Barber, 2016). The group of reactions that happen when carbohydrates are exist in a high temperature condition are called caramelisation (Quintas *et al*., 2007). The difference between caramelisation and Maillard reaction is that in caramelisation carbohydrates react by the increasing in temperature while Maillard reaction is carbohydrates reacting with amino groups. Sucrose hydrolysis is the first step in caramelisation. Under neutral conditions, sucrose hydrolyzed to D-glucose and a fructose oxocarbonium ion. Besides, abundant products could be formed by fructose carbocation, including: anhydrosaccharides, 5-hydroxymethyl-furfural (HMF) and organic acids (Clarke *et al*., 1997). Further degradation of Fructose and glucose may have further degradation to form HMF compounds. Afterwards, HMF can be further degraded to other products such as furfural by fragmentation reaction and 5-methylfurfural through reduction reaction (Clarke *et al*., 1997). Wilson and Novak(2009) reported that in their research the high absorbance in UV 254 nm at 220℃ was caused by caramelisation instead of Maillard Reaction due to lacking of amino acids.

Humic substances

Humic substances (HS) are heterogeneous organic compounds that include humic acid, fulvic acids and humin (Klavins *et al*., 1999). HS have high molecular weight, brownish color and are resistant to further reaction(Stevenson, 1994). Humic substances are present wildly in the environment and is the major component that comprises soil organic matter (Nardi *et al*., 2016). Many functional groups are related to HS, such as carboxyl groups, hydroxyl groups and methyl groups (Klavins *et al*., 1999). Stuckey and McCarty (1984) and Hodge (1953) indicated that humic acids may be produced through THP resulting in lower bioconvertibility. In accordance with the similar structure of melanoidins and humic acid, melanoidins was considered as humic substances by many research (Hedges, 1978; Ishiwatari *et al*., 1986).

Protein hydrolysis

Tanaka *et al*. (1997) defined that 41% of COD in WAS was protein, which means that protein is the largest fraction of wastewater organic material. During AD, proteins are hydrolyzed to peptides and individual amino acids, and individual amino acid can be degraded to short-chain fatty acids in turn with the subsequent release of ammonium (Wilson & Novak, 2009). At the THP operation temperature below 220℃, VFA produced from protein is around 15%-30% of that from lipid in THP pretreatment (Wilson & Novak, 2009). At the THP operation temperature between 130℃ to 150℃, around 60% of proteins are above 30 kDa, and 30% of proteins present in the range of 10 to 30 kDa. Meanwhile, Lu *et al*. (2014) observed that the size of proteins decreases with increasing temperature. Additionally, when treating THP sludge, ammonia is released by the hydrolysis of proteinaceous material and leads to inhibition of methanogenesis (Lay *et al*., 1998). Wilson & Novak (2009) found that lower temperature(<170℃) could reduce the inhibition of ammonia within AD. However, the inhibition reduction may also depend on the adaption of the biomass to the condition.

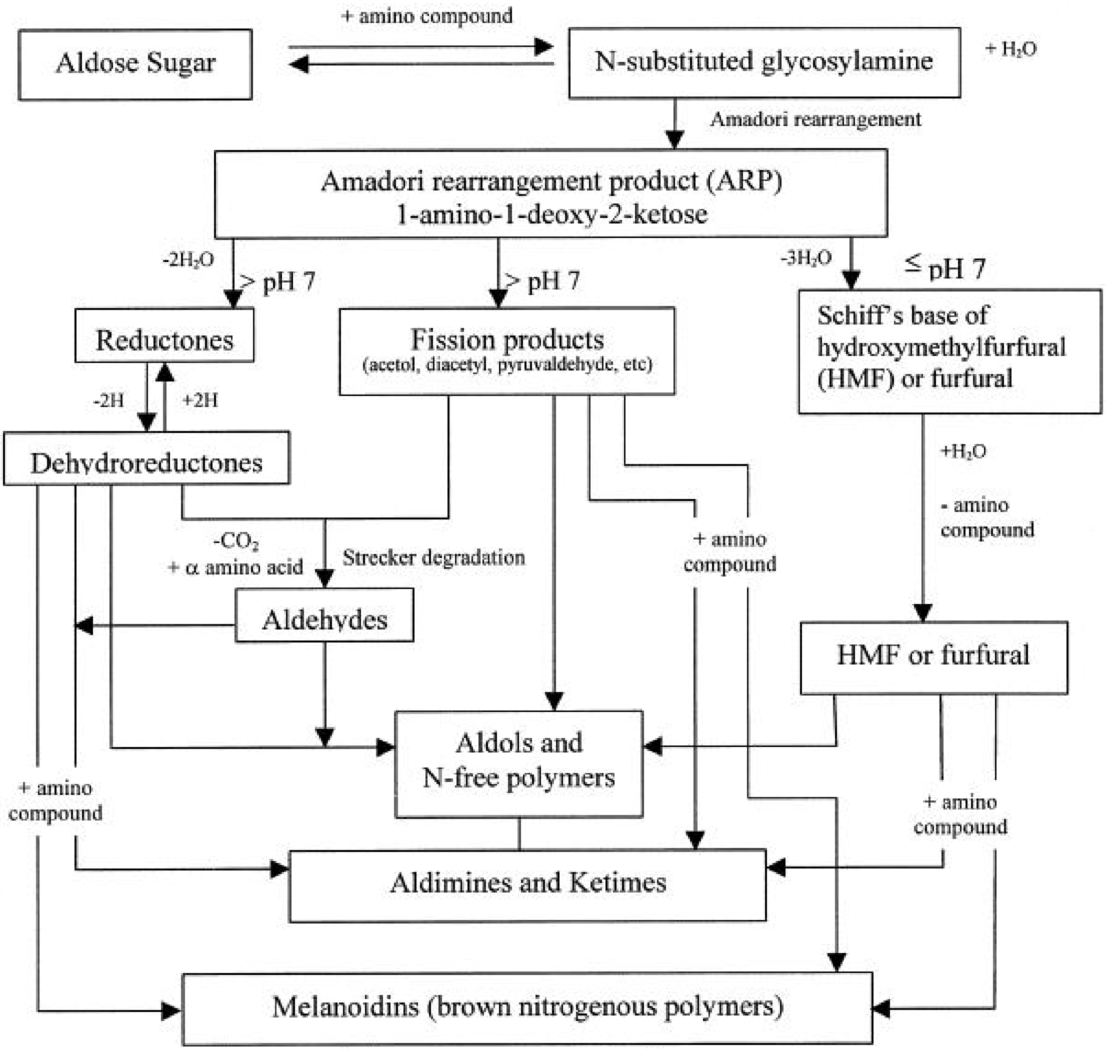

Fig 2.2 Scheme of melanoidins production (Barber, 2016).

2.2 Partial nitritation/anammox (PN/A) process

Nitrogen can be converted into different chemical species by the action of diverse microorganisms. Fig 2.3 shows the biological nitrogen cycle, which includes anammox, nitrification, denitrification, and dissimilatory nitrate reduction to ammonium (DNRA). The first three steps are explained in detail in the following sub-sections.

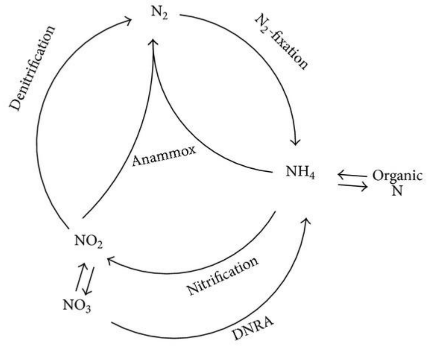

Fig 2.3. Simplified scheme of biological nitrogen cycle (Ni & Zhang, 2013).

2.2.1 Anammox

Anaerobic ammonium oxidation (anammox) was firstly detected in a denitrifying fluidized bed in 1995 by Mulder *et al*. (1995) and then it gained the interest by the researchers all over the world. The sludge treatment plant Sluisjesdijk, Rotterdam, The Netherlands installed the first full-scale anammox process in 2002. Such application has been expanding quickly recently, and full-scale anammox processes are being established increasingly in the world.

Anammox is an innovative and cost-effective method to remove nitrogen from wastewater (Gu *et al*., 2018). Under the anoxic condition, autotrophic anammox bacteria can oxidize ammonium to molecular nitrogen, using nitrite as the final electron acceptor (Jin *et al*., 2012).

The stoichiometry of the anammox process calculated by Mare *et al*. (1998) using mass balance is widely accepted. Eq 2.1 shows the stoichiometry of the anammox reaction.

$$NH_4^+ + 1.32NO_2^- + 0.66HCO_3^- + 0.13H^+ \rightarrow 0.066CH_2O_{0.5}N_{0.15} + 1.02N_2 \\ + 2.03H_2O + 0.26NO_3^- \tag{2.1}$$

The possible metabolism pathway for anammox is shown in Figure 2.4. Ammonium works as the electron donor, nitrite works as the electron acceptor and CO_2 is used as the sole carbon source. Hydroxylamine (NH_2OH) and hydrazine (N_2H_4) are formed as the intermediates. According to Jetten *et al*. (2001), firstly, hydroxylamine is formed from nitrite, and then it reacts with ammonium to form hydrazine as intermediate. While N_2H_4 generates the molecular nitrogen, it will supply electrons to reduce nitrite to NH_2OH. Anammox reactions are intracellular in a specific organelle called anammoxosome, which is a unique feature of B. anammoxidans.

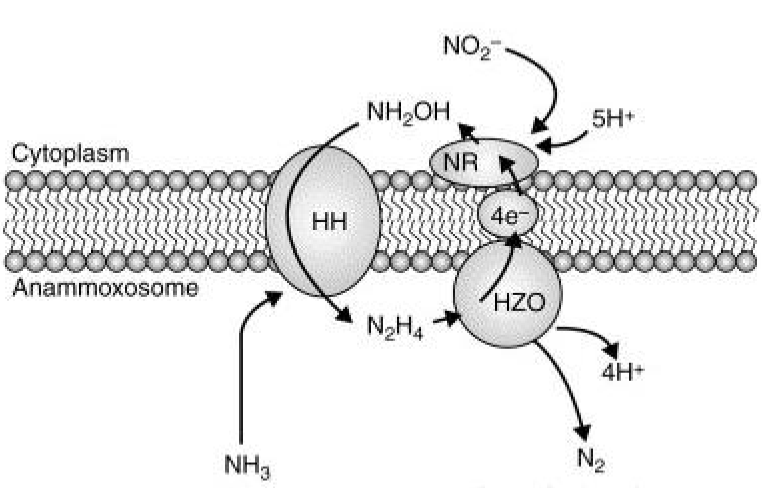

Figure 2.4 Mechanism of anaerobic ammonium oxidation. NR is a enzyme which could reduce nitrite (NH_2OH is the assumed product); HH (hydrazine hydrolase) form hydrazine using ammonia and hydroxylamine; HZO is a enzymewhich could oxidize hydrazine (Jetten *et al*., 2001).

The advantages of anammox compared to conventional biological nitrogen removal methods are the lower operation costs, higher nitrogen removal rate, and smaller space requirements (Joss *et al*., 2009). However, based on the thermodynamic calculations, the maximum growth rate of anammox is only 0.003 h^{-1} while nitrification has the growth rate of 0.04 h^{-1} (Jetten *et al*., 2001). The low growth rate of anammox makes it sensitive to the inhibition and hard to retrieve from the inappropriate conditions.

Inhibitors of anammox microorganisms

Numerous researchers focus on inhibitors of anammox bacteria. One of the inhibitors recognized is free ammonia (FA). A 50% inhibition concentration of 38 mgNH_3-N/L was noticed by Fernández *et al*. (2012) in short term tests which are in the range of 13-90 mgNH_3-N/L reported by Waki *et al*. (2007) that may inhibit the biomass activity. Fernández *et al*. (2012) suggested the optimal concentration of FA in long term tests is lower than 20 mgNH_3-N/L. However, Jung et al. (2007) provided a much lower concentration of 2 mgNH_3-N/L in a UASB reactor treating piggery wastewater. The difference between the concentration of FA in different research may due to the divergent process conditions and sludge characteristics. The concentration of FA could be calculated based on pH and temperature (Eq 2.2) (Ganigue *et al*., 2007). The mechanism of FA inhibition is reported that FA can be produced by high pH and cause changes of intracellular pH leading to the death of biomass cell (Jin *et al*., 2012; Mosquera-Corral *et al*., 2005).

$$FA(mgN/L)=\frac{TAN(mgN/L)}{1+(10^{-pH}/e^{-6344/(273+T(℃))})} \qquad (2.2)$$

Where: TAN=total ammonium as nitrogen

Although nitrite is one of the substrates of anammox biomass, a high concentration of nitrite can also cause inhibition of anammox bacterial growth (Dapena-Mora *et al*., 2007; Jin *et al*., 2012; Oshiki *et al.,* 2011). Dapena-Mora *et al*. (2007) and Oshiki *et al*. (2011) reported 50% inhibition with a concentration of 350 mgNO_2-N/L and 224 gNO_2-N/L in batch tests, respectively. Much lower anammox inhibition threshold values for nitrite were proposed by Marc *et al*. (1999) as 98 mgNO_2-N/L and 30 mgNO_2-N/L adding nitrite twice in the tests by Bettazzi *et al*. (2010). Jung *et al*. (2007) provided a similar concentration of 35 mgNO_2-N/L in a continuous reactor. The disparity between the concentration of nitrite reported in different research may because of the divergent experiment methods. Fernández *et al*. (2012) proposed that the mechanism of nitrite inhibition was caused by free nitrous acid (FNA, HNO_2). According to this hypothesis, pH has a critical impact on the balance of HNO_2/NO_2^-. The 50% inhibition concentration of HNO_2 found by Fernández *et al*. (2012) was 11 µgHNO_2-N/L. However, only a small part of researches recorded the concentration of HNO_2 in their results.

Organic matter may also lead to a suppression of anammox activity(Tang *et al*., 2010). The effect of distinct organic matters on different anammox species was summarized in Table 2.2. Three mechanisms are proposed for the inhibition effect. The first mechanism is that the existence of organic matter together with ammonium and nitrite may cause the growth of heterotrophic bacteria, and heterotrophic bacteria may lead to lower anammox activity due to substrate competition (Chamchoi *et al*., 2008). Substrate competition causes increase of substrate affinity constant but remains the maximum specific growth rate (Zhang *et al*., 2018). The other mechanism is that anammox can use organic matter as a different metabolic pathway (Güven *et al*., 2005; Kartal *et al*., 2008). Güven *et al*. (2005) found that anammox bacteria can oxidize propionate to CO_2 as the electron donor. Acetate was also considered as a possible substrate for anammox bacteria (Kartal *et al*., 2008). The different microbial pathway may be influenced by specific anammox bacteria species. On the other hand, toxic organic matter can cause irreversible inhibition of anammox bacteria. Güven *et al*. (2005) reported that alcohol and methanol (0.5 mM) have an negative effect on the activity of anammox bacteria, while another study posted that anammox activity reduced 70% by exposing to 5mM methanol in batch experiments(Isaka *et al*., 2008).

Table 2.2 Anammox inhibition caused by organic matter.

Organic matter	Anammox bacteria species	Concentration	Effect	Reference
Glucose	-	0.5-3mmol/L	Not significant	(Güven *et al*., 2005)
	Candidatus Brocadia sinica	1 mmol/L	Not significant	(Oshiki *et al*., 2011)

Acetate	*Candidatus Brocadia sinica*	1 mmol/L	Not significant	(Oshiki *et al.*, 2011)
	-	0.5-3mM	Not significant	(Güven *et al.*, 2005)
	Candidatus Brocadia fulgida	3mM	Use as substrate	(Kartal *et al.*, 2008)
Propionate	*Candidatus Brocadia sinica*	1 mmol/L	Not significant	(Oshiki *et al.*, 2011)
	-	0.5-3mM	Not significant	(Güven *et al.*, 2005)
Ethanol	*Candidatus Brocadia sinica*	1 mmol/L	-91%	(Oshiki *et al.*, 2011)
	Candidatus Kuenenia stuttgartiensis	3.3mM	-10%	(Isaka *et al.*, 2008)
	-	1mM	-30%	(Güven *et al.*, 2005)
Methanol	*Candidatus Brocadia sinica*	1 mmol/L	-86%	(Oshiki *et al.*, 2011)
	Candidatus Kuenenia stuttgartiensis	12.5mM	-77%	(Isaka *et al.*, 2008)
	-	0.5mM	-100%	(Güven *et al.*, 2005)

Effect of species on specific anammox activity (SAA)

In the anammox reactor, one species of anammox bacteria dominates solely (Hu *et al.*, 2010; Van Der Star *et al.*, 2008). It was reported that Demon reactor gained more *C. Brocadia* than CANON reactor (Gonzalez-Martinez *et al.*, 2015). Higher organic acid oxidation rates compared with other anammox species makes *C. Brocadia* easily enrich in the organic-rich system (Kartal *et al.*, 2008). The biodiversity of anammox biomass might be driven by the type of reactors and the source of wastewater. The value of SAA reported in previous literature were summarized in Table 2.3

Table 2.3 Specific anammox activity for distinct anammox species.

Anammox species	SAA (gN/g VSS/day)	Reactor condition	Process type	Reference
C. Brocadia	0.55	Granular sludge at 30℃	Anammox reactor	(Lotti *et al.*, 2015)
	0.45	Granular sludge at 34℃	Anammox reactor	(Lotti *et al.*, 2012)
C. Jettenia	0.16	Granular sludge	One step PN/A reactor	(Lotti *et al.*, 2015)
C. Kuenenia	0.28	Granular sludge at 30℃	-	(Dapena-Mora *et al.*, 2007)
	0.57	Granular sludge at 35℃	Anammox-EGSB reactor	(Chen *et al.*, 2011)

2.2.2 Nitrification

Nitrification is an aerobic microbial process which could convert ammonium into nitrite and nitrate. Two steps are involved in the nitrification process, and each step has its own group of autotrophic nitrifying bacteria. In the first step of nitrification, ammonia is oxidized into nitrite by AOO. The catabolic reaction is presented in Eq 2.3. In the subsequent step, NOO oxidize nitrite into nitrate based on Eq 2.4 (Sharma & Ahlert, 1977). Both AOOs and NOOs are chemolithoautotrophic bacteria, which means that they obtain energy from nitrogen oxidation and use carbon dioxide as carbon source for the synthesis of cellular material.

$$NH_3 + O_2 \rightarrow NO_2^- + 3H^+ + 2e^- \tag{2.3}$$

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^- \tag{2.4}$$

Nitrification coupled with denitrification is used as an effective and economical method to decrease the nitrogen content in municipal wastewater around the world (Jaramillo *et al.*, 2018). Low ratio of COD/N is expected in the reject water, as a result, anammox instead of the traditional biological treatment is applied in PN/A pretreatment (Kumar & Lin, 2010).

2.2.3 Denitrification

Denitrification is a process in which anoxic microorganisms reduce nitrate to molecular nitrogen(N_2) using organic matter as electron donor and source of carbon. Denitrification includes a series of intermediate process, nitrate is firstly converted into nitrite by the enzyme nitrate reductase, and then to nitric oxide(NO) through the enzyme nitrite reductase. Hereafter, nitrous oxide(N_2O) formed by the enzyme nitric oxide reductase and finally to molecular nitrogen(N_2) by the enzyme nitrous oxide reductase (Rodríguez *et al.*, 2011). The half-reactions related to the process are shown in the following Eq 2.5-Eq 2.8.

$$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O \tag{2.5}$$

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O \tag{2.6}$$

$$2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O \tag{2.7}$$

$$N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O \tag{2.8}$$

Denitrification is the second step of the conventional nitrogen removal method in municipal wastewater (Jaramillo *et al.*, 2018).

2.2.4 PN/A process

PN/A process is a completely autotrophic process which contains two steps: partial nitritation and anammox. In the first step, ammonia is partially oxidized to nitrite by the work of AOO. In the subsequent step, the remaining ammonium and the nitrite produced react producing molecular nitrogen (N_2) (Sliekers *et al.*, 2002; Van Dongen *et al.*, 2001). The most common strategy used in full-scale is one step reactor with granular biomass, because its lower investments costs high rate conversion (associated to high concentration of biomass) and robustness (Sliekers *et al.*, 2002).

The overall catalytic reaction is the combination of partial nitrification and anammox and it is shown in Eq 2.9 (Marina *et al.*, 2016). These two steps can work either in one single reactor or two separate reactors.

$$NH_4^+ + 0.85O_2 \rightarrow 0.44N_2 + 0.11NO_3^- + 1.43H_2O + 1.14H^+ \tag{2.9}$$

No organic carbon is needed for the growth of microorganisms which means PN/A process does not need the addition of organic matter as a source of neither carbon, nor energy. One reactor process has the benefit of lower investment cost and lower risk for nitrite inhibition (Cho *et al.*, 2011).

The operation of PN/A process need to balance the activity of AOO, anammox and NOO (Laureni *et al*., 2016). The excessive AOO could lead to the accumulation of nitrite, therefore inhibit the activity of anammox (Laureni *et al*., 2016). NOO should be inhibited in the PN/A process to avoid the conversion of nitrite to nitrate with the subsequent drop in efficiency. Three methods are applied to inhibit NOO activity in full-scale. The first method is based on the sludge age due to the fact that nitrosomonas(AOO) has a higher growth rate than that of nitrobacter (NOO) when the temperature higher than 20°C. In addition, nitrobacter is more sensitive to free ammonia (FA) so, a rise in the pH leads to the inhibition of it. Oxygen is a limiting factor for the growth of both nitrobactor and nitrosomonas. At low oxygen concentration, nitrosomonas can grow faster than nitrobacter thus nitrite production is enriched (Rosenwinkel & Cornelius, 2005). Laureni *et al*. (2016) reported that with the oxygen concentration at 0.18 mgO_2/L, the activity of NOO could be decreased firmly.

In the PN/A system, granular sludge is applied widely. The advantage of using granular sludge is that granules could keep the retention of biomass (Hubaux *et al*., 2015). As a consequence of the low growth rate, anammox biomass tends to aggregate together with AOO and denitrification to form granules (Laureni *et al*., 2016). Anammox biomass grows in the inner layer of the granules, and AOO and denitrifiers grow in the outer layer with the contact with oxygen (Hubaux *et al*., 2015). However, AOO prefers to remain as flocs in order to reduce the mass transport limitation (Laureni *et al*., 2016). The combination of flocs and granular sludge could lead to the decline of the process efficiency and stability (Hubaux *et al*., 2015).

Compared with conventional nitrification-denitrification, PN/A has evident advantages. The main one is that it needs no extra electron donor (organic matter) and this can lead to cost-saving and lower biomass production. In addition, the aeration can be decreased by 60% because only half of the ammonium should be oxidized to nitrite which can also lower the energy demand (Kartal *et al*., 2011; Van Hulle *et al*., 2010). In recent years, PN/A process has been widely implemented in landfill leachate and reject water from THP digestate dewatering (Ganigué *et al*., 2008; Zhang *et al*., 2016).

Previous research has shown that after THP, high molecular weight organic compounds (higher than 10kDa) are found in reject water, these compounds may inhibit the activity of anammox and/or AOO (Figdore *et al*., 2011). Zhang *et al*. (2018) found that soluble compounds existing in reject water were the main inhibitors of anammox, while AOO were limited by diffusional limitation, which was caused by particulate and colloidal organics. In addition, nitrification and heterotrophic bacteria may be associated together in the PN/A process resulting in the wash-out of anammox(Gu *et al*., 2018). To compensate for the inhibitory effect on PN/A process, higher dissolved oxygen(DO) set-point(1mgO_2/L), longer aeration time and efficient anammox retention ca be applied (Zhang *et al*., 2016).

2.3 Conversion rate of biomass

Kinetic models

The most popular model expressing microbial growth rate is Monod's microbial growth kinetics (Chen *et al.*, 2011; Zhang *et al.*, 2018). The model is represented as follows:

$$\mu = \frac{\mu_{\max} \cdot S}{S + K_S} \tag{2.10}$$

Where:

μ : Specific growth rate (d^{-1})

μ_{max}: Maximum specific growth rate (d^{-1})

S: Substrate concentration (mg/L)

K_S: Apparent affinity coefficient for S (mg/L)

A feature of Monod's kinetics is that each substrate acts independently as a growth-limiting factor. One of the limitations of Monod's equation is that the model depends on the limitation of other substrate (Han & Levenspiel, 1988). If other substrate also has negative effect on the biomass, Monod's kinetics could be restricted. Many studies have focused on the kinetic models to simulate anammox process based on Monod equation. The maximum specific growth rate and affinity coefficient of nitrite and ammonium have been studied widely for anammox bacteria (Chen *et al.*, 2011; Puyol *et al.*, 2013). Table 2.4 shows some results of kinetic parameters estimated in anammox bacteria. From Table 2.4, the range of these three parameters may due to fitting strategies and the substrate transportation limitation due to the size of sludge. Continuous experiments (Chen *et al.*, 2011; Puyol *et al.*, 2013) have higher substrate affinity coefficient (less affinity for the substrate) for both nitrite and ammonium, than batch experiments (Strous *et al.*, 1999; Van Der Star *et al.*, 2008). In these cases, the diffusivity of substrate into the biomass may be determined by the size of the granular biomass taking into account the mass transport limitations.

Table 2.4 Kinetic parameters for anammox bacteria

$\mu max(d^{-1})$	$KsNO_2^-$(mM)	$KsNH_4^+$(mM)	Reference
-	0.35	0.64	(Puyol *et al.*, 2013)
0.732	0.047	2.625	(Chen *et al.*, 2011)

0.2246	-	6.6	(Ni *et al.*, 2012)
1.1	<0.007	<0.007	(Strous *et al.*, 1999)
0.23	0.0002-0.003	-	(Van Der Star *et al.*, 2008)
0.098	0.086	0.028	(Chen *et al.*, 2011)
0.08	0.02	0.02	(Dapena-Mora *et al.*, 2004)

Conversion rate of biomass

Conversion rate can be described as following (Chen *et al.*, 2011):

$$r_{SU} = \frac{1}{Y}\mu X \tag{2.11}$$

Where:

r_{su}: Rate of substrate uptake (mg substrate/(L • d))

Y: Biomass/ substrate yield (mg VSS/mg substrate)

X: Biomass concentration (mg VSS/L)

Assuming that the stoichiometric synthesis yield of anammox is constant(0.066 C-mol/mol NH_4^+) (Strous *et al.*, 1998), both specific growth rate(μ) and biomass concentration(X) have an effect on the rate of substrate uptake. Besides, other parameters(substrate concentration and affinity coefficient) may influence the rate of substrate uptake by reducing specific growth rate(μ). It is hard to separate the effect by each factor or how THP influences the substrates conversion rate of PN/A microorganisms when using THP reject water. From the engineering point of view, a clear understanding of the conversion rate is more useful that the populations involve in the PN/A process. As a result, this research focuses on the conversion rate of substrate, instead of independent factors that might influence the microbial populations.

3.Materials and methods

3.1 Sampling of PN/A biomass

Reject water from AD dewatering process after struvite recuperation (influent of PN/A process) and PN/A microorganisms (along with effluent) were collected from six wastewater treatment plants (WWTPs) in The Netherlands. The samples were taken from Sluisjesdijk (Rotterdam), Tilburg, Olburgen, Apeldoorn, Hengelo and Amersfoort in The Netherlands. All of these WWTPs had PN/A side stream treatment process to remove ammonium from rejected water. Moreover, thermal hydrolysis process (THP) was applied as pretreatment of AD in four of these plants (*i.e.* Tilburg, Apeldoorn, Hengelo and Amersfoort), and for the case of Sluisjesdijk and Olburgen, no AD pre-treatment was applied. After sampling, reject water and PN/A supernatant were filtered through 0.45 µm filter and stored at 4°C for further analysis. The characteristics of THP and PN/A process for the studied WWTPs were summarized in Table 4.1.

Table 3.1 THP characteristics and PN/A process characteristics for Sluisjesdijk, Olburgen, Tilburg, Hengelo, Apeldoorn and Amersfoort. (a: for anammox reactor; - : no information; T: temperature)

	Sluisjesdijk	Olburgen	Tilburg	Hengelo	Apeldoorn	Amersfoort
THP company	No THP	No THP	Cambi System	Cambi System	Sustec System	Lysotherm System
T(℃) for THP	No THP	No THP	165	165	140	140-170
PN/A technique	Sharon/Anammox	CANON	ANAMMOX	NAS-ONE (Colsen)	Demon	Demon
Waste water source	Reject water	Potato industry and reject water	Reject water	Reject water	Reject water	Reject water
T(℃) for PN/A reactor	35	35	-	-	30	30
Aeration	-	Continuously	Intermittent	-	Intermittent	intermittent

		aerated				
Reactor type	Internal circulation	Airlift reactor	-	-	Micrograules	Granular sludge
DO(mg/L)	0^a	1.5-2	2	1.0	0.3	0.3
Reference	(Lotti, Kleerebezem, Van Loosdrecht, & bioengineering, 2015)	(Lackner et al., 2014; Lotti et al., 2015)		(Desloover et al., 2011)	(Lackner et al., 2014; Lotti et al., 2015)	(Castro-Barros, Jia, van Loosdrecht, Volcke, & Winkler, 2017; Lackner et al., 2014)

3.2 Biomass preparation

In order to compare the potential impact of the compounds present in the different PN/A reactors, two sets up of experiments were carried out for specific anammox activity (SAA) and specific denitrification activity (SDA). In one experimental set up, the biomass was rinsed with the culture medium without substrate three times in order to avoid the impact of possible inhibitions present in the reject water, and measure the conversion rates. In the other set up biomass was left with half of the original broth present in the PN/A reactor. For AOO activity, and NOO activity, only the tests with washing were applied. The reagents to prepare the culture medium for anammox biomass, denitrification activities and AOO activity and NOO activity are shown in Table 3.2, Table 3.3, and Table 3.4 respectively. If using granular anammox sludge, in the final washing half of the volume of the culture media was left along with the biomass in order to handle it easily. Sluisjesdijk had separate reactors for partial nitritation and anammox. AOO and NOO existed in the form of suspended biomass, so washing step was bypassed.

Table 3.2. Culture medium for anammox biomass (Van de Graaf *et al.*, 1996).

Reagents	Concentration in the culture medium (mg/L)
$KHCO_3$	500
KH_2PO_4	27.2
$MgSO_4*7H_20$	300

$CaCl_2*2H_2O$	180
EDTA	20
$FeSO_4$	5
$ZnSO_4*7H_2O$	0.43
$CoCl_2*6H_2O$	0.24
$MnCl_2*4H_2O$	0.99
$CuSO_4*5H_2O$	0.25
$NaMoO_4*2H_2O$	0.22
$NiCl_2*6H_2O$	0.19
$NaSeO_4*10H_20$	0.21
H_3BO_4	0.014

Table 3.3. Culture medium for denitrification biomass (Rustrian *et al.*, 1997)

Reagents	Concentration in the culture medium (mg/L)
Peptone	50
Yeast extract	50
NH_4Cl	38
$MgSO_4*7H_2O$	600
$CaCl_2*2H_2O$	70
EDTA	100
KH_2PO_4	50
K_2HPO_4	90
$FeCl_3*6H_2O$	3

HBO_3	0.3
$CUSO_4*5H_2O$	0.06
KI	0.06
$MnCl_2*4H_20$	0.24
$Na_2MoO_4*2H_20$	0.12
$ZnSO_4*7H_2O$	0.24
$CoCl_2*6H_2O$	0.3

Table 3.4. Culture medium for ammonia (AOO) or nitrite (NOO) oxidizers biomass (Campos*et al.*, 2001)

Reagents	Concentration in the culture medium (mg/L)
KH_2PO_4	250
$MgSO_4$	60
NaCl	1000
$NaHCO_3$	5000
$FeSO_4*7H_2O$	2.7
$MnCl_2*4H_2O$	0.1
$CoCl_2*6H_2O$	0.024
$NiCl_2*6H_2O$	0.024
$CuCl_2*2H_2O$	0.017
$ZnCl_2$	0.068
Na_2MoO_4	0.024

3.3 Substrates conversion rates measurement

In order to determine the maximum conversion rates of the respective substrate in the anammox, denitrifiers, AOO and NOO biomass present in PN/A reactors, ex situ anoxic and aerobic batch experiments were performed. Anammox and denitrification activities were performed in anoxic batch experiments in Oxitop bottles (M Xylem Analytics, Germany) as shown in Fig 3.1a, the pH was adjusted at the beginning of the experiments between 7.45-7.55. The batch tests were performed using one dry incubator with a stirring system at 35°C (the same temperature as in full-scale) Aerobic batch experiments were carried out in a chamber(circa 130 mL) with pH adjusted to 7.45-7.55 at the beginning of the experiments. Two syringes were used to add substrate at bottom and discharge extra liquid on the top. Temperature was controlled using a water bath at 35±1.5°C to simulate the same temperature as in the full-scale PN/A reactors.

Fig 3.1 Oxitop devices used for measuring specific anammox activity and specific denitrification activity..

3.3.1 Specific anammox activity (SAA) and Specific denitrification activity (SDA) measurement

Manometric batch tests were used to measure the biomass SAA(gN_2-N/ gVS/day) and SDA(gN_2-N/gVS/day). The concentration of nitrite and ammonium added as substrate in the Oxitop bottles for SAA tests were both 80 mg N/L($(NH_4)_2SO_4$ and $NaNO_2$) which below the inhibition concentration proposed by Dwyer *et al*. (2009) of 98 mg NO_2-N/L. The concentration of sodium acetate and nitrite added as substrate in the Oxitop bottles for SDA tests were 300 mg COD/L($NaCH_2COOH*3H_2O$) and 30 mg N/L($NaNO_3$), respectively. The increased pressure in the bottles was considered as pure dinitrogen produced by anammox biomass. And for SDA, the dinitrogen production was corrected by the gas composition. Regarding to the high doubling time for anammox biomass, no biomass growth was assumed during the tests.

In order to ensure the anoxic conditions, the bottles were caped tightly and flushed in the headspace with Argon gas for 1min using the lateral orifices. The Oxitop bottles were put on a stirrer plate inside an incubator, at 35°C. The substrates were added using a syringe from the lateral orifices and the pressure normalized with the atmospheric. Once the tests were finished, the gas composition, volatile solids and pH were measured in all the reactors.

3.3.2 Specific AOO&NOO activity measurement

Respirometry method was implemented to know the maximum conversion rate of the substrates that are oxidized using oxygen as final electron acceptor. In order to measure the respirometry, the chamber was completely filled with culture media and the biomass using the valves present in it. The substrates which were NH_4Cl (80 mgN/L) to measure AOO activity, and $NaNO_2$ (80 mgN/L) for NOO activity measurement were added using a syringe. The reaction chamber was placed into a water bath to maintain constant temperature of 35±1.5°C. To measure the endogenous respiration consumption, no substrate was added in the chamber and the oxygen consumption was measured. During the reaction, DO was detected every ten seconds using dissolved oxygen sensor (FDO 925, WTW, Germany) and the data were collected using a laptop connected to the probe. The tests were finished when DO reached 3mg/L to avoid the oxygen limitation. Once the tests were finished, volatile solids were measured in the reactors.

3.4 Mathematical modelling of specific microbial activities

3.4.1 Calculations of SAA and SDA

The conversion rates of anammox biomass and denitrification biomass were calculated based on the maximum dinitrogen production rate in each bottle during the reaction time. Moles of dinitrogen produced were calculated based on the overpressure and the headspace volume in each bottle(Eq 3.1) (Sander, 2015). Henry’s law was used to consider the dissolved amount of the dinitrogen in the liquid phase(Eq 3.2) (Sander, 2015). In Eq 3.1, X equals to 1 for SAA calculation since the pressure in the bottles increased only caused by the production of denitrogen. For SDA, X should be determined by measuring the gas composition in the headspace. The mass of dinitrogen produced was calculated based on the number of moles(Eq 3.3).

$$n_i = \left(\frac{(P_i - P_A) * V_{Hi}}{R * T} + (P_i - P_A) \cdot K_{(35℃)} * R_V\right) * f_{VP} * X \tag{3.1}$$

$$K_{(35℃)} = K_{(25℃)} e^{A\left(\frac{1}{T(25℃)} - \frac{1}{T}\right)} \tag{3.2}$$

$$M_i = n_i * 28 \tag{3.3}$$

Where：

n_i=Number of moles of N_2 produced in the i-th bottle (mol)

P_i= Absolute pressure in the i-th bottle (mbar)

P_A=Atmospheric pressure in the initial point (mbar)

V_{Hi}=Headspace volume in the i-th bottle (L)

R= Ideal gasses constant 83.14472(mbar*L/mol/K)

T= Absolute Temperature (273.15K+35K)

$K_{(25℃)}$= Henry's law constant at 25℃ (advised $6.4*10^{-7}$mol/L/mbar)

$T_{(25℃)}$= Absolute Temperature of the original Henry's constant (273.15K+25K)

A= Factor to correct the Henry's constant by the temperature (K) (advised 1300 K)

R_V= Volume of reaction (L)

f_{VP}=Correction factor for vapour pressure based on Antoine's equation (dimensionless) *0.9446 for 308.15K

X=Molar fraction of dinitrogen in the headspace (dimensionless)

M_i=Mass of N_2 i-th bottle (g)

Considering the mass of dinitrogen produced over the time, the maximum production rate can be calculated as the slope of dinitrogen production(Eq 3.4). SAA is commonly expressed in g N_2-N/gVS/day, so the solids concentration was measured after the tests were finished. Based on the biomass mass(Eq 3.5) in the Oxitop bottles and the maximum rate of dinitrogen production, conversion rate can be derived by Eq 3.6. The tests were carried out in triplicate and the standard deviation was calculated among them to determine the variability.

$$\frac{dM(t)}{dt} \approx \frac{M_{i+1} - M_i}{t_{i+1} - t_i} \approx Slope(\begin{matrix} M_{i-1}, M_i, M_{i+1} \\ t_{i-1}, \ t_i, \ t_{i+1} \end{matrix}) \tag{3.4}$$

$$X_M = X_R * R_V \tag{3.5}$$

$$Conversion\ rate = MAX(\frac{\frac{dM_{(t)}}{dt}}{X_M})(gN_2 - N/day/VS) \tag{3.6}$$

Where:

X_M= Anammox biomass mass (gVS)

X_R= Concentration of Anammox biomass in the reactor (gVS/L)

3.4.2 Calculations of AOO and NOO activities.

The conversion rates of nitrogen were calculated in accordance with the stoichiometry of nitrification (Sharma & Ahlert, 1977). The conversion rates of AOO and NOO were derived from the oxygen consumption rate as Eq(7) and Eq(8) show.

$$\frac{d[NH_4^+ - N]}{dt} = \frac{d[O_2]}{dt} * \frac{-1}{3.43gNH_4^+ - N} \tag{3.7}$$

$$\frac{d[NO_2^- - N]}{dt} = \frac{d[O_2]}{dt} * \frac{-1}{1.14gNO_2^- - N} \tag{3.8}$$

Anabolism of AOO and NOO should be counteracted since only energy produced was considered in these tests. The endogenous oxygen consumtion rate of AOO and NOO were calculated in the same way without substrate and substracted from the final conversion rate. Specific AOO and NOO activities were expressed in $gN_{converted}$/day/gVS. Equation 3.9 shows the maximum conversion rate

$$MAX(\frac{\frac{d[A]}{dt} - \frac{d[A_{endogenous}]}{dt}}{X_M * R_V})(\frac{gN_2 - N}{day * gVS}) \tag{3.9}$$

Where:

$\frac{d[A]}{dt}$ = Conversion rate due to substrate uptake (mg/L/s)

$\frac{d[A_{endogenous}]}{dt}$ = Conversion rate due to endogenous respiration (mg/L/s)

3.4.3 Mathematical modelling description

Two different mathematical models were used to simulate the dinitrogen cumulation in the Oxitop bottles: the first-order kinetic model and Gompertz model.

The basic first-order kinetic equation is described as following(Kafle & Chen, 2016):

$$\frac{dC}{dt} = -KC \tag{3.10}$$

Where:

C= Biodegradable substrate concentration

t= The time(day)

K= First order rate constant (d^{-1})

Derivation of the model with the dinitrogen measurement instead of substrate consumption is described as following (Kafle & Chen, 2016):

$$G(t) = G_0 \times (1 - e^{(-Kt)}) \tag{3.11}$$

Where:

G(t)= Cumulation of dinitrogen at the time t (mL/g VS)

G_0= Maximum dinitrogen potential of the substrate (mL/g VS)

Gompertz model is a practical non-linear model. The Gompertz model used for simulate the dinitrogen production could be expressed as following (Kafle & Chen, 2016):

$$G(t) = G_0 \cdot \exp\left\{ -\exp[\frac{R_{\max} \cdot e}{G_0}(\lambda - t) + 1] \right\} \tag{3.12}$$

Where:

G(t)= Cumulation of dinitrogen at the time t (mL/g VS)

G_0= Maximum dinitrogen potential of the substrate (mL/g VS)

R_{max}= Maximum dinitrogen production rate (mL/g VS/d)

λ=Lag phase (day)

e= exp (1) = 2.7183

Both the models were applied to simulate the cumulation of dinitrogen. R^2 was considered as the evaluation criteria which means that the specific activities was calculated using the model with a higher R^2. In this study, the first-order kinetic was chosen to express the results of the conversion rates.

3.5 Chemicals and analysis methods

3.5.1 Chemicals

Detailed information of the reagents used for batch experiments and for calibration methods are listed in Appendix 1.

3.5.2 Analytical methods

The pH was measured with pH probe model SenTix® 940 (WTW, Germany). Electric conductivity was measured with conductivity sensor model LF 413T IDS(Xylem, Germany). Real color was analyzed in a spectrophotometer Thermo Spectronic (GENESYS, Germany) spectrophotometer at 475 nm wavelength. Brown color intensity can be compared based on the absorbance at this wavelength. UV absorbance was measured on a Thermo Spectonic (GENESYS, Germany) spectrophotometer at 254 nm in a quartz 1 cm path length cuvette.

Reject water was characterized using commercial kits (Hach, USA) were used for analyzing the concentration of ammonium (LCK 303), nitrite (LCK 342), nitrate (LCK 340), total nitrogen (LCK 338), ortho-P, total phosphate (LCK 350), soluble COD (LCK 514) and soluble TOC(LCK 386) measured by kits (DR3900, Hach, USA). Protein and humic substances were detected based on the Lowry method and the modified protocol described by Fr *et al.* (1995). The concentration of furfural and hydroxymethyl-furfural were measured with sulfuric acid (5mM) as eluent by HPLC (Agilent, USA) according to protocole developed by Yuan & Chen (1998). The volatile solids(VS) concentration was measured in triplicate according to the US standard methods 1684. Gas composition and volatile fatty acids (VFAs) were detected by GC (Agilenttech 7890A) with the carrier gas of helium. The sample for volatile fatty acids were pretreated by formic acid (10 µL). The protocol used for VFAs were modified by Zhang *et al.*

4.Results and discussion

4.1 Characteristics of WWTPs

A variety of characterisation methods were used to determine the effect of THP pretreatment on RW. Table 4.1 contains the characteristics of the influent and effluent of PN/A system from six full-scale PN/A plants, which are Sluisjesdijk, Olburgen, Tilburg, Hengelo, Apeldoorn, and Amersfoort. The information of the studied plants were reported in Table 3.1. For Sluisjesdijk, the influent of Sharon reactor (RW), influent of one anammox reactor (AnIn) and effluent of one anammox reactor were measured. And for Olburgen, Tilburg, Hengelo, Apeldoorn and Amersfoort, the characteristics of reject water (RW) and effluent of PN/A reactors were measured.

It was evident that, compared with the RW of Sluisjesdijk and RW of Olburgen, RW of Tilburg, Hengelo, Apeldoorn and Amersfoort contained a much higher concentration of COD(Table 4.1) which fitted with the researches that COD solubility increased due to THP pretreatment (Bougrier *et al.*, 2008; Xue *et al.*, 2015). Xue *et al*. (2015) reported that the soluble COD concentration increased 30% when treating the sludge under 140℃ for 20min and increased 38% under the condition of 160℃ for 15min. The different values of COD concentration in RW between Tilburg, Hengelo, Apeldoorn and Amersfoort may be caused by the different THP operational conditions. Tilburg and Hengelo have the Cambi system and the operational temperature is 165℃ which is higher than Apeldoorn(140℃) and Amersfoort(140-170℃). Besides, Pereboom *et al*. (2014) reported that less COD through THP was expected using the Sustec system due to the low temperature(140°C) compared with other commercial THP pretreatment. In the meanwhile, VFA contributed to 12.7%, 2% and 23% of the total COD in Tilburg, Hengelo, and Apeldoorn, respectively which could be explained by the poor operation of AD. However, no VFA was observed in Amersfoort both in RW and effluent. VFAs were produced in THP pretreatment by lipid and protein hydrolysis. For instance, Wilson and Novak (2009) observed 492 mg COD/L at 170℃ by glyceryl trilinolenate hydrolysis. Afterward, VFA could be digested in AD (Xue *et al*., 2015). A high concentration of VFA in the reject water in Tilburg, Hengelo, and Apeldoorn indicated the instability of AD which may be caused by THP pretreatment (Gavala *et al*., 2003).

The color adsorption present in Sluisjesdijk and Olburgen were much lower than the one in Tilburg, Hengelo, Apeldoorn and Amersfoort (Table 4.1). A high concentration of high aromatic and complex molecular structures contributed to the high value of color adsorption (Dwyer *et al*., 2008). Previous work found that in Oxley creek WWTP, Australia, the color of the THP effluent increased from 85 to 3837 mg-Pt-Co/L applying THP at 140℃ and increased to 3 times of the value before when the operation temperature raised to 165℃ (Dwyer *et al*., 2008). Therefore, the high value of color in Tilburg, Hengelo, Apeldoorn and Amersfoort may be linked to the application of THP pretreatment. It had been documented that melanoidins were the main component of the refractory organic compounds which were attributed to the brownish of the reject water (Bougrier *et al*., 2008; Liu *et al*., 2012). UV254 is a common method to indicate the amount of melanoidins (Barber, 2016). The absorbance of UV254 of RW in Tilburg, Hengelo, Apeldoorn and Amersfoort were more than 5 times higher than the one of Sluisjesdijk and Olburgen (Table 4.1) which proved that melanoidins could be produced by THP pretreatment. Melanoidins was accepted as humic substances due to the similarity with humic acids (Yamamoto & Ishiwatari, 1989), so the concentration of humic substances could indicate the concentration of melanoidins in the liquid. Fig 4.1 shows the correlation of the concentration of humic substances with the absorbance of UV254. The R^2 is 0.9433 which indicated the concentration of humic substances has a positive relationship with the concentration of melanoidins measured as UV 254nm absorbance in this research.

Specific ultraviolet absorbance(SUVA) is a suitable parameter to understand the aromaticity and condensation state of the carbon present in the organic matter. The values of SUVA in RW ranged from 0.36 to 1.88 L/mg/m (Table 4.1). High values of SUVA was in the RW in Hengelo, Apeldoorn and Amersfoort (Table 4.1) and the values was comparible with SUVA in Merrimac effluent (1.609 L/mg/m) reported by Dwyer et al. (2008). A higher SUVA was in the effluent than the one in the RW in Hengelo, Apeldoorn and Amersfoort. This indicated compounds which could be considered as melanoidins with highly heterocyclic and aromatic structure present in the RW and remained after the PN/A process (Dwyer *et al*., 2008).

Xue *et al*. (2015) reported that 10 times of soluble protein concentration could be detected at 160℃ than that of raw sludge. Afterward, the produced protein could be consumed in AD. Although higher concentration of protein may be produced due to the installation of THP pretreatment, no protein could be determined in the reject water after AD in this research. The absence of proteins was probably because part of the soluble proteins were used to produce refractory compounds when presented with sugar at the same time in the reactor and since the samples were taken after anaerobic digestion in a place where the proteins are already consumed by the anaerobic microorganisms. Besides, when the temperature above 150℃, amino acids broke up to form VFA and release ammonium (Wilson & Novak, 2009).

Table 4.1 shows that the concentration of total ammonia as nitrogen (TAN) in RW in Tilburg, Hengelo, Apeldoorn and Amersfoort was higher than that in Sluisjesdijk and Olburgen. Extra ammonium produced in the plants with THP installation by aminoacids splitting and an increase of protein solubilization (Wilson & Novak, 2009). According to Wilson and Novak (2009), 1400 mgN/L (150℃) and 1690 mgN/L (170℃) of ammonium could be produced by protein hydrolysis through THP pretreatment based on the assumption that 40% of total COD (approximately 150000 mg/L) was proteins in the sludge. Besides, Wett *et al*. (2006) reported that around 0.02 g NH_3-N/g TSS was released through conventional AD. As a result, a higher concentration of ammonium can be estimated in Tilburg, Hengelo, Apeldoorn and Amersfoort due to the application of THP pretreatment.

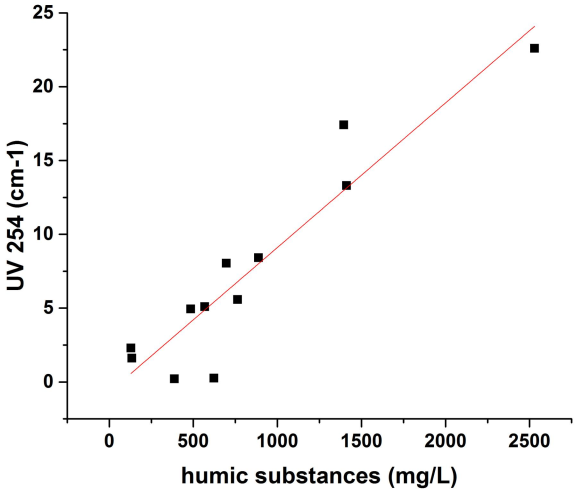

Fig 4.1 Correlation of the concentration of humic substances with the absorbance of UV254 in the analyzed samples.

Table 4.1 Overview of the characteristics of the influent and effluent of the PN/A process(average±standard deviation). ShIn was the influent of the Sharon reactor in Sluisjesdijk. AnIn was the influent of the anammox reactor in Sluisjesdijk. RW was the reject water which entered into the PN/A reactors. Effluent was the effluent of the PN/A reactors. Nd: not detected.

Characteristics	Sluisjesdijk			Olburgen		Tilburg		Hengelo		Apeldoorn		Amersfoort	
	RW	AnIn	Effluent	RW	Effluent	RW	Effluent	RW	Effluent	RW	Effluent	RW	Effluent
THP Company	No THP			No THP		Cambi System		Cambi System		Sustec System		Lysotherm System	
Electric conductivity (mS/cm)	10.45	6.79	1.746	6.35	4.84	13.5	2.85	14.94	3.61	13.7	6.2	13.54	5.87
Color (mgPt-Co/L)	237±2	308±9	281±14	71±2	63±24	2563±91	1262±82	5311±46	4785±0	1249±39	1374±52	2361±158	2611±99
UV254(cm^{-1})	1.075±0.001	2.30±0.04	1.617±0.006	0.261±0.002	0.21±0.01	13.3±0.2	5.1±0.2	22.6±0.2	17.41±0.04	5.58±0.05	4.94±0.01	8.41±0.05	8.04±0.07
COD(mg COD/L)	470±17	1059±5	315±2	109±2	71±5	3152±6	822±42	3527±20	1875±15	1709±32	811±2	1602±18	1168±6
TOC(mg-C/L)	130±5	151±8	107±3	73±4	67.7±0.4	2020±20	296±7	1203±24	685±12	528±3	267±5	506±11	361±10
SUVA(L/mg/m)	0.83	1.52	1.51	0.36	0.31	0.66	1.72	1.88	2.54	1.06	1.85	1.66	2.23
Ortho-P	21.9±0.2	28.0±0.2	28.3±0.2	2.9±0.1	18.4±0.2	103±1	57±2	107±3	23±1	36.7±0.6	175±2	29.7±0.5	51±6
TP	22.7±0.3	30.9±0.3	32.4±0.2	3.0±0.6	18.6±0.1	131±4	67±2	117±2	29±2	33±0	172±1	32.3±0.5	52±3
NH$_4^+$(mg N/L)	1190±119	590±20	55±2	244±19	22±2	1970±135	101±9	2400±40	199±3	1663±21	204±0	1910±10	581±22
NO$_3^-$(mg N/L)	nd	185.8±22.3	73.2±0.3	nd	23.2±0.4	10.17±0.06	4±0.1	17±0.4	14.1±0.2	nd	nd	6.3±0.2	18±3
NO$_2^-$(mg N/L)	nd	644±7	nd	9.76±0.05	1.21±0.0	nd	nd	nd	nd	nd	nd	nd	57±2

					1								
Proteins(mg/L)	-	9±6	6±7	nd	nd	nd	nd	nd	nd	nd	nd	120±71	nd
HS(mg/L)	-	129±11	134±6	623±18	388±12	1411±1	568±4	2530±142	1395±27	763±22	484±12	888±10	696±27
VFA(mg COD/L)	9±8	nd	nd	nd	nd	400±7	14±4	70±21	61±2	396±11	18±7	nd	nd

Table 4.2 PN/A process characteristics for Sluisjesdijk, Olburgen, Tilburg, Hengelo, Apeldoorn and Amersfoort. (a: for anammox reactor)

	Sluisjesdijk	Olburgen	Tilburg	Hengelo	Apeldoorn	Amersfoort
Technology	Sharon/Anammox	CANON	ANAMMOX	NAS-ONE(Colsen)	Demon	Demon
Waste water source	Reject water	Potato industry and reject water	Reject water	Reject water	Reject water	Reject water
Temperature	35	35	-	-	30	30
References	(Lotti *et al.*, 2015)	(Lackner *et al.*, 2014; Lotti *et al.*, 2015)		(Desloover *et al.*, 2011)	(Gonzalez-Martinez *et al.*, 2015; Lackner *et al.*, 2014)	(Castro-Barros *et al.*, 2017; Lackner *et al.*, 2014)

4.2 Experiments with PN/A sludge on microbial activities

4.2.1 Effect of THP on specific anammox activity of PN/A sludge

Manometric batch tests run with 80 mgN/L nitrite and 80 mgN/L ammonium were used to measure the biomass specific anammox maximum activity (SAA, gN$_2$-N/ gVS/day). The substrate consumption rate could be calculated based on the dinitrogen production rate and indicated the activity of anammox biomass. The tests were conducted on anammox biomass sampled from six distinct WWTPs. The information of these WWTPs are summarized in Table 4.2.

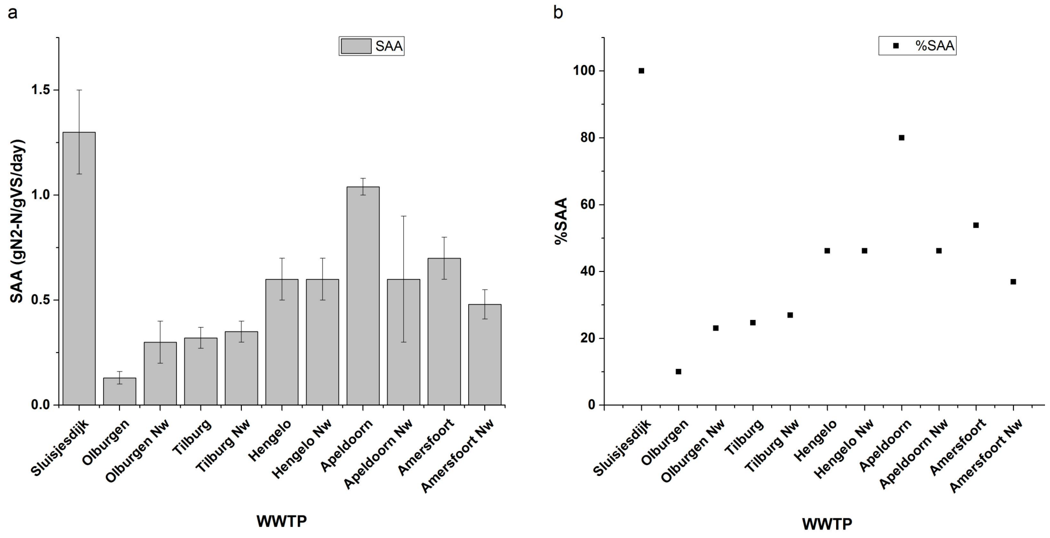

Fig 4.2 a: biomass specific anammox activity (SAA) measured for Sluisjesdijk anammox reactor, Olburgen, Tilburg, Hengelo, Apeldoorn and Amersfoort with and without washing the biomass with washing medium. b: SAA values normalized by the value in Sluisjesdijk anammox reactor. Nw=no washing.

Although the total ammonium as nitrogen (TAN) concentration in the RW exceeded 1000 mgN/L in the five WWTPs, more than 90% ammonium could be removed by the PN/A processes in these WWTPs, indicating that the anammox biomass had superior performance, except in Amersfoort where 70% nitrogen removal could be achieved. The values of SAA for different WWTPs are shown in Fig 4.2. The maximum SAA was in Sluisjesdijk one anammox reactor (1.3 ± 0.2 gN$_2$-N/gVS/day) in where had separate Sharon/anammox reactors, meaning that the biomass tested was just anammox, contrary to the other installations where AOO were also present, automatically lowering the anammox activity that is measured per gram VS. Lower values were reported as 0.45 gN$_2$-N/gVS/day and 0.55 gN$_2$-N/gVS/day by Lotti *et al*. (2012; 2015) in the same plants. The difference may be caused by the operational conditions at different time. The value of SAA for Olburgen in which one step PN/A process was installed was only 10% of the maximum SAA in Sluisjesdijk one anammox reactor, although no THP pretreatment was applied in Olburgen. One possible reason is that the granular biomass in Olburgen PN/A reactor which was the coagulation of anammox, denitrifiers, and AOO lead to the low concentration of anammox biomass per VS resulting in a low value of SAA. Besides, the potato industrial wastewater together with the reject water may also cause the low value of SAA in Olburgen by the adjustment of different dominant species in the reactor. The similar value of SAA was reported by Lotti *et al*. (2012) as 0.16 gN_2-N/gVS/day. SAA values in other WWTPs where had THP pretreatment were in the range of 0.16-1.8 gN_2-N/g VS/day which was also found by other researches in different treatments (Dapena-Mora *et al.*, 2007).

Spearman correlation between SAA and process parameters

The Spearman correlation coefficient could demonstrate the strength of the correlation between two different parameters. Fig 4.3 showed a Spearman correlation analysis illustrated the correlation of the parameters including color, UV254, COD, NH_4^+, HS and VFA in the reject water and effluent from PN/A process with SAA. The average values of the triplicate tests were used in this analysis.

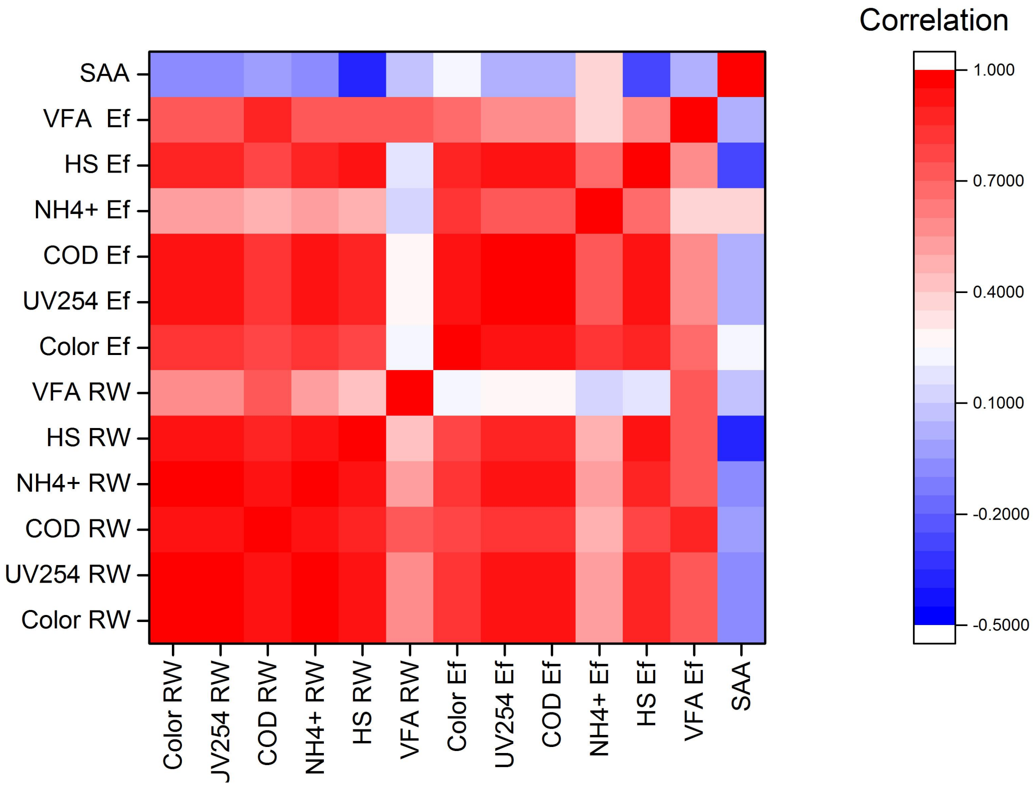

Fig 4.3 Spearman correlation analysis demonstrated the correlation of the parameters including color, UV254, COD, NH_4^+, HS and VFA in the reject water and the effluent from PN/A process with SAA.

From Fig 4.3, negative correlations of SAA with the absorption of color, UV254, and the concentration of COD, NH_4^+ and humic substances in the reject water were analyzed. However, the Spearman correlations were lower than 0.4, which means the correlations were weak. Moreover, the significant p-values for these correlations were above 0.5, indicating a weak evidence that the correlations were correct. On the contrary, SAA had positive correlations with the absorption of color, UV254 and the concentration of COD, NH_4^+, and VFA. However, the significant p-values for the analyzed correlation were also above 0.5. More data from different plants should be collected to get significant correlations. The correlation between different parameters and SAA were discussed in the following.

Effect of refractory compounds

It has been documented that refractory compounds could be formed due to the THP pretreatment (Liu *et al*., 2012; Stuckey & McCarty, 1984). The presence of refractory compounds especially melanoidins may cause a negative effect on anammox activities. A low anammox activity was also observed by Zhang *et al*. (2016) at a high concentration of COD (3000 mgCOD/L). The production of refractory compounds in THP pretreatment was shown in Table 4.1 by the value of color, UV254 and humic substances. Comparing SAA values in the studied plants, lower SAA value was observed at a higher value of humic substances (Fig 4.4). The data from Olburgen was not exhibited in the figure since Olburgen used both potato wastewater and reject water. The different parameters of the influent may lead to different performance of biomass. The Spearman correlation of color and SAA for other five studied plants was -0.9 which was a strong negative correlation (ρ=0.1). And the same correlation was found for UV254 and humic substances. For the same plant, SAA value decreased 60% when the biomass exposed to the original RW which has low concentration of humic substances (750-1000 mg/L) than exposed to the culture medium. While the high concentration of humic substances (above 1500 mg/L) didn’t show inhibition on anammox activity (Fig 4.4). Similar trend was also observed for color and UV254.

Fulvic acids and humic acids are hardly removed by biological technologies (*i.e*. nitrification and denitrification) in the main stream (Renou *et al*., 2008; Zhang *et al*., 2016) and remain in the reject water after AD. Anammox biomass is autotrophic bacteria, as a result, no COD is needed for anammox growth. Therefore, the effect of refractory compounds on anammox activity was not directly caused by competing for refractory compounds as a substrate. However, the direct effect of humic substances on anammox activity was not clearly clarified, humic substances have influences on the interaction of S and N compounds by acting as redox mediators (Rikmann *et al.*, 2012). There are three possible mechanisms for the reduction of anammox activity:(1) other bacteria could use refractory compounds for growth and compete substrates with anammox biomass; (2) the complex compounds could limit the substrate diffusion in to the granular sludge; (3) the refractory compounds were toxic to anammox biomass. Although denitrifiers cannot use fulvic acids directly, fulvic acids could be mineralized to biodegradable organics (Liang & Liu, 2008). From table 4.3, the removal efficiency of color, UV254, and humic substances indicated that denitrifiers and/or other heterotrophic bacteria existed in the system consuming humic substances and may result in competing substrates with anammox bacterium. The toxicity level of COD on anammox activity proposed by Zhang *et al.* (2016) was 800 mg COD/L which was lower than the COD concentration in the plants containing THP pretreatment. Therefore, the high concentration of COD may be a reason for low SAA value in the studied plants with THP.

Table 4.3 Removal efficiency of color, UV254 and humic substances in Sluisjesdijk, Olburgen, Tilburg, Hengelo, Apeldoorn and Amersfoort. (+) means the value in effluent was higher than that in influent.

	Sluisjesdijk	Olburgen	Tilburg	Hengelo	Apeldoorn	Amersfoort
Color removal (%)	9	75	51	10	+	+
UV254 removal (%)	30	20	62	23	11	4
Humic substances removal(%)	+	38	60	45	37	22

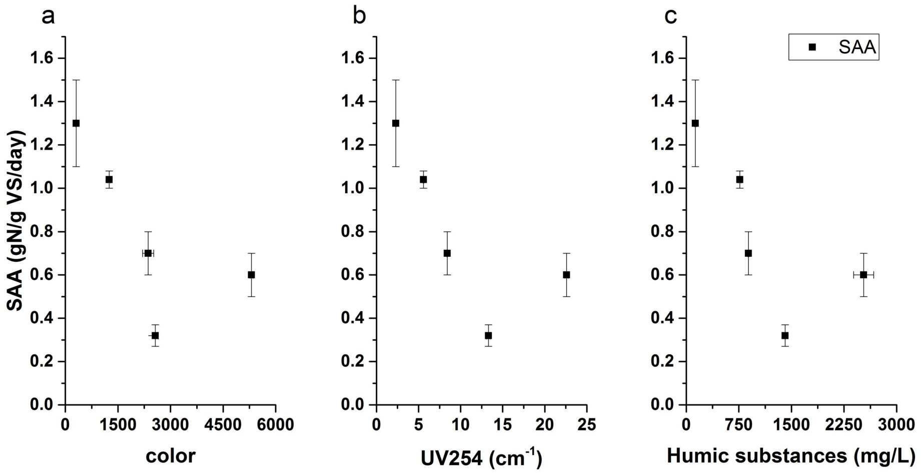

Fig 4.4 Effect of color, UV254 and humic substances on the specific anammox activity(SAA)(squares) measured in the reject water of Sluisjesdijk, Olburgen, Tilburg, Hengelo, Apeldoorn and Amersfoort. The value of SAA was measured with washing the biomass with culture medium.

Effect of VFA

VFA could be produced by THP and anaerobic digestion and residual VFA could be detected in the reject water (Table 4.1). Therefore, the effect of VFA on anammox activity was analyzed. Although high concentration of VFA was measured in the RW in Tilburg, Hengelo and Amersfoort, low concentration of VFA was in effluent. And no VFA was observed in Sluisjesdijk, Olburgen and Amersfoort. No clear effect of VFA in the anammox activity was found in this research comparing the tests washing with the culture medium and tests without washing. It has been documented that higher anammox activity took place when exposed to 1 mM acetate in batch experiments, while anammox activity decreased in the long term experiments (Dapena-Mora *et al.*, 2007; Van de Graaf *et al.*, 1996). The possible reason is that acetate influences the activity of anammox by increasing nitrite formation (Van de Graaf *et al.*, 1996), however the concentration of acetate in VFA was low in the effluent in these WWTPs, the nitrite formation could be neglected. On the other hand, even if there was an increase of anammox activity caused by VFA, the increase was possibly counteracted by other inhibition characteristics, for instance, the presence of refractory compounds.

Effect of ammonium

A higher concentration of ammonium could be produced by THP pretreatment due to protein hydrolysis (Wilson & Novak, 2009). From Fig 4.3, SAA had very weak positive correlation with NH_4^+ concentration in the influent and weak negative correlation with NH_4^+ concentration in the PN/A reactor, however the p-values for both Spearman correlation were 0.5 meaning the weak confidence of the correlation. The concentration of TAN in the PN/A reactor measured in this study was lower than 1000 mgN/L which was the concentration that inhibition might happen (Strous *et al.*, 1999). However, the concentration of free ammonia (FA) rather than TAN may have negative effect on the anammox performance.

In the batch tests pH was set at 7.5 and the temperature was controlled at 35℃, as a result, 3.4% of the ammonium could be calculated that existed as uniozied ammonia. In the tests, FA was in the range of 0.8 to 20 mgN/L for distinct WWTPs (Table 4.4). Previous literature proposed that FA above 13 mgN/L may have negative impact on the anammox performance (Waki *et al.*, 2007). In this way, lower SAA values in the tests without washing with the culture medium for Amersfoort compared with the the one washing with the medium possibly explained by the higher concentration of FA in the bottles. No clear decreasing of SAA for Tilburg and Hengelo was observed, suggesting that FA might have no inhibition effect in the anammox activity at low concentration (<13 mgN/L). As a result, FA might not be the reason for the anammox activity decrease in this study. Other compounds existed in the reject water from Apeldoorn and Amersfoort were presumed to have negative effect on the performance of anammox biomass.

Table 4.4 Concentration of free ammonium in the effulent in Sluisjesdijk anammox reactor, Olburgen, Tilburg, Hengelo and Apeldoorn.

	Sluisjesdijk	Olburgen	Tilburg	Hengelo	Apeldoorn	Amersfoort
FA(mgN/L)	2	0.8	3	7	7	20

4.2.2 Effect of THP on specific denitrification activity of PN/A sludge

30% to 80% COD removal was observed through PN/A process in Sluisjesdijk, Olburgen, Tilburg, Hengelo, Apeldoorn and Amersfoort, hypothesizing the existence of denitrifiers and other heterotrophs in the PN/A system.

In the presence of organic matter and nitrate produced by AOO in PN/A system, nitrite or nitrate could be converted to dinitrogen by denitrification because of higher standard free energy (ΔG^0=-843 KJ/mol) of denitrification than that of anammox (ΔG^0=-297 KJ/mol), improving the nitrogen removal efficiency (Kumar & Lin, 2010; Ni, Ni, Hu, & Sung, 2012). SDA value for distinct WWTPs and removal efficiency of COD are shown in Fig 4.5. The ratio of produced NO_3^--N and removed total ammonium nitrogen (TAN_{rem}) was calculated to estimate the performance of denitrification (Table 4.5). Comparing with the theoretical value of NO_3^--N_{prod}/TAN_{rem} (0.11) based on the stoichiometry of PN/A process, lower ratio of NO_3^--N_{prod}/TAN_{rem} (Table 4.5) were observed in these WWTPs, indicating other pathway of nitrogen removal happened in the process. Highest ratio of NO_3^--N_{prod}/TAN_{rem} was 0.1 in Olburgen which had lowest denitrification activity as 0.06±0.01 gN_2-N/gVS/day. For Tilburg and Hengelo, the concentration of NO_3^- decreased through the PN/A process confirming higher SDA. The WWTPs using THP pretreatment had much lower ratio of NO_3^--N_{prod}/TAN_{rem} than the WWTPs where no pretreatment of AD was applied, indicating higher denitrification in Tilburg, Hengelo, Apeldoorn and Amersfoort.

Comparing the tests using the biomass washed with the culture medium and the tests that without washing, a higher SDA value for the biomass which exposed to the original effluent than the biomass washing with culture medium was measured in this study (Fig 4.6). This could be explained by the suspended denitrifiers remained in the effluent without washout by the culture medium, so that more denitrifiers per VS in the bottles. In order to lower the mass transport, denitrifiers tended to remain suspended in the reactor (Laureni *et al*., 2016). Because of a higher value of SDA without washing with culture medium than the one washing with the culture medium, negative effect of the compounds existed in the effluent was not obvious in the tests.

The ratio of COD/N is another parameter to assess the ability of denitrification (Kumar & Lin, 2010). Desloover *et al*. (2011) reported that denitrification could be inhibited at the ratio of COD/N of 2, because nitrate could be converted to ammonium as dissimilatory nitrate reduction to ammonia (DNRA) at high ratio of COD/N (Dalsgaard *et al*., 2005; Van Rijn *et al*., 2006). The suitable range of COD/N for denitrification is 0.8-1.6 reported by Kumar and Lin (2010). The ratio of COD/N in Tilburg, Hengelo, Apeldoorn and Amersfoort were in the range of 0.8-1.6 shown in

Table 4.3, indicating higher contribution of denitrifcation as same as the calculation results. In Sluisjesdijk, the ratio of COD/N of 1.8 resulting in a slightly low activity of denitrification may be caused by the competition of denitrification and DNRA. Lowest SDA was observed in Olburgen which had the lowest ratio of COD/N of 0.45 lower than the suitable ratio of 0.8. The possible reason for the lowest SDA in Olburgen may be the insufficient COD in the reject water. In conclusion, the concentration of COD and the ratio of COD/N are the two main parameters for the influence of denitrification activity by THP pretreatment.

Previous research proposed that denitrification could compete with anammox (Dong & Tollner, 2003). For the purpose of verifying the relationship of denitrifiers and anammox in the PN/A process, contribution of denitrification and anammox to nitrogen removal was calculated based on the stoichiometry of denitrification and anammox mentioned in literature review. Assuming half of the ammonium in the reject water was converted to nitrite due to partial nitritation. Small fraction of ammonium and COD was consumed for biomass synthesis (Chamchoi *et al*., 2008). The contribution of each process is shown in Fig 4.6. Anammox contributed less than 90% of the total nitrogen removal in Tilburg, Hengelo, Apeldoorn and Amersfoort, while in Sluisjesdijk and Olburgen, more than 95% of the total nitrogen removal was caused by anammox. The possible explanation was the high concentration COD due to THP pretreatment may inhibit the activity of anammox. Chamchoi *et al*. (2008) reported that anammox activity was inhibited when the COD concentration higher than 300 mg/L. Due to THP pretreatment, more than 1000 mg/L COD could be detected in the reject water in Tilburg, Hengelo, Apeldoorn and Amersfoort, which was much higher than the concentration leading to inhibition of anammox inhibition.

In accordance with the contribution of denitrification on nitrogen removal, the concentration of COD that consumed in denitrification process could be calculated (Fig 4.7). Beside denitrification process, COD could be removed by extra oxygen as electron donor. And the extra oxygen used to consume COD was an important term for energy consumption in WWTPs. Only in Apeldoorn and Amersfoort, more than 50% COD could be removed by denitrification. In Tilburg, 1741 mg COD/L should be removed by extra oxygen which cost the most energy demand in the studied WWTPs.

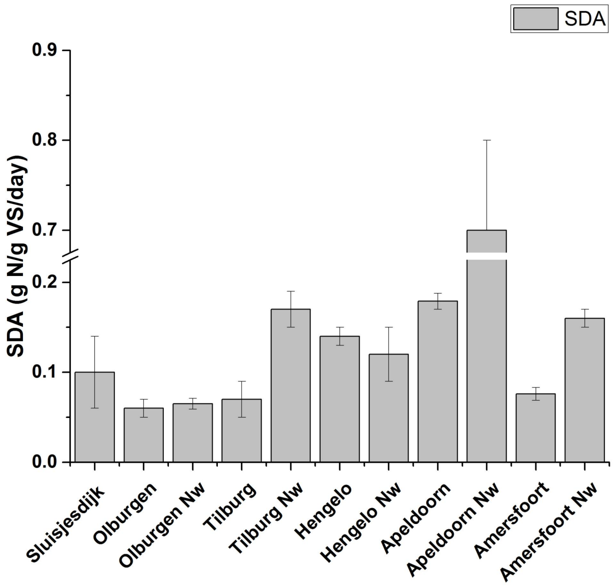

Fig 4.5 Specific denitrification activity(SDA) measured for Sluisjesdijk one anammox reactor, Olburgen, Tilburg, Hengelo, Apeldoorn and Amersfoort and Specific denitrification activity(SDA) without washing with the culture medium measured for Olburgen, Tilburg, Hengelo, Apeldoorn and Amersfoort.

Table 4.5 The ratio of COD/N, ratio of produced NO_3^--N to removed total ammonium nitrogen and total ammnium removal removal for Sluisjesdijk, Olburgen, Tilburg, Hengelo, Apeldoorn and Amersfoort.

	Sluisjesdijk	Olburgen	Tilburg	Hengelo	Apeldoorn	Amersfoort
COD/N	1.8	0.45	1.6	1.47	1.03	0.84
NO_3^--N_{prod}/ TAN_{rem}	0.06	0.1	<0	<0	0	0.009
TAN removal	95%	91%	95%	92%	88%	70%

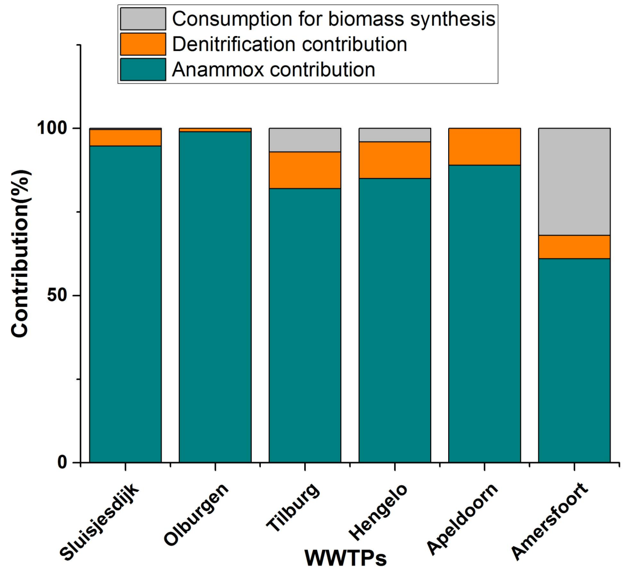

Fig 4.6 Contribution of anammox, denitrification and biomass synthesis on ammonium removal for Sluisjesdijk anammox reactor, Olburgen, Tilburg, Hengelo, Apeldoorn and Amersfoort.

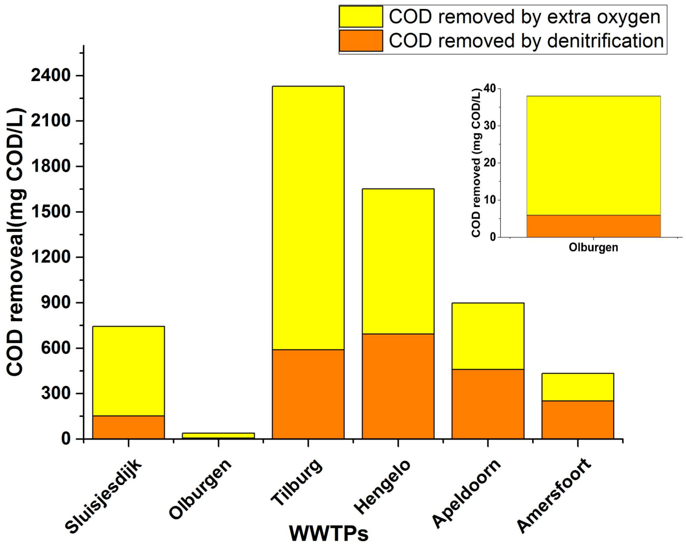

Fig 4.7 COD removal by denitrification and extra oxygen for Sluisjesdijk, Olburgen, Tilburg, Hengelo, Apeldoorn and Amersfoort.

4.2.3 Effect of THP on AOO and NOO activity of PN/A sludge

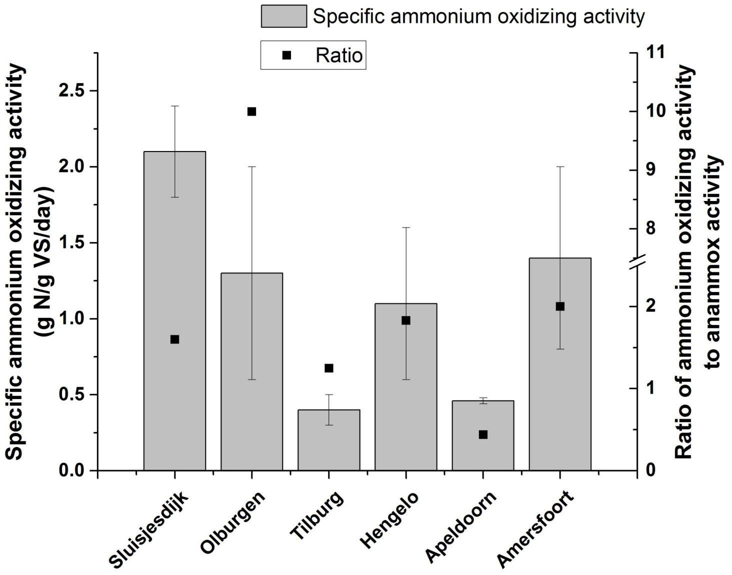

Fig 4.8 Specific ammonium oxidizing activity measured for Sluisjesdijk Sharon reactor, Olburgen, Tilburg, Hengelo, Apeldoorn and Amersfoort and the ratio of ammonium oxidizing activity to anammox activity in each WWTPs. The column is the specific ammonium oxidizing activity and the dot is the ratio of ammonium oxidizing activity to anammox activity.

Specific ammonium oxidizing activity(gN_2-N/gVS/day) was measured in this research by respirometry (Fig 4.8) in order to know the ammonium conversion capacity in the studied WWTPs. Sluisjesdijk Sharon reactor had the highest value of specific ammonium oxidizing activity of 2.1±0.3 gN_2-N/gVS/day due to separate PN/A process meaning that higher concentration of AOO per VS, which was higher than 1.2 gN_2-N/gVS/day in a Sharon reactor reported by Fux *et al*. (2002). The possible reason was the higher concentration of ammonium in the influent of Sharon reactor as the ammonium concentration for Sluisjesdijk was 1190±119 mgN/L, while 659±56 mgN/L was measured in the research by Fux *et al.* (2002). Compared with Sluisjesdijk Sharon reactor, other WWTPs had single reactor for PN/A process, and lower activity of ammonium oxidizing was observed due to less concentration of AOO in the granular biomass. Lower activity of ammonium oxidizing in Tilburg, Hengelo, Apeldoorn which had THP pretreatment, indicating negative effect may be caused by THP pretreatment on AOO activity. NOO activities was neglected in this study since the activities were low in the studied WWTPs.

Partial nitritation process by AOO could provide nitrite for anammox process. The ratio of ammonium oxidizing activity to anammox activity for distinct WWTPs are shown in Fig 4.6. For Sluisjesdijk, Tilburg, Hengelo and Amersfoort, the ratio were 1.6, 1.25, 1.8 and 2, respectively. In accordance with the stoichiometry of deammonification mentioned before, the ratio of nitrite to ammonium is 1.32. As a result, for these four WWTPs, AOO played superior performances cooperating with anammox in PN/A process. For Olburgen, ammonium oxidizing activity was ten times than anammox activity. It may be caused by flocculent biomass increasing nitrite in the system (Vlaeminck *et al.*, 2008). For Apeldoorn, the ratio of ammonium oxidizing activity to anammox activity was less than 1, while no limitation of nitrite on anammox activity was observed. The flocculent biomass may be washed out for Apeldoorn leading to lower specific ammonium oxidizing activity. Vlaeminck *et al.* (2008) reported that four times higher of specific ammonium oxidizing activity of flocs than granular biomass.

Inhibition of FA concentration on nitritation was reported as 10-150 mg/L (Anthonisen *et al.*, 1976). In this research, Tilburg, Hengelo, and Apeldoorn where had lower than 10 mg/L FA in the reject water (Table 4.3), so FA might not be the reason for the low activity of AOO which in accordance with the research by Figdore et al. (2011) that no inhibition of FA on AOO activity. This could also be indicated by the Spearman correlation that was calculated as ρ=-0.48 for FA and ammonium oxidizing activity.

Higher concentration of VFA in Tilburg, Hengelo, Apeldoorn and Amersfoort were observed in the reject water due to THP pretreatment. The Spearman correlation was calculated as ρ=-0.9 for VFA and ammonium oxidizing activity, indicating inhibition of VFA on ammonium oxidizing activity. Previous researches was proposed that the inhibition of VFA on AOO activity happened at the concentraion of 2000-6000 mg COD/L (Gome *et al.*, 2000; Leyva-Díaz *et al.*, 2015). However, in this study, the concentration of VFA was much lower than this range. Moreover, Eilersen, *et al*. (1994) and Zhang *et al*. (2016) reported that no clear inhibition was observed of VFA on AOO activity. As a result, conclusion on the effect of VFA on AOO activity could not be drawn based on the data gained in this research.

In has been documented that production of refractory compounds was considered to decrease the oxygen consumption rate of AOO by limiting oxygen diffusion (Zhang *et al.*, 2016; Zhang *et al.*, 2018). The Spearman correlation was calculated as ρ=-0.6 for humic substances and ammonium oxidizing activity and ρ=-0.48 for UV254 and ammonium oxidizing activity, indicating refractory compounds may had negative effect on ammonium oxidizing activity. While, the decrease of ammonium oxidizing activity due to inhibition of refractory compounds or limitation of oxygen diffusion could not be determined in this research.

4.3 Oxygen demand in PN/A process

According to the theory, 50% to 60% of the oxygen could be saved in PN/A process as the consequence of only half of the ammonia converted to nitrite (Fux & Siegrist 2004). In order to verify the oxygen saving in PN/A process, the oxygen demand was calculated in accordance with the fraction of denitrification activity and ammonia oxidizing activity analyzed aforementioned. Part of the COD in the reject water was removed by denitrification and the remained COD should be removed by oxygen as electron acceptor. Besides, AOO used oxygen to convert NH_4^+ to NO_2^-. The actual value was shown in Fig 4.9. Due to the high concentration of NH_4^+ caused by protein hydrolysis, the oxygen demand for nitritation was high in Tilburg and Hengelo. Higher concentration of COD was found in Tilburg and Hengelo which resulted in higher oxygen demand for COD removal. The fraction of oxygen demand of COD removal and partial nitritation could be found in Fig 4.10. In Tilburg, more than 35% of oxygen should be used for COD removal. And in Sluijuesdijk, Hengelo, Apeldoorn and Amersfoort, approximate 12% of oxygen should be provided for COD removal. In general, the installation of THP could boost the demand of oxygen in PN/A process due to the high concentration of NH_4^+ and COD. However, other compounds which could be used as electron acceptor were not considered in the analysis.

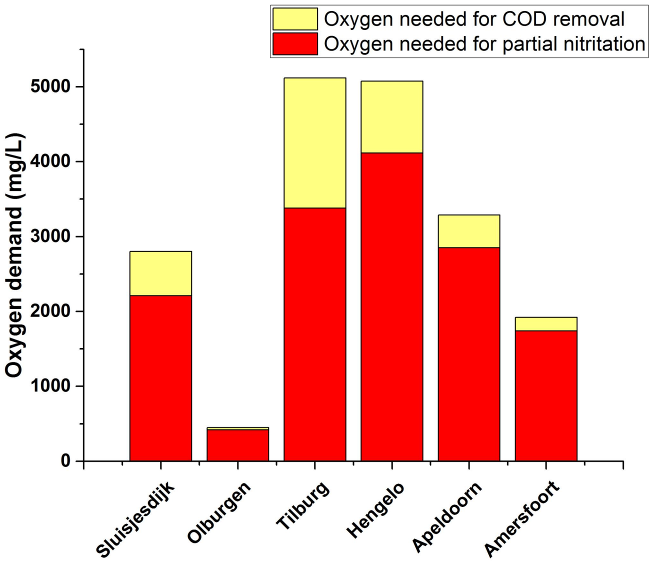

Fig 4.9 Oxygen demand for COD removal and partial nitritation in Sluisjesdijk, Olburgen, Tilburg, Hengelo, Apeldoorn and Amersfoort.

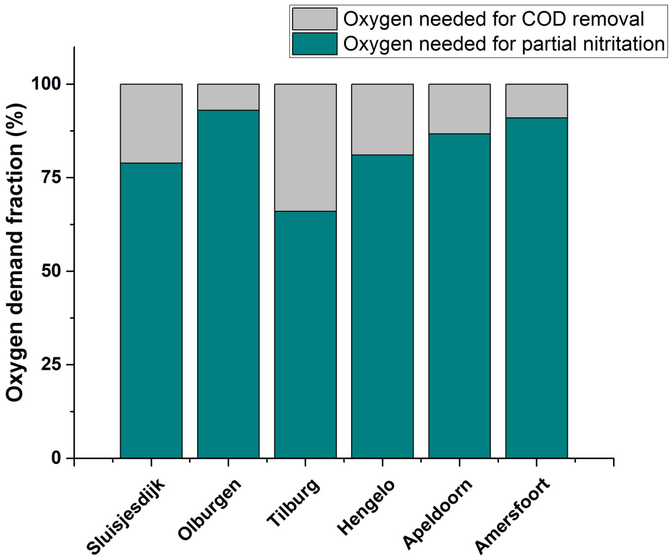

Fig 4.10 The fraction of oxygen demand for COD removal and partial nitritation in Sluisjesdijk, Olburgen, Tilburg, Hengelo, Apeldoorn and Amersfoort.

5.Conclusion

In this chapter, the findings of characteristics and microbial activities in six WWTPs were summarized. The specific research objectives for this study were:1) Identification of the differences in the conversion processes in full-scale PN/A systems with/without THP; 2) Determination of the biochemical charaterisation of the reject water with/without THP installation; 3) Identification of the oxygen demand in the PN/A process.

- Different characteristics between the studied plants with/without THP. In the WWTPs with THP installation (*i.e*. Tilburg, Hengelo, Apeldoorn and Amersfoort), a higher concentration of COD, TAN and refractory compounds in the reject water could be observed than the one in the WWTPs without THP (*i.e*. Sluisjesdijk and Olburgen).
- THP could suppress the activity of anammox. The granular biomass could be a reason for lower anammox activities in Olburgen, Tilburg, Hengelo, Apeldoorn and Amersfoort. Additionally, low values of specific anammox activity could correspond to high concentration of melanoidins indicated by the similar trend of anammox activity with color, UV254 and humic substances, although Tilburg had low anammox activities at low concentration of melanoidins. FA and VFA were considered to have no effect on anammox activities due to the low concentration in the effluent.
- High concentration of COD and suitable COD/N due to the application of THP boost the denitrification activity and lead to higher contribution of denitrifiers on nitrogen removal in the PN/A process.
- Lower AOO activities were measured in the WWTPs with THP than the one in Sluisjesdijk Sharon reactor. One possible reason was the one step PN/A process had lower concentration of AOO than Sharon reactor. The effect of refractory compounds generated in the THP could not be determined in this study.
- More oxygen were needed in the PN/A process in WWTPs with THP due to high concentration of COD and TAN.

Two main recommendations for further study were stated as following:

1) The predominant anammox species in distinct WWTPs should be clarified. In accordance with the research, different predominant species could have different specific anammox activity. As a result, the different values of SAA in distinct WWTPs could be influenced by the different predominant anammox species in the PN/A reactor. Molecular techniques should be provided to define the dominant anammox species in the studied WWTPs.

2) Isolation of the effect of distinct compounds on microbial activities should be

determined. In this study, possible mechanisms of the effect of the compounds generated in THP were proposed. Further study on the effect of specific compounds on microbial activities should be researched.

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Appendix 1 Modelling parameters for SAA and SDA

Fig A.1.1 Modelling paramters for SAA in Sluisjesdijk anammox reactor, Olburgen, Tilburg, Hengelo, Apeldoorn and Amersfoort. Nw: no washing biomass.

	Gompertz model			First-order model	
	Lag phase (day)	Maximum dinitrogen potential of the substrate (mL/g VS)	Maximum dinitrogen production rate (mL/g VS/d)	Maximum dinitrogen potential of the substrate (mL/g VS)	First order rate constant (d^{-1})
Sluisjesdijk	0.000	0.028	0.218	0.028	12.002
	0.000	0.031	0.240	0.031	12.001
	0.000	0.024	0.185	0.024	12.002
Olburgen	0.057	0.019	0.055	0.021	3.037
	0.073	0.021	0.056	0.023	2.634
	0.030	0.022	0.066	0.023	3.810
Olburgen Nw	0.000	0.020	0.116	0.020	7.196
	0.000	0.025	0.329	0.025	16.387
	0.000	0.023	0.175	0.023	10.820
Tilburg	0.000	0.021	0.034	0.021	2.496
	0.000	0.022	0.056	0.022	4.030
	0.000	0.021	0.056	0.021	4.256
Tilburg Nw	0.000	0.020	0.059	0.021	4.601
	0.000	0.020	0.056	0.020	4.429
	0.000	0.021	0.069	0.021	5.336
Hengelo	0.000	0.029	0.073	0.031	3.615
	0.000	0.029	0.127	0.030	6.915
	0.000	0.029	0.123	0.030	6.610
Hengelo Nw	0.000	0.032	0.641	0.032	29.102
	0.002	0.029	0.991	0.029	48.652
	0.000	0.029	0.874	0.029	48.345
Apeldoorn	0.005	0.028	0.338	0.028	17.086
	0.000	0.028	0.254	0.028	14.539
	0.001	0.028	0.316	0.028	17.750
Apeldoorn Nw	0.001	0.013	0.650	0.013	68.875
	0.002	0.024	1.534	0.024	89.144
	0.003	0.031	2.159	0.031	92.024
Amersfoort	0.000	0.027	0.362	0.027	21.470
	0.000	0.028	0.449	0.028	25.419
	0.000	0.028	0.429	0.028	24.524
Amersfoort Nw	0.000	0.025	0.143	0.026	8.860

	0.000	0.027	0.207	0.027	11.922
	0.000	0.022	0.202	0.022	14.315

Fig A.1.2 Modelling paramters for SDA in Sluisjesdijk anammox reactor, Olburgen, Tilburg, Hengelo, Apeldoorn and Amersfoort. Nw: no washing biomass.

	Gompertz model			First-order model	
	Lag phase (day)	Maximum dinitrogen potential of the substrate (mL/g VS)	Maximum dinitrogen production rate (mL/g VS/d)	Maximum dinitrogen potential of the substrate (mL/g VS)	First order rate constant (d^{-1})
Sluisjesdijk	0.111	0.010	0.018	0.688	0.018
	0.065	0.014	0.028	0.044	0.548
	0.072	0.016	0.025	0.510	0.040
Olburgen	0.061	0.011	0.035	0.019	1.588
	0.070	0.013	0.033	0.039	0.682
	0.024	0.014	0.037	0.019	2.260
Olburgen Nw	0.000	0.009	0.073	0.009	11.134
	0.000	0.009	0.086	0.009	13.855
	0.000	0.009	0.084	0.009	13.942
Tilburg	0.022	0.011	0.025	0.016	1.693
	0.009	0.009	0.031	0.010	4.061
	0.141	0.008	0.045	0.015	1.324
Tilburg Nw	0.045	0.011	0.069	0.013	5.313
	0.037	0.011	0.081	0.012	6.864
	0.045	0.011	0.059	0.013	4.475
Hengelo	0.006	0.012	0.040	0.012	5.042
	0.025	0.008	0.031	0.008	6.006
	0.000	0.011	0.039	0.012	5.302
Hengelo Nw	0.000	0.007	0.086	0.007	18.644
	0.000	0.012	0.160	0.012	20.933
	0.000	0.010	0.159	0.011	23.545
Apeldoorn	0.000	0.011	0.057	0.011	8.587
	0.000	0.010	0.058	0.010	8.560
	0.000	0.010	0.047	0.010	7.518
Apeldoorn Nw	0.003	0.006	0.269	0.006	59.927
	0.003	0.008	0.372	0.008	61.543
	0.004	0.007	0.406	0.007	70.253
Amersfoort	0.000	0.009	0.037	0.010	5.906
	0.000	0.009	0.031	0.009	5.257
	0.000	0.009	0.033	0.009	5.454
Amersfoort Nw	0.000	0.010	0.066	0.010	10.743
	0.000	0.009	0.073	0.010	11.627

	0.000	0.009	0.069	0.010	11.543