Influence of humic substances created in THP on partial nitritation step of PN/A reactors

By

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Abstract

Anaerobic digestion (AD) is an effective and economical in secondary sludge management. Thermal hydrolysis process (THP) is a reliable pre-treatment method to improve the biodegradability of sludge. Partial nitritation and anammox process (PN/A) is a suitable method used to remove nitrogen in reject water which has a low COD/N ratio. However, some organic compounds produced in THP process, such as melanoidins, have negative effects on AOO and anammox bacteria. Considering that humic substances occupy a large part of the soluble organics in reject water of THP-AD process. Therefore, this study aimed to investigate the acute and long-term toxicity of three types of humic substances (commercial humic acid, melanoidins and reject water) on partial nitritation step of PN/A process. The biodegradability of three types of humic substances under aerobic and anaerobic conditions, and the evolution of humic substances during the biodegradability assays were also analysed. The results showed that all three types of humic substances had higher biodegradability under aerobic condition than that under aerobic condition, and melanoidins had more biodegradable compounds than reject water and humic acid. During the AD process, humic substances acted as electron acceptors, decreasing the cumulative methane production of all three types of humic substances compared to the blank. Also, small MW fractions were transformed to large MW fractions in all three types of humic substances during both aerobic and anaerobic digestion. Moreover, during aerobic and AD, both fulvic acid-like and humic acid-like substances were formed. In addition, the results of acute toxicity tests showed commercial humic acids had a stronger inhibitory effect than reject water and melanoidins on AOO biomass. In addition, a positive correlation between humic substances concentration and AOO activity loss was observed. The long-term operation of PN reactor was failed due to the formation of granular sludge in the reactor and the growth of AOO biomass in the tubing and culture medium.

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Abbreviations

AD：	Anaerobic digestion
AOO:	Ammonium oxidizing organisms
AMO:	Ammonia monooxygenase
AHO:	Hydroxylamine oxidoreductase
BMP：	Biomethane potential
BOD:	Biochemical oxygen demand
COD:	Chemical oxygen demand
EEM:	Excitation-emission matrix
MW:	Molecular weight
NOO:	Nitrite oxidizing organisms
OUR:	Oxygen uptake rate
PN/A:	Partial nitritation / anammox
SEC-HPLC:	Size exclusion ehromatography- High performance liquid chromatography
SUVA:	Specific ultraviolet absorbance
TOC:	Total organic carbon
THP:	Thermal hydrolysis process
TS:	Total solids
VS:	Volatile solids

Content

1. Introduction

1.1 Research background and motivation

AD is an effective and economical method in secondary sludge management as it can reduce sludge volume and produce biogas for energy recovery (Khanh et, al., 2021). However, sewage sludge has low biodegradability due to the presence of complex floc-like structures and a large amount of high molecular weight organic matter (Khanh et al., 2021). Therefore, the AD performance is limited by the low biodegradability of sludge. To improve the biodegradability of sludge, numerous pre-treatment processes prior to the AD have been developed, including physical, mechanical, chemical, thermal, and biological methods (Khanh et, al., 2021). Among the pre-treatments, THP is a reliable method used to enhance anaerobic digestion, which has been commercialized for more than 25 years (Baber, 2016). Over the past 20 years, more than 82 full-scale THP facilities either in operating or in the various stage of design around the world (García-Cascallana et al., 2021). THP in general terms includes a 30 – 60 min hydrolysis operated at high temperature (140 – 180 ^{o}C) and high pressure (4.8 – 12.6 bar), followed by a sudden decompression to break down the organic matter (Zhang et al., 2020; Baber, 2016). It has been documented that THP could significantly: i) improve the biodegradability and dewaterability of sludge (Xue et al., 2015, Phothilangka et al., 2008, Higgins et al., 2017); ii) reduce the viscosity of sludge (Higgins et al., 2017); iii) reduce odour and pathogens regrowth from the dewatering step (Chen et al., 2011). Nevertheless, one of the challenges of the treatment of THP-AD centrate (dewatering liquor from the AD) is that the centrate contain a higher concentration of ammonium and organic matters compared to those without THP treatment (Cao et al., 2021). Most of these organic matters are recalcitrant and are mainly produced by Maillard reaction during THP process. These recalcitrant organics have been proven to have negative effects on AOO and anammox bacteria (Cao et al., 2021). In addition, the relative low COD/N ratio of this THP-AD centrate makes it difficult to remove nitrogen by directly recycling it into the influent of the wastewater

treatment plant (Han et al., 2020). Therefore, treating AD-THP centrate separately not only helps to meet the discharge standard but also reduces the nitrogen load of the mainline process.

PN/A has been considered as a suitable method for removing TAN from reject water and mature landfill leachate with low COD/N ratio (Qian et al., 2021, Wang et al., 2019). PN/A includes two steps: i) ammonia-oxidizing organisms (AOO) oxidize about 50% of ammonium to nitrite; ii) anammox organisms convert nitrite and ammonium to dinitrogen gas. External carbon source dosing is not needed in the PN/A process and the requirements of oxygen are about 40% lower than the nitrification/denitrification process (Ferna´ndez ed al., 2016). In the past 10 years, the PN/A process has been investigated in several lab-scale and full-scale studies for removing nitrogen in THP-AD centrate (Figdore et al., 2012, Zhang et al., 2018, Gu et al., 2018, Cao et al., 2021, Wang et al., 2021, Han et al., 2020). However, most of these studies have reported that the organic compounds produced in THP-AD cause inhibition on PN/A performance (Figdore et al., 2012, Zhang et al., 2018, Gu et al., 2018, Cao et al., 2021,). Although many studies observed the inhibitory effect of recalcitrant organics on microorganism activity, only few studies have investigated the recalcitrant organic compounds and the mechanism of inhibition. Besides, previous studies showed AOO are more sensitive to recalcitrant organics than Anammox bacteria (Zhang et al. 2016, Cao et al., 2016).

1.2 Previous studies and research gaps

Previous studies have shown that the recalcitrant organic compounds produced in THP-AD have inhibitory effect on AOO and anammox bacteria activity. According to Figdore et al. (2012), the soluble fraction of recalcitrant organic compounds lowers the AOO activity in PN/A process. Also, Cao et al. (2021) performed batch tests to identify the short-term inhibitory effects of humic substances on AOO biomass and anammox bacteria. They found AOO and anammox bacteria lost 40.6% and 41.5% of activity when exposed to only 20% THP-AD centrate. Zhang et al., (2018) also performed batch

tests to identify the short-term inhibitory effects of humic substances on AOO biomass and anammox bacteria, and they found AOO is more sensitive to recalcitrant organics than Anammox bacteria (Zhang et al., 2016). The partial nitritation serves as the first step in the PN/A process, and the inhibition of HS on it would significantly limit the performance of the whole process. Therefore, further studies on inhibitory effect of HS on partial nitritation are necessary. In addition, recent studies have almost exclusively focused on the inhibitory effect of organic matter, only few studies have focused on the identification and evolution of soluble organics in the THP-AD centrate during the PN/A process. Gu et al. (2018) performed EEM to characterize organic compounds in regular centrate and THP-AD centrate. The results demonstrated that THP-AD centrate contained a higher concentration of fluvic acid-like substances and humic acid-like substances than those without THP treatment, indicating that THP-centrate contained a higher content of recalcitrant organic matter. EEM was also performed by Cao et al. (2021) to characterize organic compounds produced in the THP-AD, and their results were similar to Gu's (2018) result. They observed fluvic acid-like substances and humic acid-like substances were detected in the THP-AD centrate, and its fluorescence intensity was much higher than regular centrate. Cao et al. (2021) also found that some fluvic acid-like and humic acid-like substances were removed or transformed during the aerobic process in the PN reactor by measuring the changes in 3D-EEM. Although these studies have identified soluble organics in the THP-AD centrate and demonstrated part of them could be removed and transformed during the PN/A process. It is still unclear how these organic compounds are transformed and removed; and subsequently impact the performance of the PN/A process. Therefore, further studies on organics transformation during PN/A process and the inhibition mechanism of these organic matters are also necessary.

1.3 Research questions and objectives

Considering that humic substances occupy a large part of the soluble organics in reject water of THP-AD process, the focus of our present work will be on the humic substance's transformation and its influence on the AOO. Therefore, this study aimed

to investigate the acute and long-term toxicity of three types of humic substances (commercial humic acid, melanoidins and reject water) on partial nitritation step of PN/A process. The biodegradability of three types of humic substances under aerobic and anaerobic conditions was investigated, and the evolution of humic substances during the biodegradability assays was also analysed.

The following four questions will be answered in this study:

1. What is the biodegradability of humic substances under anaerobic and aerobic/anoxic conditions?
2. How do humic substances transform during the biodegradation process? How do the structure and molar weight of humic substances change during the process?
3. Do humic substances affect the oxygen uptake rate of AOO biomass?
4. In the long-term running PN reactor, is the humic substances' inhibition on the microorganism caused by its complexation with trace elements?

1.4 Thesis structure

To answer the above research questions, a literature review and laboratory research was applied. This master thesis could be divided into seven chapters.

Chapter 1: Introduction

Chapter 1 introduces the general background information of this research, the relevant research questions and research objectives are also provided in this chapter.

Chapter 2: Literature review

The literature presented in Chapter 2 is firstly carried out to understand the thermal hydrolysis of sewage sludge and the partial nitritation/anammox (PN/A) process. When the research started, the collected experimental data were analyzed and explained based on the literature to ensure the theoretical accuracy of the results.

Chapter 3: Materials and methods

To answer the research questions, a series of laboratory experiments are conducted. The research method and analysis techniques are presented in Chapter 3.

Chapter 4: Results and discussions

Chapter 4 plots and illustrates the finding of the experimental results.

Chapter 5: Conclusion

Chapter 5 answers the research questions. Conclusion and recommendation are also presented in this chapter.

2. Literature review

2.1 Thermal hydrolysis process

AD is commonly used around the world to treat sewage sludge, since it can not only reduce sludge quantities but also recover energy (Khanh et al., 2021). However, sewage sludge has complex organics composition, comprising the complex floc-like structures (extracellular polymeric substances) and a large amount of high molecular weight organic matter (Khanh et al., 2021). The complex organic composition of the sludge lead to longer retention times and larger reactor volumes, and result in less biogas production (Khanh et al., 2021). Consequently, numerous pre-treatment processes prior to AD have been developed to improve the biodegradability of the sludge, including physical, mechanical, chemical, thermal, and biological method (Khanh et al., 2021). Among the developed pre-treatment process, THP has been shown to be a reliable technology and has been commercialized for more than 20 years (Baber, 2016).

During THP, sewage sludge is treated in two ways in series (shown in figure 1.1): i) High-temperature and high-pressure conditions can accelerate water's activity with polymers or other high-molecular-weight substances and convert them into simpler compounds (Hii et al., 2014); ii) a steam explosion generated by rapid decompression disintegrates the sludge by causing a rupture of the cell walls (Yan et al., 2022). After THP treatment, the complex compounds and cellular content of sewage sludge are grinded. The intracellular material and water are released, which makes sludge easier to digest. Consequently, THP enhances the biodegradability of sewage sludge and improves the performance of biogas production. The increase in biogas production compared to AD without THP varies from 30% to 70% in waste activated sludge (Phothilangka et al., 2008, Oosterhuis et al., 2014).

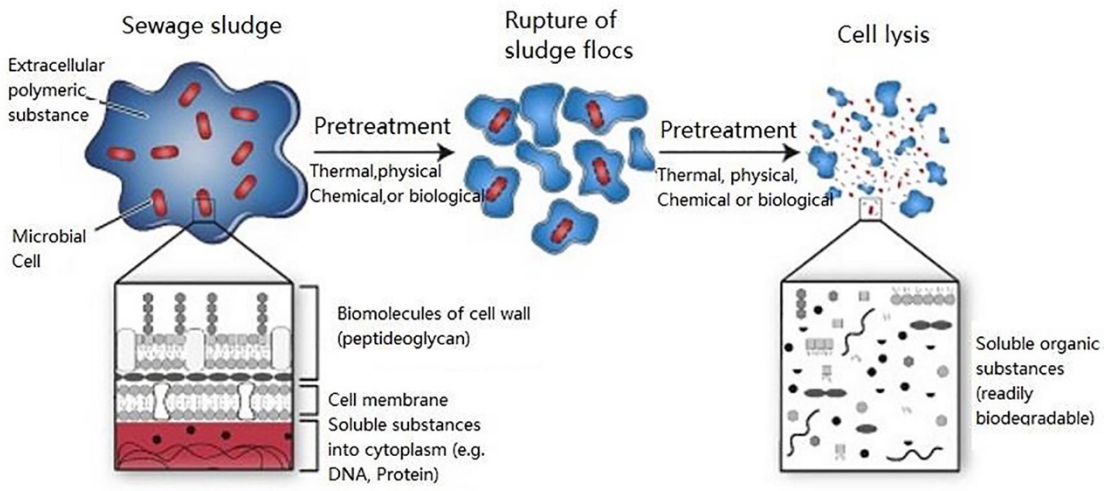


Figure 1.1 Mechanism of thermal hydrolysis process (Yan et al., 2022)

THP is usually performed at 140 °C to 180 °C and pressure of 6 to 11 bars, with a time span of 30 – 60 minutes (Phuong et al., 2021, Penaud et al., 2000). Temperature is the main factor that influences THP, and the properties of pre-treated sludge are significantly different under different temperatures (Phuong et al., 2021, Yan et al., 2013, Dwyer et al., 2008). Theoretically, a higher temperature increases the reaction kinetics of hydrolysis and thus increases the solubilization of sludge (Baber, 2016). However, the use of THP produces brown recalcitrant compounds in the soluble fraction at temperatures of 140-165 °C (Baber, 2016). The generation of recalcitrant compounds is attributed to Maillard reactions. Melanoidins are believed to be recalcitrant to biodegradation because of their complex structure (Chandra et al., 2008).

2.1.1 Production of Melanoidins

One drawback of using THP in the pre-treatment process is the higher concentration of refractory organic compounds in the THP-AD centrate compared to the centrate without THP treatment. The production of these brown recalcitrant compounds in the THP pre-treatment is mainly attributed to Maillard reaction (Dwyer et al., 2008). Maillard reaction is a non-enzymatic reaction between amino compounds and carbonyl moiety of sugar molecular, which was first described in 1912 (Maillard, 1912). It is reported that sewage sludge (primary, activated sludge and digested sludge) contains 20-40% polysaccharides and 30-50% proteins, making it a suitable reactant for the Maillard

process (Jimenez et al., 2013). The Maillard reaction is a series of reactions (shown in figure 1.2), which could be divided into three stages (Hodge, 1953). In the first stage, amino groups in proteins and carbonyl groups in sugars react to produce a N-substituted glycosylamine, which is then transformed to 1-amino-1-deoxy-2-Ketose under Amadori rearrangement (Nursten, 2005). The products of the first stage continue to be dehydrated and fragmented in the middle stage, producing a variety of intermediates, like reductones, and fission products. The intermediates undergo condensation in the final step, producing melanoidins (Nursten, 2005). The production of melanoidins has a positive correlation with the increase in temperature between 140 and 165 °C, which is typically the temperature range of THP process (Baber., 2016).

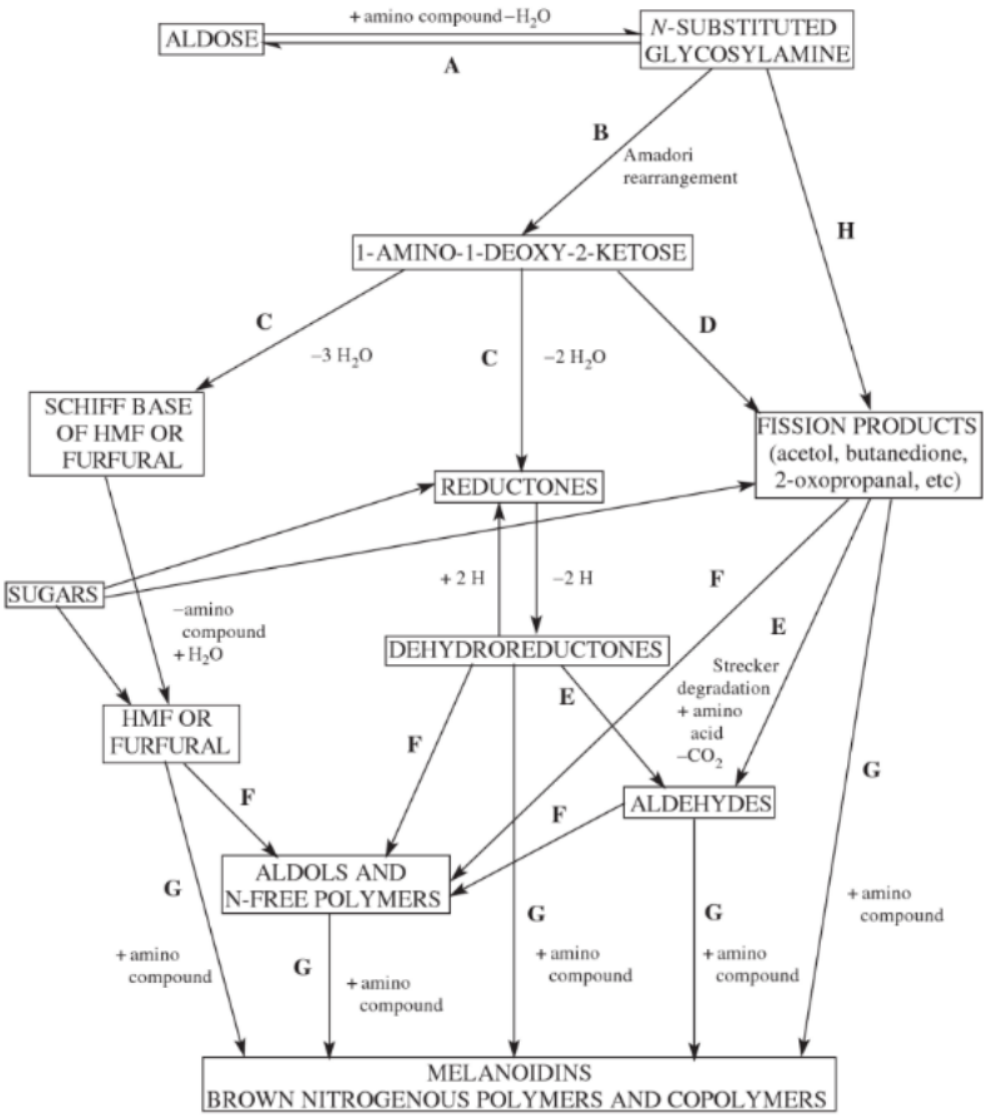


Figure 1.2 Mechanisms of Millard reaction (Nursten, 2005)

2.2 Biological ammonium removal methods

2.2.1 Nitrification/denitrification

Ammonium is converted to nitrate in the nitrification process (Schloesing and Müntz, 1877). Nitrification mainly includes two steps: i) ammonium is oxidized to nitrite; ii) produced nitrite is then oxidized to nitrate. Both AOO and NOO are chemolithoautotrophic bacteria, which use inorganic carbon as the carbon and energy source.

The catabolic reaction equation for AOO is shown in equation 1:

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + H_2O + 2H^+ \qquad \text{Eq. 1}$$

The catabolic reaction equation for NOO oxidizes nitrite is shown in equation 2:

$$NO_2^- + 1.5O_2 \rightarrow NO_3^- \qquad \text{Eq. 2}$$

Denitrification is the process of converting nitrate to dinitrogen gas by denitrifying bacteria following the pathway introduced by Payne (1973):

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

Nitrification/denitrification is a conventional biological nitrogen removal method widely used in domestic wastewater treatment and industrial wastewater treatment (Razia, 2014). During denitrification process, the microorganisms use organic matter as carbon source and electron doner. However, it has some drawbacks, such as a high oxygen requirement for nitrification and a need for organic carbon for denitrification, which cause high cost (Li et al., 2018).

2.2.2 Anammox

Anammox has gained much attention around all over the world as a viable biological nitrogen removal technique because of its high nitrogen removal efficiency, low energy consumption, low requirement for exogenous organics, and low sludge production (Zhao et al., 2021). In the anoxic ammonium oxidation (anammox) process, anammox bacteria covert ammonium to dinitrogen gas by using nitrite as the final electron acceptor. The stoichiometric equation for anammox process is shown in Eq. 3 (Strous et al., 1998):

$$NH_4^+ + 0.13H^+ + 1.32NO_2^- + 0.66HCO_3^- \rightarrow 0.066CH_2O_{0.5}N_{0.15} + 1.02N_2 + 0.26 NO_3^- + 2.03H_2O \quad Eq.3$$

2.2.3 PN/A process

Since 1995, a novel biological nitrogen removal technology based on partial nitritation and anammox process has become an attractive solution for municipal wastewater centrate (with low COD/N ratio) nitrogen removal (Fernández et al., 2016). PN/A includes two steps: i) ammonia-oxidizing organisms (AOO) oxidizes about 50% ammonium to nitrite; ii) anammox organisms convert nitrite and ammonium to dinitrogen gas. The possible metabolism pathway of partial nitritation/anammox process is shown in Figure 1.3. The predominant thinking of ammonium oxidation, which is primarily based on investigations of the model AOO Nitrosomonas Europaea, is that it is a two-step enzymatic process (Vajrala et al., 2013). Ammonium is first oxidized to NH_2OH, catalyzed by AMO. NH_2OH is subsequently oxidized to nitrite by a multiheme enzyme, HAO (Caranto et al., 2017). In the anammox process, nitrite produced in PN is converted to dinitrogen gas. According to Kartal et al. (2016), nitrite conversion occurs in three sequential redox reactions with two intermediates, NO and N_2H_4. First, nitrite is reduced to NO, catalyzed by nitrite reductase. Then, NO combined with NH_4^+ are converted to N_2H_4, catalyzed by hydrazine synthase. In the final step, N_2H_4 is oxidized to N_2, and hydrazine dehydrogenase is hypothesized to be the catalyst for this step.

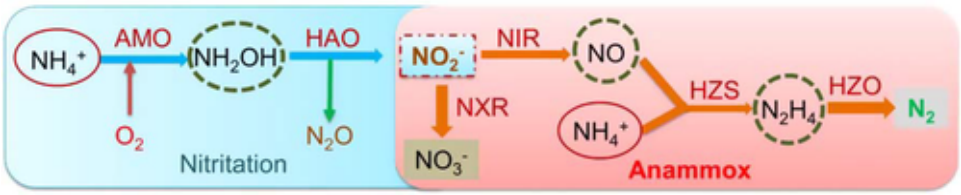


Figure 1.3 Metabolic mechanism of partial nitritation-anammox process performed by AOO and anammox bacteria (Li et al., 2018)

Compared to the conventional biological nitrogen removal process (nitrification/denitrification), PN/A has three advantages: i) oxygen demand is lower

than the conventional nitrogen removal process via nitrification and denitrification since only 50% ammonium needs to be partially oxidized to nitrite (Cao et al., 2017); ii) Both AOO and Anammox bacteria are autotrophic, therefore the biodegradable organic matter is not necessary for PN/A process; iii) excess sludge could be reduced by 80% due to the small biomass yield of Anammox bacteria (Cao et al., 2017). Considering the less oxygen requirement and less excess sludge treatment demand, the cost for PN/A (0.22 €/kg N) is three times cheaper than the conventional nitrification/denitrification process (Fernández et al., 2016). PN/A has gained interest by researchers all over the world due to its significant advantages, and more than 200 full-scale reactors has been operating in Asia and Europe (Lackner et al. 2014).

2.2.3.1 One-stage and two-stage PN/A reactors

The PN/A process can be accomplished by one-stage or two-stage systems (shown in Figure. 1.4). In the one-stage system, partial nitritation and anammox are integrated in one reactor, low DO is maintained in the reactor to protect Anammox bacteria and provide favorable conditions for partial nitritation (Zhao et al., 2019). In the two-stage system, half of the ammonium is oxidized to nitrite by ammonia-oxidizing organisms (AOO) in a partial nitritation reactor, and subsequently is fed into anammox reactors with other half unoxidized ammonia (Zhao et al., 2019). Several PN/A reactor configurations have been developed since 1995 (Lackner et al., 2014). For better control of partial nitritation, most of the earlier full-scale PN/A installations used two-stage configuration reactors (Lackner et al., 2014). More experience was gained with the installation of full-scale PN/A. Then, the focus was shifted from two-stage configuration reactors to one-stage configuration reactors which are much easier to operate and has lower investment costs (Lackner et al., 2014). Consequently, one-stage configuration has been applied in over 88% of full-scale PN/A installations (Lackner et al., 2014).

The first full-scale two-stage configuration PN/A installation was SHARON-ANAMMOX, which was established in Rotterdam Dokhaven WWTP (Hellinga et al., 1998). For the one-stage full-scale PN/A installation, DEMON® and CANON®

configurations are commonly used (Corbalá et al., 2016). DEMON® configuration is based on a SBR system with a pH-based control strategy (Wett et al., 2007). In PN/A process, partial nitritation process reduces the pH, while anammox reaction increases the pH; therefore, pH can be used as control parameter to characterize the current state of reactions in SBR reactors. In the DEMON® configuration, the duration of aeration in SBR system is controlled by pH to maintain a balance between partial nitritation reaction and anammox reaction. CANON® configuration is based on a SBR system with a DO-based control strategy (Lv et al., 2019). Under oxygen limiting conditions, a balance between partial nitritation reaction and anammox reaction was achieved.

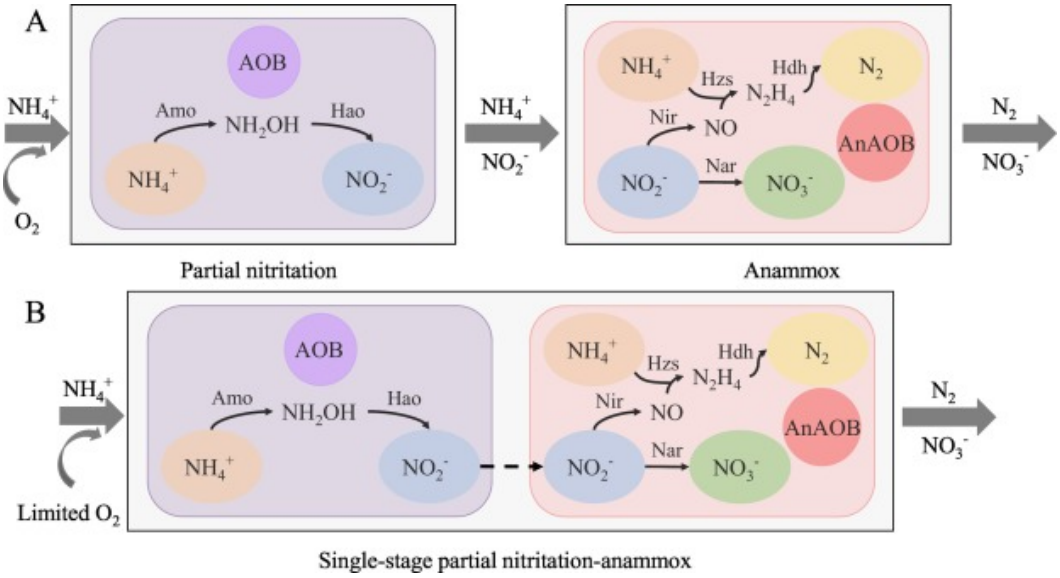


Figure1.4 Metabolic mechanism of PN/A process involved in both one-stage and two-stage reactor (Zhao et al., 2019).

2.2.3.2 Microbial community in PN/A

The microbial community in PN/A reactors is mainly composed of AOO, NOO, anammox bacteria, and heterotrophic bacteria (Cao et al., 2017). AOO and Anammox bacteria are the microorganisms responsible for nitrogen removal in the PN/A. NOO are difficult to separate from AOO in the environment, so they inevitably appear in the PN/A process (Cao et al., 2017). Three kinds of HB are reported to be found in PN/A

when wastewater contains a high C/N ratio, comprising heterotrophic denitrifiers, glycogen-accumulating organisms and phosphorus-accumulating organisms (Cao et al., 2017). However, the presence of NOO and HB in the reactor will affect the performance of PN/A process. The species of AOO, NOO, and Anammox bacteria described in the literatures are shown in Table 1.1.

16 species of AOO have been described and named since 1980s (Koops et al., 2005). Three AOO genera have been reported to perform partial nitritation reactions in PN/A process, comprising Nitrosospira (Qiu et al., 2019; Siripong and Rittmann et al., 2007), Nitrosomonas (Luo et al., 2017; Blackburne et al., 2008b) and Nitrosococcus (Qiu et al., 2019). Nitrosomonas genus is reported as the most abundant AOO bacterial group in PN/A process, which is primarily due to its high substrate and oxygen affinities (Shourjeh et al., 2021).

The NOOs are phylogenetically affiliated with a specialized group of Proteobacteria. The species of Proteobacteria responsible for nitrite oxidation are from genera such as Nitrospina (Lucker et al., 2013), Nitrobacter (Han et al., 2018), Nitrospira (Siripong and Rittmann et al., 2007), and Nitrococcus (Mohamed et al., 2010). Nitrospira is reported as the dominant NOO species in the wastewater system (Siripong and Rittmann et al., 2007; Cao et al., 2017).

Anammox reactions are carried out by the bacteria from mainly six candidate genera (Pereira et al., 2017), including Candidatus Anammoxoglobus (Liu et al., 2008; Kartal et al., 2007), Candidatus Jettenia (Quan et al., 2008; Nikolaev et al., 2015), Candidatus Brasilis (Viancelli et al., 2011), Candidatus Kuenenia (Schmid et al., 2000; Wang et al., 2018), Candidatus Scalindua (Schmid et al., 2003) and Candidatus Brocadia (Kartal et al., 2008).

Table 1.1 AOO, NOO, and anammoxdescribed in the literature (modification after Cao et al. (2017) and Pereira et al. (2017)).

AOO Genus	Habitat	References
Nitrosospira	Municipal wastewater	Siripong and Rittmann, et

	treatment system	al. (2007)
Nitrosomonas	PN/A wastewater treatment system	Qiu et al. (2019) Blackburne et al., 2008b Luo et al. (2017)
Nitrosococcus	PN/A wastewater treatment system	Qiu et al. (2019)
NOO Genus		
Nitrospina	Marine	Lucker et al., (2013)
Nitrobacter	Fertilization treatment system	Han et al., (2018)
Nitrospira	Municipal wastewater treatment system	Siripong and Rittmann (2007)
Nitrococcus	Marine	Mohamed et al., (2010)
Anammox Genus		
Candidatus Anammoxoglobus	Wastewater treatment system	Liu et al., (2008) Kartal et al., (2007)
Candidatus Jettenia	Wastewater treatment system	Quan et al., (2008) Nikolaev et al., 2015
Candidatus Brasilis	Wastewater treatment system	Viancelli et al., 2011
Candidatus Kuenenia	Wastewater treatment system	Schmid et al., (2000) Wang et al., (2018)
Candidatus Scalindua	Wastewater treatment system	Schmid et al., (2003)
Candidatus Brocadia	Wastewater treatment system	Kartal et al., (2008)

2.2.3.3 Factors influencing PN/A process

2.2.3.3.1 DO

DO is critical to start and operate the partial nitritation process since it could significantly impact the diversity and kinetics of AOO and NOO (Cao et al., 2017). The

optimal dissolved oxygen level in the PN process should promote the growth of AOO while suppressing NOO (Li et al., 2018). The suppression of NOO mainly depends on the high oxygen affinity of AOO compared to that of NOO under oxygen limetted conditions. Several studies demonstrated that the growth rate of NOO is slower than that of AOO under low dissolved oxygen concentrations, DO <0.5 mg O_2/L is commonly used to specifically inhibit NOO growth (Li et al., 2018; Gilbert et al., 2015; Blackburne et al., 2008). However, recent research demonstrated that partial nitritation might also benefit from high DO concentrations (>1.5 mg O_2/L) with an intermittent aeration strategy (Wett et al., 2013; Li et al., 2018). According to Cao et al. (2016), when DO was kept between 1.5 and 2.0 mgO_2/L during the aerobic phase, the nitrogen removal performance increased. This finding suggests that intermittent aeration with relatively high DO could support AOO growth while suppressing NOO.

2.2.3.3.2 Temperature

The effect of temperature on growth rate among different groups of microorganisms makes it another important factor in the PN process (Li et al., 2018). Applying optimal temperature could maintain the balance between AOO, Anammox bacteria, and NOO, which thus improves the performance of PN/A (Shourjeh et al., 2021). AOO grow more quickly than NOO at temperatures higher than >25 °C (Xu et al., 2015). Therefore, NOO could be suppressed at high temperatures between 30 and 40 °C with a short SRT of around 1.5 days (Li et al., 2018). Furthermore, it has been suggested that temperatures between 30 and 40 °C are ideal for anammox-based technologies to achieve adequate nitrogen removal efficiency (Shourjeh et al., 2021). Typically, most of the studies on PN/A was carried out at temperature 30-35 °C (Li et al., 2018)

The drop in temperature has a great impact on the composition of the microbial community, which may therefore have an impact on the performance of the PN/A process. As temperature drops, the decrease rate of NOO growth rate is less than AOO's, which might lead to NOO outcompeting AOO within the range of 10 to 20 °, affecting the PN/A performance (Li et al., 2018). According to Lackner et al. (2015), AOO and Anammox bacteria growth rate decreased at a temperature lower than 12 °C, which led to a decrease in the ammonia conversion rate and an increase in nitrate accumulation in

both, SBR and MBBR reactors.

2.2.3.3.3 pH

According to Feng et al. (2017), the optimal pH for AOO and NOO activities is predicted to be 7.4 and 6-7.5 at 30°C, respectively. However, another study found that a pH variation between 7 and 8 would be a suitable range to suppress NOO. In recent studies on the PN/A process, pH was adjusted to 7~7.5 (Li et al., 2018)

2.2.3.3.4 Inhibitors

- Free ammonia

Free ammonia inhibits the growth of AOO bacteria if free ammonia concentration exceeds the threshold. Different threshold values were proposed for nitritation inhibition (Razia, 2014). According to the study of Li et al. (2012), nitritation step was inhibited when free ammonia concentration exceeded 20 mgN/ L. Razia (2014) reported the inhibition of free ammonia on AOO activity occurred when the concentration of ammonia was between 8 and 120 mg N/L. And AOO lost all activity when the concentration of ammonia was higher than 120 mg N/L. Another study said the inhibition on AOO activity occurred when the concentration of free ammonia was higher than 2 mg N/L.

- Free nitrous acid

Different threshold values were proposed for free nitrous acid inhibition. Egli et al. (2001) reported that anammox lost all activity when the nitrite concentration was higher than 185 mg N/L. Me at al. (2016) reported anammox lost all activity when the nitrite concentration was higher than 280 mg N/L. However, according to the study of Depena-Mora et al. (2007), anammox only lost 50% activity when the nitrite concentration was 350 mg N/L

pH variations have a great impact on occurrence of free ammonia and free nitrous acid, which work together to inhibit the bacteria.

2.3 THP's influence on PN/A process

Recently, the feasibility of treating THP-AD centrate with PN/A have been investigated in several lab-scale and full-scale studies (Zhang et al., 2018, Gu et al., 2018, Cao et al., 2021, Wang et al., 2021, Han et al., 2020). These studies mainly focus on two aspects: i) the inhibition on microorganism activity by recalcitrant organic compounds produced in THP-AD. Ii) the possible control strategies for mitigating the effect of recalcitrant organics on PN/A process performance.

Wang et al. (2021) conducted a one-stage PN/A process for treating THP-AD centrate. A nitrogen removal rate of 0.58 ± 0.06 g N/(L·d) was achieved in treating anaerobic digester effluent from THP-AD, which was much lower than the nitrogen removal rate (0.94 ±0.39 g N/(L·d)) of start-up period (synthetic wastewater). Also, according to Figdore et al. (2012), the performance of a pilot-scale DEMON process was significantly reduced with the addition of THP-AD centrate. Furthermore, Chan et al. (2005) found that THP-AD reject water inhibits anammox more severely than conventional AD reject water in a full-scall PN/A application. Han et al. (2020) investigated the start-up and long-term nitrogen removal performance of the full-scale PN/A process for treating THP-AD centrate. The full-scale plant could treat 2500 m^3 THP–AD sludge filtrate every day, and the average nitrogen removal rate of the PN/A process was 0.21-0.28 g N/(L·d); however, unstable performance was observed during the operation of this full-scale PN/A process. The unstable performances and lower nitrogen removal rate observed in these studies were ultimately attributed to the acute toxicity of recalcitrant organic compounds (Cao et al., 2022).

Several studies further focused on the composition of recalcitrant organic compounds in the THP-AD centrate and the possible inhibition mechanism (Table 1.2). Batch test were performed by Zhang et al. (2018) to investigate the effect of THP-AD centrate on the activity of AOO and Anammox bacteria. They found that AOO and Anammox bacteria lost more than 80% activity when they were exposed to 50% THP-AD centrate. Then, they divided the content of organics in THP-AD centrate into four sub-fractions

(particulate COD, small colloidal COD, large colloidal COD, and soluble COD) and used a microbial kinetic model to estimate how each component inhibits the activity of microorganisms. The results showed that soluble carbon compounds in the centrate could directly inhibit AOO. Also, the particulate and colloidal organic could inhibit AOO by causing diffusion limitation on the AOO containing flocs. Lackner et al. (2014) reported polymer residue could cause foaming issues and potentially diffusion limitations. Cao et al. (2021) also performed batch tests to investigate the effect of THP-AD centrate on the activity of AOO. The results showed that AOO lost its activity by 62.7% when exposed to 60% THP-AD centrate, and anammox bacteria lost 100% of activity when exposed to 60% THP-AD centrate. They also used the size-exclusion chromatography-organic carbon detect inorganic nitrogen detection (LC—OCD-OND) to characterize the soluble organics in the PN/A process. The analysis showed that the organic compounds in the centrate could be divided into five sub-fractions, including high molecular weight protein (HMW-PN, MW>20kDa), high molecular weight polysaccharide (HMW-PS, MW>20kDa), building blocks (300-500kDa), low molecular weight neutrals (LMW neutrals, MW< 350 Da) and low molecular weight acids (LMW acids, MW< 350 Da). The results showed the building blocks that relate to the fraction of humic-like substances were the major organic compounds in THP-AD centrate. Also, EEM was performed by Gu et al. (2018) to characterize organic compounds in THP-AD centrate, which showed Fluvic acid-like substances and humic acid-like substances were the major organic compounds in THP-AD centrate.

Table 1.2 Mechanism of inhibition of recalcitrant organic compounds

Inhibitor	Inhibition mechanism	Reference
Soluble COD	Direct inhibition	Zhang et al. (2016)
Soluble organic	Direct inhibition	Cao et al. (2021)
Particulate and large colloidal COD	Indirect inhibition: limiting the diffusion of substrate (e.g. Oxygen, ammonium)	Zhang et al. (2016)
Polymer residue	Causing foaming issues and	Lackner et al.

	potentially diffusion limitations	(2014)

To mitigate the inhibition, several studies focused on the control strategies of PN/A process. Cao et al. (2021) found the inhibition effect of recalcitrant organic compounds could be mitigated by an intermittent aeration strategy. Figdore et al. (2012) recommended that a 1:1 dilution of the THP-AD centrate could maintain a stable operation. Gu et al. (2018) fed the PN/A reactors with diluted regular THP-AD centrate, and the load was increased gradually to undiluted centrate. The results demonstrate that inhibition was observed during 70% THP-AD centrate feeding and the concentration of ammonium and nitrite in the effluent increased rapidly and synchronous, which indicates the dilution of centrate could achieve stable PN/A performance. A similar experiment was carried out by Cao et al. (2021), and the ideal nitrogen removal rate was achieved when the feeding proportion of THP-AD centrate was below 60%. The nitrogen removal rate was 0.55 g N/(L·d), which was quite close to the nitrogen removal rate of AD without THP.

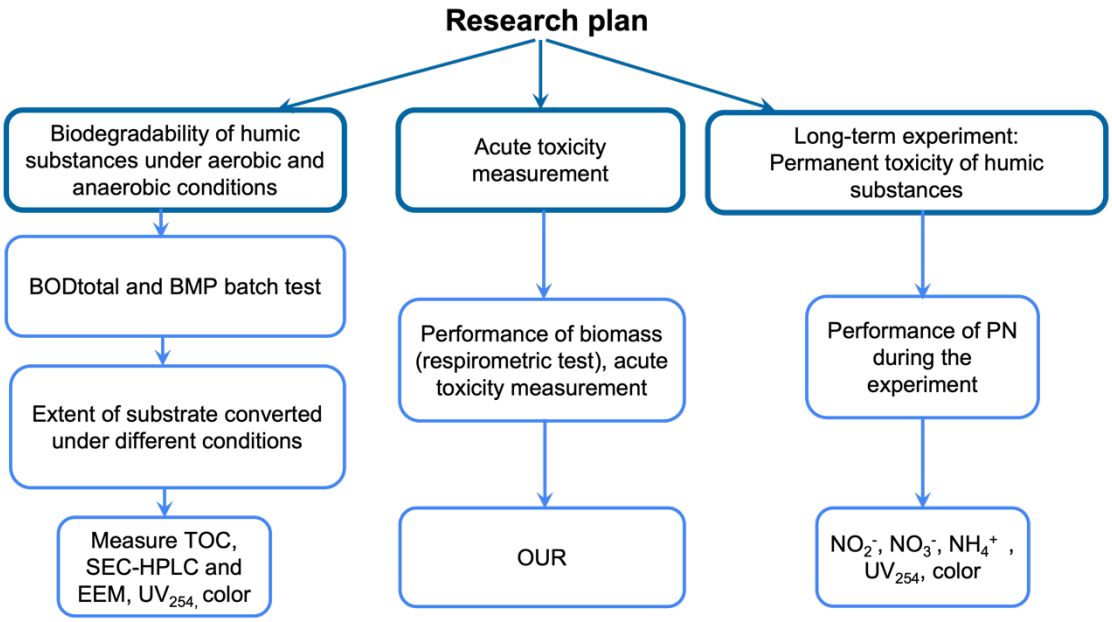
2.4 Literature evaluation

Literature review shows THP could improve AD digestion performance. However, THP-AD produces AD-refractory organic compounds, which would cause the unstable performance of the PN/A process. Earlier studies, as well as current work, focus on the operation strategy of PN/A process, such as intermittent aeration and dilution to mitigate the inhibition of microorganisms by recalcitrant organic compounds. Only a few studies focus on the identification and evolution of these recalcitrant organic compounds in the THP-AD centrate during PN/A process. These studies have identified soluble organics in the THP-AD centrate and demonstrated part of them could be removed or transformed during the PN/A process. However, no study to date has reported how these organic are transformed and removed and subsequently impact the performance of PN/A process. To bridge the gap of the evolution of these recalcitrant

organic compounds during biodegradation, the evolution of organic compounds along PN-anammox processes is required to be investigated further. In addition, the mechanism of inhibition of microbial activity by these recalcitrant organic substances has also been little studied.

3. Materials and methods

Research plan



Humic substances preparation

Commercial humic acid (CAS number: 1415-93-6, Sigma-Aldrich, USA), synthetic melanoidins and reject water were used as HS in this study. The melanoidins used in the study were prepared by mixing 0.25 M glycine, 0.25 M glucose, and 0.125 M sodium bicarbonate and autoclaving for 3 hours at 121°C (Migo et al., 1993). Reject water was obtained from the dewatering step of a THP-AD process in Hengelo wastewater treatment plant (WWTP), The Netherlands. For acute toxicity tests, reject water was heated to 55 °C and dinitrogen was bubbled to strip the high concentration of ammonium in it.

3.1 Acute toxicity tests

To evaluate the acute toxicity of incremental concentration of humic substances (1, 2, and 3g COD/L), on the AOO biomass activity, commercial humic acid, melanoidins

and reject water were used in this study. The activity of AOO biomass under different concentration (1, 2, and 3 gCOD/L) of humic substances was measured with respirometric tests, and then compared with control groups in absence of HSs. The respirometric test were used because they could provide the assessment of specific ammonium oxidizing activity (gN_2-N/gVS/day) of AOO biomass to express the activity of AOO biomass. The respirometry tests were performed in a batch reactor (shown in Figure 3.1), using pre-concentrated AOO biomass which was sampled from a SHARON reactor located at Sluisjesdijk WWTP, The Netherlands. The pre-concentrated AOO biomass was prepared through centrifugation (20 min at 7000rpm). To measure the maximum conversion rate of the ammonium that is oxidized using oxygen as final electron acceptor, the sealed batch reactor was firstly filled with culture medium (shown in Table 3.1). The pH of the culture medium was adjusted to 7.45-7.55 at the beginning of the experiment. A temperature- controlled water bath was used to keep the temperature constant at 35±1°C. The reactor chamber was stirred open to reach DO saturation concentration. HS were added to the reactor to reach different concentrations (1, 2, and 3 gCOD/L). Pre-concentrated AOO biomass was then added to the batch reactor with a syringe to reach a concentration of 0.2 gVS/L in the batch reactor. The substrate (NH_4Cl) was added to the batch reactor through syringes to reach a concentration of 80 mg TAN in the reactor. In addition, 10mM $NaClO_3$ was added to the reactor to inhibit NOO biomass activity (Fu et al., 2018). During the experiment DO was online measured with a dissolved oxygen probe (Multi 3630-ids, WTW, Germany), and the data were stored in the laptop connected to the multimeters (shown in Figure 3.1). The reaction was stopped when the DO concentration reached 3mg/L, to prevent the influence of the oxygen affinity constant. To measure the endogenous respiration consumption, the oxygen consumption was measured in the batch reactor without substrate and discounted from the measurements with substrate.

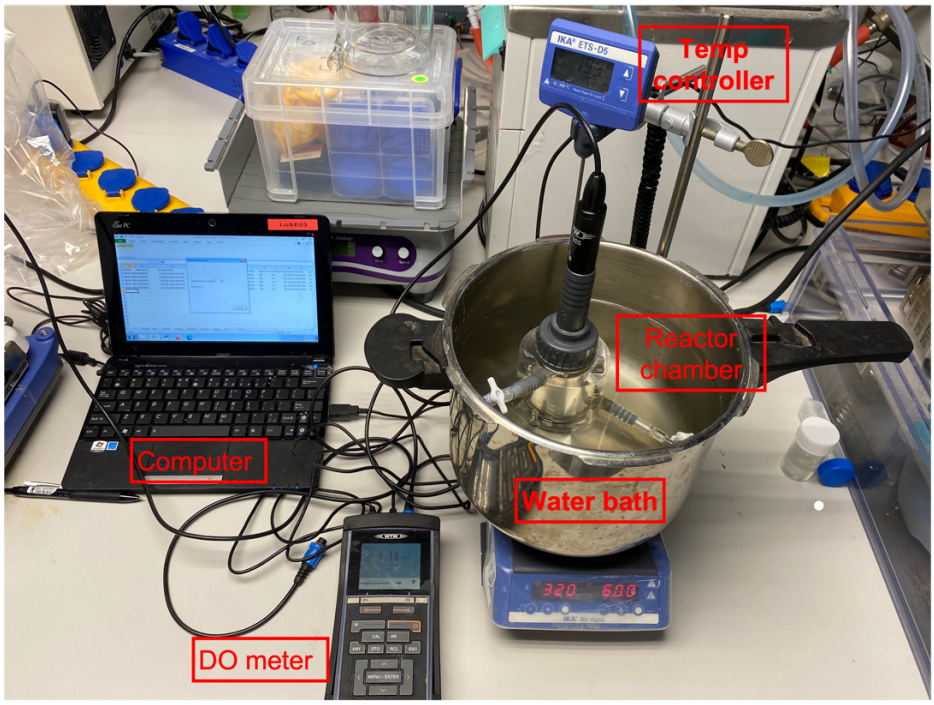


Figure 3.1 Set-up of respirometry test

To calculate the AOO conversion activities, it was needed to calculate the maximum rate of nitrogen conversion in each reactor and the endogenous respiration rate. The maximum nitrogen conversion rate of AOO could be derived from OUR as shown in Eq.1. The OUR was derived from the slope of the linear fitting regression curve of DO curve using 3 points.

$$\frac{D[NH_4^+\text{-N}]}{dt} = \frac{d[DO]}{dt} * \frac{-1}{3.43 \frac{gCOD}{gNH_4^+\text{-N}}} \qquad \text{Eq. 1}$$

The specific ammonium oxidizing activity was expressed in the mass of nitrogen converted per mass of AOO biomass and per unit of time (gN_2-N/gVS/day). The maximum specific ammonium oxidizing activity could be calculated from Eq.2.

$$\text{S-AOO} = \text{MAX}\left(\frac{\frac{d[NH_4^+\text{-N}]}{dt} - \frac{d[A_{endogenous}]}{dt}}{X_M}\right)\left(\frac{gN_2\text{-N}}{day*gVS}\right) \qquad \text{Eq. 2}$$

Where,

S-AOO: Specific AOO activity

$\frac{d[NH_4^+\text{-N}]}{dt}$: Conversion rate of ammonium (mg NH_4^+-N/L/s)

$\frac{d[A_{endogenous}]}{dt}$: conversion rate due to endogenous respiration (mg NH_4^+-N/L/s)

X_M: mass of AOO biomass in the reactor (g VS)

Table 3.1 Composition of culture medium used for respirometry tests.

MINERAL MEDIUM I	
NaCl	1000 mg/L
$MgSO_4$	60 mg/L
KH_2PO_4	250mg/L
MINERAL MEDIUM II	
$NaHCO_3$	5 g/L
TRACE ELEMENTS	
$FeSO_4 \cdot 7H_2O$	2.7 mg/L
$MnCl_2 \cdot 4H_2O$	0.1 mg/L
$CoCl_2 \cdot 6H_2O$	0.024 mg/L
$NiCl_2 \cdot 6H_2O$	0.024 mg/L
$CuCl_2 \cdot 2H_2O$	0.017 mg/L
$ZnCl_2$	0.068 mg/L
Na_2MoO_4	0.024 mg/L

3.2 Anaerobic biodegradability assays

The anaerobic biodegradability of three types of humic substance (melanoidins, reject water and commercial humic acid) was evaluated by performing a BMP test. The BMP test was conducted with an Automatic Methane Potential Test System II (AMPTS II) (BPC Instruments, Sweden). AMPTS II is an analytical instrument that allows online

measurement of the (ultra-low) biomethane flow generated during anaerobic digestion of any biodegradable substrate. AMPTS II consists of three parts, incubation bath, CO_2 removing unit and CH_4 volume measuring equipment.

Figure 3.2 Instrument set-up of BMP test

In the incubation part, inoculum (digested sludge, TS=33.2±0.4gTS/L; VS= 24.0±0.4gVS/L), substrate and medium solution (phosphate buffer, micro-nutrients and macro-nutrients shown in Table 3.2) were filled in 500 mL Duran bottle (Fig 3.2), with a total reaction volume of 400 mL. Tests were performed in triplicates. To compare the biodegradability of the three different types of humic substances, the COD in all the reaction vessels was same (2.3 g COD/L). The substrate/inoculum ratio in the reaction vessels was 0.5 g COD/g VS (Eq.3). The volume of inoculum and substrates needed to be added into each reaction bottles were calculated according to the Eq3, 4 and 5. To achieve anaerobic environment, all reaction vessels were flushed with N_2 for 2 minutes before the test started. All reaction vessels were incubated in a temperature-controlled shaker (Innova 44 shaker, New Brunswick Scientific) at 35°C and 120 rpm for 30 days. Reaction vessels were connected to wash bottles which were filled with 3M NaOH solution to remove CO_2 before methane quantification. Finally, gas was analysed by the methane gas volume measuring equipment and the exact CH_4 production was recorded to the computer. The experiment was run for around 30 days until the cumulative CH_4 production remained stable.

$$\frac{V_{sub} \times VS_{sub}}{V_{sludge} \times VS_{sludge}} = 0.5 \text{ g COD/g VS} \quad \text{Eq. 3}$$

$$V_{sub} + V_{sludge} + V_{demi-water} = 0.4 \text{ L} \quad \text{Eq. 4}$$

$$\frac{V_{sub} \times VS_{sub}}{V_{sludge} + V_{substrate} + V_{demi-water}} = 2.3 \text{ g COD/L} \quad \text{Eq. 5}$$

The biodegradability of substate is defined as the actual CH_4 production from the BMP test and the theoretical methane yield considering 350 ml CH_4/g COD at 0°C and 1.013 bar.

Table 3.2 Composition of culture medium used for biomethane potential tests.

Phosphate buffer	
$K_2HPO_4 \cdot 3H_2O$	0.2 M
$NaH_2PO_4 \cdot 2H_2O$	0.2 M
30.5 ml A + 19.5 ml per liter medium to obtain 10 mM phosphate buffer at pH 7.	
Macronutrients	
NH_4Cl	160 g/L
$CaCl_2 \cdot 2H_2O$	8 g/L
$MgSO_4 \cdot 7H_2O$	9 g/L
6 ml per liter medium	
Micronutrients	
$FeCl_3 \cdot 4H_2O$	2 mg/L
$CoCl_2 \cdot 6H_2O$	2 mg/L
$MnCl_2 \cdot 4H_2O$	0.5 mg/L
$CuCl_2 \cdot 2H_2O$	30 mg/L
$ZnCl_2$	50 mg/L
HBO_3	50 mg/L
$(NH_4)_6Mo_7O_2 \cdot 4H_2O$	90 mg/L
$Na_2SeO_3 \cdot 5H_2O$	100 mg/L
$NiCl_2 \cdot 6H_2O$	50 mg/L
EDTA	1 g/L
HCl 36%	1 ml/L
Resazurine	0.5 g/L
Yeast extract	2 g/L
0.6 ml per litre medium	

3.3 Aerobic biodegradability assays

The aerobic biodegradability of melanoidins, reject water and commercial humic acid was evaluated by measuring the biochemical oxygen demand.

Inoculum, substrate and dilution water (phosphate buffer and nutrients, shown in Table 3.3) were filled in 250 ml Duran bottles All samples and blank were performed in triplicates. The procedures was conducted as follows: Humic acid, melanoidins and reject water samples were diluted 20, 100 and 2 times with dilution water, respectively. In addition, a nitrification inhibitor (Allylthiourea, 5mg/L) was also added (Wang et al.,

2017). Then, samples were saturated (higher than 7 mgO_2/L) with oxygen by aerating with compressed air. Diluted samples were then transferred to Duran bottles with specific filling volume (shown in Table 3.4). Furthermore, two sodium hydroxide pellets were added in a rubber stopper to absorb carbon dioxide produced by the microbial degradation. Then, all bottles were sealed and incubated in a temperature-controlled shaker (Innova 44 shaker, New Brunswick Scientific, Germany) at 20°C and 120 rpm. The negative pressure in bottles caused by the consumption of oxygen was manually measured by a pressure sensor every day to calculate the BOD value according to the Eq7. The experiment was run for around 30 days until the BOD remained stable.

$$BOD = \frac{M(O_2)}{R \cdot T_m} \cdot \left(\frac{V_{tot} - V_I}{V_I} + a \frac{T_m}{T_0} \right) \cdot \Delta p(O_2) \qquad \text{Eq.7}$$

Where,

$M(O_2)$: Molecular weight of oxygen (32000 mg/mol)

R: Gas constant (83,144 L·hPa/(mol·K))

T_0: Absolute Temperature (273.15 K)

T_m: Measuring temperature (293.15 K) for BOD_5

V_{tot}: Bottle volume [mL]

Vl: Sample volume [mL]

A: Bunsen absorption coefficient (0.03103)

$p(O_2)$: Difference of the partial oxygen pressure [hPa]

Figure 3.3 Set-up of BOD test

Table 3.3 Composition of dilution water used for BOD tests.

Phosphate buffer	
$K_2HPO_4 \cdot 3H_2O$	21.75 g/L
KH_2PO_4	8.5 g/L
$Na_2HPO_4*7H_2O$	33.4 g/L
NH_4Cl	1.7 g/L
1 ml per liter dilution water	
Nutrients	
$MgSO_4*7H_2O$	22.5 g/L
$CaCl_2$	27.5 g/L
$FeCl_3 \cdot 6H_2O$	0.25 g/L
1 ml per liter dilution water	
Inhibitor	
Allylthiourea, 5mg/L	

Table 3.4 Filling volume and dilution factors

	Bottle volume (ml)	Dilution factor	Filling volume (ml)	COD (g/L)
Blank	250	0	160	
Melanoidins	250	100	50	0.63
Humic acid	250	20	100	0.49
Reject water	250	2	50	1.57

3.4 Evolution of humic substances during anaerobic and aerobic assays

To investigate the evolution of melanoidins, humic acid and reject water during aerobic and anaerobic assays. In both BMP and BOD test, there is another identical control group (3 bottles for the blank; 3 bottles for melanoidins; 3 bottles for humic acid; 3 bottles for reject water) was just used to take samples. During the BMP test, samples were taken at day1, 6, 11, 17, 24. During the BOD test, samples were taken at day1, 4, 8, 11, 17, 24. TOC, UVA254 and colour of samples were measured. SEC-HPLC and EEM were also performed to investigate these organics' transformation and evolution.

3.5 Long-term effect of HS on AOO biomass activity

To investigate whether the inhibitory effect of HS on AOO biomass is due to a scarcity of trace elements as a result of complexation between HS and trace elements, a long-term experiment was conducted. Figure 3.4 shows the diagram of the long-term experiment. A chemostat with an effective working volume of 2L was used in this study. The reactor was kept at 35°C and equipped with pH, DO and temperature probes (Endress+Hauser, Switzerland) for online monitoring of pH, DO and temperature. The pH was controlled at around 7.2 by automatically adding CO_2 or Na_2CO_3 according to the online control system. CO_2 and Na_2CO_3 were also used as the carbon source. DO was controlled at around 1mg/L by automatically changing the aeration rate. One peristaltic pump was used to supply influent through the bottom of the reactor, and the effluent was withdrawing from the top of the reactor with another peristaltic pump. The hydraulic retention time (HRT) was fixed at 1.5 days. The influent comprised $(NH_4)_2SO_4$ and mineral nutrients (shown in Table 3.5).

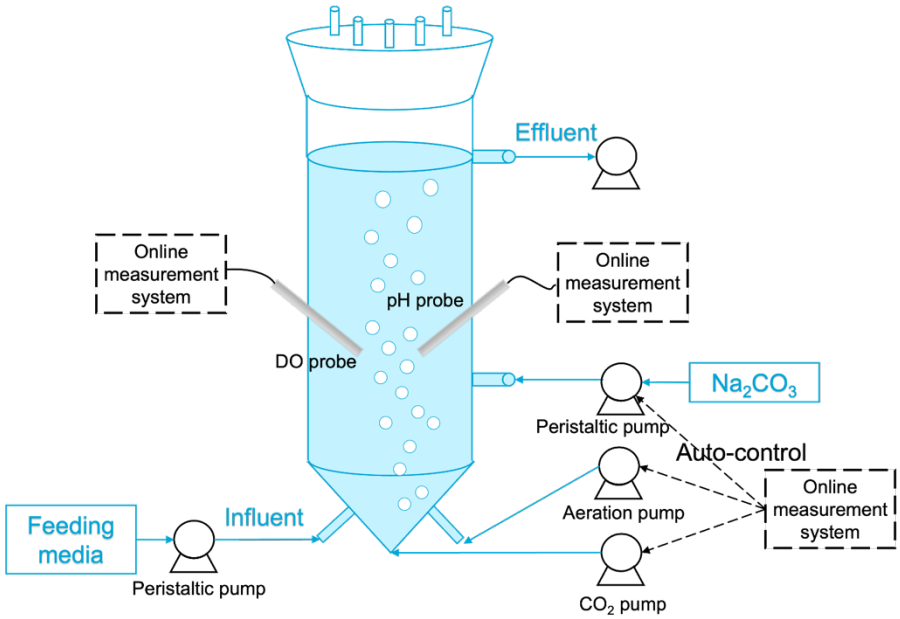


Figure 3.5 Schematic diagram of long-term experiment

Table 3.5 Culture medium used in long-term operation

Substrate	
$(NH_4)_2SO_4$	4.71 g/L
Macro-nutrients	
$MgSO_4*7H_2O$	6 g/L
KH_2PO4	12.5 g/L

$CaCO_3$	2.72 g/L
10 ml per liter feeding media	
Trace elements	
$FeSO_4 \cdot 7H_2O$	5 g/L
$CoCl_2 \cdot 6H_2O$	1.57 g/L
$MnCl_2 \cdot 4H_2O$	5.06 g/L
$CaCl_2 \cdot 2H_2O$	7.35 g/L
$ZnCl_2$	1.04 g/L
$(NH_4)_6Mo_7O_2 \cdot 4H_2O$	1.05 g/L
$CuSO_4 \cdot 5H_2O$	50 g/L
Na_2EDTA	50 g/L
1 ml per liter feeding media	

The long-term operation was divided into three phases (shown in Table 3.6): i) in the start-up period, the reactor was operated to achieve stable performance; ii) after the reactor has stable performance, 1g COD/L humic substances (Humic acid, melanoidins and reject water) was added into the reactor through the feeding media; iii) additional trace elements were added into the reactor. The concentration of NH_4^+-N, NO_2^-N and NO_3^-N in the influent and effluent were analysed every day to measure the performance of reactor. Concentration of UV_{254} and colour of effluent were planned to measure during the long-term operation. To investigate whether the complexation between HS and metal ions caused the scarcity of metal ions in liquid phase, the metal ions in effluent were planned to measure by using Inductively Coupled Plasma-Mass Spectrometer (ICP-MS) during the experiment.

Table 3.6 Operation strategy of long-term experiment

Phase	Operation period (d)	Strategy
Phase I	1-14	No HS
Phase II	15-28	1g COD/L HS in influent.
Phase III	29-42	1g COD/L HS in influent + additional trace elements

3.5 Analysis methods

Ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) were measured with Ion Chromatography (Metrohm, Netherlands). Chemical oxygen demand (COD) of humic substances was measured with Hach kits LCK541. TOC was measured with a TOC analyser (TOC-V CPH, Shimadzu, Japan). Colour was measured by colorimetry using a spectrophotometer Genesys 10S UV-Vis (Thermo Spectronic, Germany) at 475nm in 1cm path length plastic cuvettes and converted to the unit of mg Pt-Co/L using a calibration curve prepared with a standard solution. UV absorbance was measured by spectrophotometer (Genesys 10S UV-Vis, Thermo Spectronic, Germany) at 254nm using a 1cm path length quartz cuvettes.

- SEC-HPLC

SEC-HPLC was performed with SHIMADZU Liquid Chromatography (Shimadzu, Japan) to investigate the variation in the MW distribution of humic substances during BMP and BOD test. The stationary phase used in the SEC-HPLC was a YarraTM 3um SEC-2000, LC column 300 x 7.8mm (Phenomenex, USA). Polystyrene sulfonate standards were used for the calibration in several specific MW (1100, 4710, 14900, and 29100 kDa). The mobile phase comprised 10mM sodium phosphate buffer at pH 7 and acetonitrile with a ratio of 3:1, and with a constant flow rate of 1mL/min, and the injection volume was 25uL. Sample was measured with UV detector at wavelengths of 254 and 210 nm, and the total elution time was 20 minutes.

- EEM

To investigate the transformation of the HS during the BMP and BOD process, the excitation-emission matrix (EEM) spectra analysis was performed by using a spectrofluorometer. All samples were filtered with 0.45μm and diluted with ultra-pure water to 1 mg/L TOC for EEM analysis. EEM spectra were obtained by scanning at an emission (Em) wavelength range from 245.87 to 827.26 nm with a 4.65 nm increment and an excitation (Ex) wavelength range from 240 to 800 nm with a 3 nm increment.

The EEM data were discarded when the Em>Ex+20 or Em<Ex-20, to remove the impact of the first and second order Rayleigh scattered fluorescence. Fluorescence regional integration was used in this study to analyze the evolution of three excitation–emission regions of EEM spectroscopy. According to the study of Chen et al. (2003), EEM peaks are related with humic acid-like substances (Em>380nm & Ex>280nm), fulvic acid-like substances (Em>380nm & Ex<280nm), and tyrosine/tryptophan-like substances (280nm<Em<380nm & Ex<280nm). Therefore, EEM spectroscopy was divided in to three regions (shown in Fig 3.6). The integration beneath the same EEM region would represent the cumulative fluorescence of dissolved organic matter with similar properties (Chen et al., 2003). Therefore, through integrating the aera in each EEM spectra regions (Fig 3.6), cumulative fluorescence volume was calculated (according to Eq.8), which quantitively describe the content of humic acid-like substances, fulvic acid-like substances, and tyrosine/tryptophan-like substance.

$$\varphi = \int_{Ex} \int_{Em} I(\lambda_{Ex}\lambda_{Em}) d\,\lambda_{Ex} d\lambda_{Em} \qquad \text{Eq.8}$$

Where,

φ: Cumulative fluorescence volume in specific EEM spectra region

$I(\lambda_{Ex}\lambda_{Em})$: Fluorescence intensity at each excitation-emission wavelength pair

λ_{Ex}: Excitation wavelength

λ_{Em}: Emission wavelength

In addition, the cumulative fluorescence volume was normalized to a DOC concentration of 1 mg/L for comparison of EEMs from three types of humic substances (melanoidins, humic acid and reject water). The cumulative fluorescence volume was also normalized to relative regional aera (nm^2) to reduce the effects of different EEM regions area on overall interpretations (Chen et al., 2003). Therefore, the normalized cumulative fluorescence volume was expressed in AU $(nm^2)^{-1}$ $(mg\ C/L)^{-1}$.

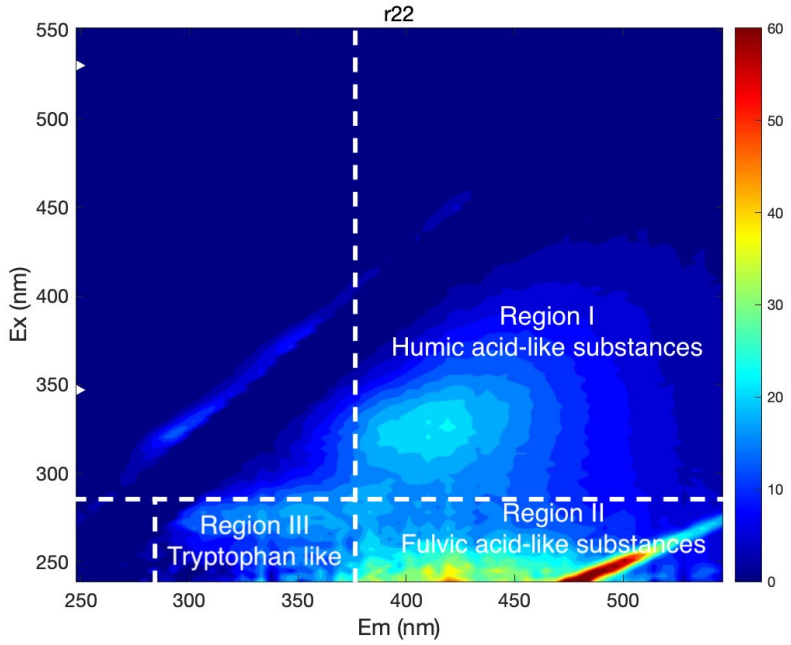


Figure 3.6. EEM spectra was divide into three regions (Region I: humic acid-like substances; Region II: Fulvic acid like substances; Region III: tyrosine/tryptophan-like substances).

- Electron delivered to humic substances

In order to elucidate whether humic substances acted as electron acceptors during anaerobic assays, sCOD/sTOC ratio was measured. In addition, the amount of electrons delivered to three types of humic substances (humic acid, melanoidins and reject water) during anaerobic assay was also estimated. Assuming transition Metals, sulfide were all reduced, COD measured was only attributed to reduced carbon. The amount of electrons delivered to humic substances was calculated through the difference of cumulative CH_4 production between humic substances and blank according to the eq.9, which is expressed in the percentage of electrons delivered to humic substances.

$$\text{Electrons delivered to HS (\%)} = \frac{CH_{4\ \text{Blank}} - CH_{4\ \text{humic substances}}}{0.35\ LCH_4 \cdot gCOD^{-1} * COD_{in\_\ \text{humic substances}}}$$

Where,

$CH_{4\ \text{Blank}}$: Cumulative CH_4 production of blank groups

$CH_{4\ \text{humic substances}}$: Cumulative CH_4 production of three types of humic substances

$COD_{in\_\ \text{humic substances}}$: Input COD of humic substances in anaerobic assays

- Statistical analysis

Analysis of Variance (ANOVA) was performed to analyze the differences between samples. ANOVA were performed by using statistical analysis software IBM SPSS 20.0. The differences between samples with $p < 0.05$ were considered to be statistically significant (Lv et al., 2019).

4. Results and discussion

4.1 Humic substances biodegradation under aerobic and anaerobic conditions

4.1.1 Biodegradability assays

Fig 4.1(a) shows the cumulative methane production of commercial humic acid, melanoidins, reject water, and blank during anerobic assays. Fig 4.1(b) shows the aerobic biodegradability of commercial humic acid, melanoidins, reject water. As can be seen from Fig 4.1(a), the cumulative methane production of melanoidins, reject water, commercial humic acid, and blank (inoculum + nutrients) after 30 days digestion was 266.4 ± 12.2 mL, 246.3 ± 7.4 mL, 184.6 ± 5.1, and 267.7 ± 3.8 mL CH_4, respectively.

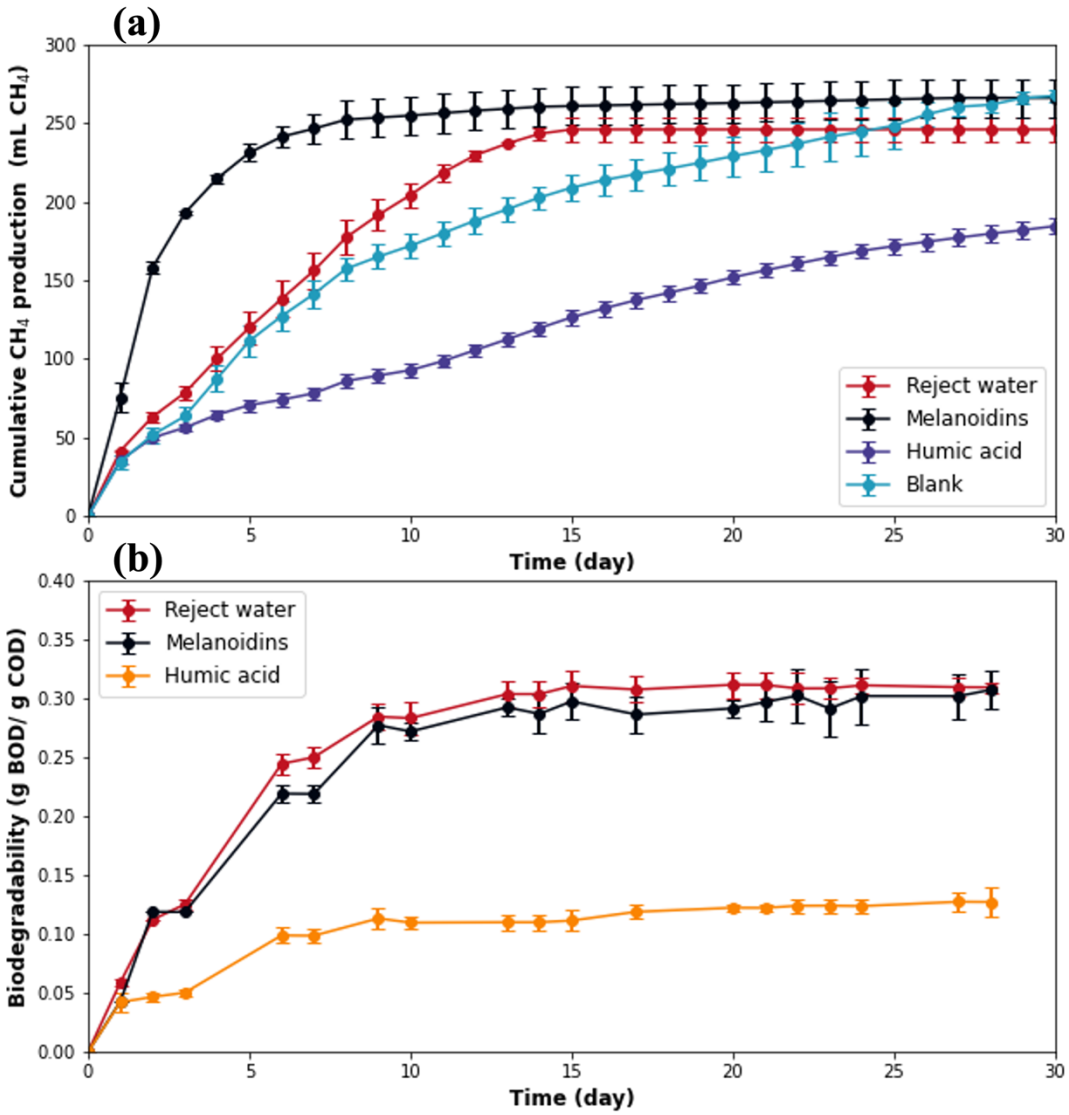


Figure 4.1 (a) Cumulative methane production of reject water, melanoidins, humic acid, and blank during BMP test. The concentration of inoculum used in this test was 4.6g VS/L. (The cumulative methane production of reject water, melanoidins and humic acid at day 30 was 231.6 ± 10.6 mL, 214.1 ± 6.5 mL, and 106.6 ± 4.5 mL CH_4/g COD, respectively.) (b) Biodegradability of reject water, melanoidins, and humic acid during BOD test.

Both melanoidins and reject water showed similar variation trend in CH_4 production rate, being higher than that of the blank group during the start-up period but lower than that of the blank group at the end (Fig 4.1a). For melanoidins group, there was a rapid increase in CH_4 production during the first 8 days digestion, then remained stable (Fig 4.1a). The fast CH_4 production in the start-up period of melanoidins could be attributed to the degradation of easily biodegradable substrates in the Melanoidins, such as aliphatic moieties of humic substances and some intermediate products of the maillard reactions. Early studies by Liu et al. (2019) and Wang et al. (2021) have revealed aliphatic and amide moieties of humic substances were easily biodegradable, and first degraded contributed to CH_4 production during the AD. Also, the intermediate products of the maillard reactions in melanoidins samples, FURFURAL and HMF, were reported to be biodegradable (Ghasimi et al., 2016). Compared to melanoidins, reject water had lower CH_4 production in the start-up period, and the trend of cumulative CH_4 production of reject water and blank was quite similar in the start-up period (Fig 4.1a), suggesting humic substances in reject water were less biodegradable than melanoidins. The less CH_4 production of reject water than melanoidins may be due to the fact that the reject water is the dewatering fluid from AD process, which may have already consumed easily biodegradable compounds. Therefore, the humic substances left in the reject water were less biodegradable. It should be noted that the cumulative CH_4 production of reject water and melanoidins was lower than the blank in the end, which is different form the results reported in the literature. Wang et al (2022) reported the cumulative methane production of melanoidins was 296.7 ml/gVS, which shows melanoidins is biodegradable.

Humic acid had a very different trend in cumulative CH_4 production variation. As can

be seen from Fig 4.1a, the cumulative CH_4 production of humic acid was much lower than melanoidins, reject water and blank throughout the anaerobic assays. An early study by Khadem et al. (2017) observed a similar phenomenon, they found the CH_4 production was reduced by 79% during the AD when 1 g/L humic acid was added. The reduction in CH_4 production could be attributed to humic substances, which were used as terminal electron acceptors during the AD. Lovley et al. (1996a) demonstrated that microorganisms could transfer electrons derived from anaerobic microbial oxidation to redox active functional groups in humic substances, such as quinones. Also, Cervantes et al. (2000a) found the reduction of active functional groups in humic substances became the preferred pathway over methanogenesis. In order to further elucidate whether humic substances acted as electron acceptors during aerobic and anaerobic assays, the evolution of COD/TOC ratio of all three types of humic substances (melanoidins, humic acid and reject water) during aerobic and anaerobic assays was plotted in the Fig 4.2. COD/TOC ratio is an indicator of average oxidation state of carbon in the compound. According to the study of Saroj et al. (2005), the average oxidation state of carbon in the compound has a negative correlation with the COD/TOC ratio. The relationship of oxidation state of carbon in organic compound and COD/TOC ratio is shown in Fig 4.3. (Saroj et al., 2005). As can be seen from the Fig 4.2(a), a general increasing trend of COD/TOC ratio of reject water and humic acid was observed during anaerobic digestion. Increase of COD/TOC ratio was also observed in melanoidins during day 5-11 and 17-24. Assuming the COD measured was only attributed to reduced carbon, the increase of COD/TOC ratio suggested the average oxidation state of carbon in humic substances decreased during anaerobic digestion. The amount of electrons delivered to humic substances was calculated through the difference of cumulative CH_4 production between humic substances and blank. The results showed the percentage of electrons delivered to melanoidins, reject water and humic acid during AD was 0.4%, 6.6% and 25.8%, respectively. This finding demonstrated humic substances were used as electron acceptors during anaerobic digestion, which was the reason why all three types of humic substances had lower cumulative CH_4 production than blank in the end. In contrast, a general decreasing trend

of COD/TOC ratio of all three types of humic substances was observed during the aerobic assay (Fig 4.2(b)). The decrease in the COD/TOC ratio means the average oxidation state of carbon in all three types of humic substances increased. According to the study of Saroj et al. (2005), the increase in oxidation state of carbon could be attributed to the degradation of aromatic compounds. The aromatic structure was broken, and aliphatic structure was formed with -CHO, -COOH and -OH functional groups. Since aliphatic compounds had higher oxidation state carbon than aromatic carbon, the average oxidation state of carbon in the compound would increase (Saroj et al., 2005).

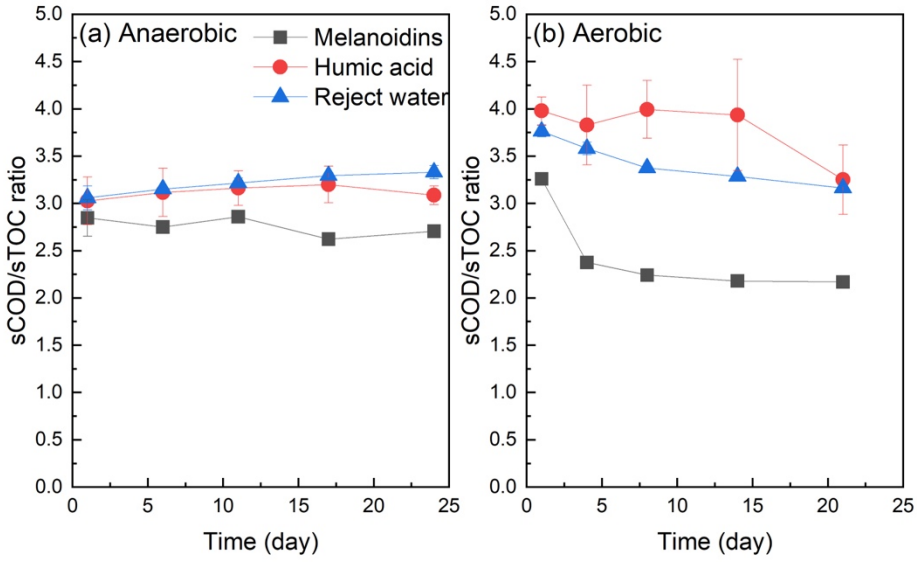


Figure 4.2. The evolution of sCOD/sTOC ratio of all three types of humic substances (melanoidins, humic acid and reject water) during anerobic (a) and aerobic assays (b).

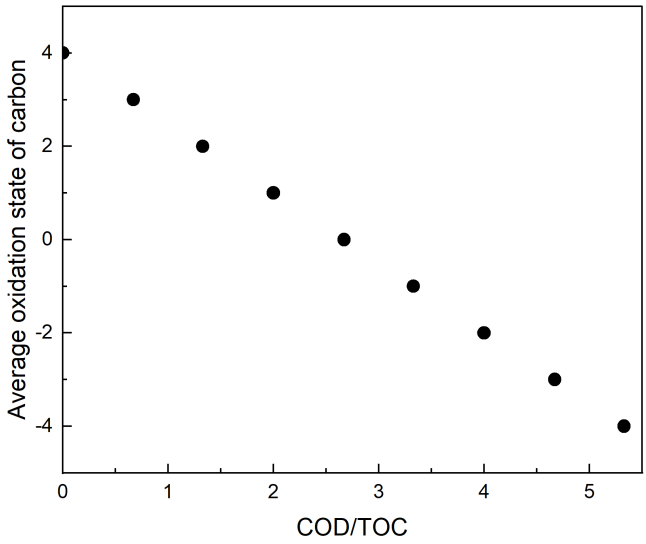


Figure 4.3 Relationship of the oxidation state of carbon in organic compound and sCOD/sTOC ratio (Saroj et al., (2005))

The aerobic biodegradability of melanoidins, humic acid and reject water was evaluated through a BOD test as shown in Fig 4.1b. During the aerobic assay, the change in the biodegradability of melanoidins and reject water was synchronous, but the biodegradability of reject water was greater than melanoidins in the end. Compared to melanoidins and reject water, humic acid had a much lower biodegradability through the BOD test. By day 30, the aerobic biodegradability of reject water, melanoidins and humic acid was 30.9% ± 0.4%, 30.7% ± 1.5%, and 12.7% ± 1.3%, respectively.

The results of biodegradability assays demonstrated that all three types of humic substances were not fully recalcitrant under aerobic conditions. Therefore, the presence of humic substances in THP-AD centrate may promote the growth of heterotrophic bacteria in the PN/A process. The growth of heterotrophic bacteria, such as heterotrophic denitrifying bacteria and NOO, competes with functional populations in PN/A (AOO and anammox bacteria) for substrate, oxygen and living space, which may affect the performance of the PN/A process (Li et al., 2020). In addition, the aerobic biodegradability assays showed the aerobic biodegradability of reject water and melanoidins were ~20% after 5 days aerobic digestion. This finding clearly suggested a short aerobic pre-treatment before AD could at least remove part of humic substances. And, the reduction in humic substances may reduce the inhibitory effects of these organic compounds on the AOO and anammox bacteria. An early study by Ghasimi et al. (2016) reported the intermediate products of the Maillard reactions, furfural and HMF, could inhibit the biomass, but they are biodegradable. Therefore, a short aerobic pre-treatment may remove these inhibitory organic compounds and reduce the inhibitory effect on AOO and anammox bacteria. Several studies have already demonstrated the aerobic biological pre-treatment was effective in reducing the inhibitory effect of organic compounds in THP-AD centrate. Valenzuela et al. (2022) reported even a 1-day aerobic biological pre-treatment of THP-AD centrate could remove the inhibitory effect of centrate on the activity of AOO biomass. Also, Zhang

et al. (2016) found that the relative activity of AOO exposed to THP-AD concentrates that had undergone a 7-day aerobic biological pretreatment was increased by ~60% compared to those directly exposed to THP-AD concentrates.

4.1.2 Evolution of humic substances during biodegradation assays

4.1.2.1 Molecular weight distribution

Fig 4.4 shows the variation in MW distribution of three types of humic substances (melanoidins, reject water and humic acid) measured at 254 nm during aerobic and anaerobic digestion. As observed from Figure 4.4, melanoidins and reject water had similar MW distribution, which were mainly comprised of fraction I and II. For reject water, fraction I accounted for ~56% and fraction II accounted for ~40% (Fig 4.4A(a)) For melanoidins (Figure 4.4A(c)), fraction I contributed to ~41% and fraction II contributed to ~58%. Compared with melanoidins and reject water, commercial humic acid had a very different MW distribution. As can be seen from Fig 4.4A(b), commercial humic acid had 4 fractions (I, II, III, and V). Fraction II and III accounted for ~33% and ~58%, respectively. Fraction I and V only accounted for ~7% and ~2%, respectively. The MW distribution of humic acid was consistent with the study of Song et al. (2010), which reported commercial humic acid has a higher content of high-MW compound. The origin of commercial humic acid was peat or coal-related substances (Kobayashi et al., 2018). Early studies reported the commercial humic acid obtained from peat was abundant in aromatic structures and low in carboxyl groups content (Huculak et al., 2018; Kobayashi et al., 2018), which was the reason why it has a higher

content of high-MW fractions.

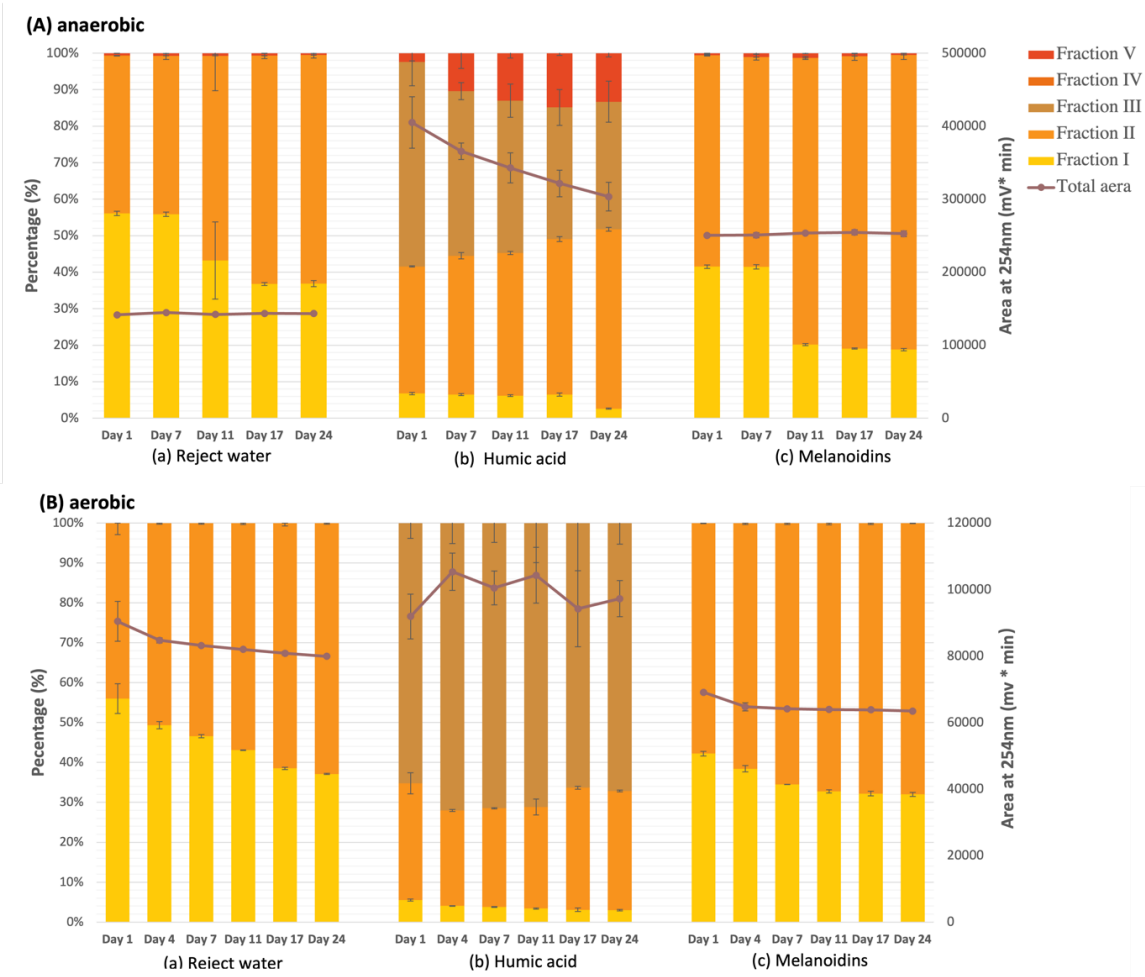


Figure 4.4 Evolution of MW distribution of three different types of humic substances (reject water, melanoidins and humic acid) measured at wavelength of 254nm during anaerobic (A) and aerobic digestion (B). (Fraction I: MW<1.1kDa; Fraction II: MW>1.1kDa & <4.71; Fraction III: >4.71kDa & <14.9kDa; Fraction IV: >14.9kDa & < 29.1kDa; Fraction V: >29.1kDa)

During the anaerobic process, there were no significant variations in total area at 254nm of melanoidin (p-vaue=0.673, ANOVA) and reject water (p-value=0.133, ANOVA) (Fig 4.4A(a)(c)), which indicated the aromatic compounds were not degraded under anaerobic process. However, there was a significant decrease (p=0.002, ANOVA) in total area at 254nm of humic acid during the AD (Fig 4.4A(b)), from 405,070.0 ± 35,392.10 to 303380.0 ± 19586.90 mV*min, which was attributed to the decrease in the content of fraction III. This finding suggested the aromatic compounds of humic

acid between MW >4.71kDa & <14.9kDa were removed during the AD. In addition, a transformation of small MW substances to large MW substances was observed in all three types of humic substances during anaerobic digestion. As can be seen in Fig 4.4A(a)(c), there was a transformation of small MW substances (fraction I) to large MW substances (fraction II) in both melanoidins and reject water. The transformation of small MW substances to large MW substances was also found in humic acid (Fig 4.4A(b)), from fraction I to fraction II and V. The increase in the content of lager MW substances suggested humification process may happened, which means humic substances were formed during the AD process.

During the aerobic process, the total area at 254nm of melanoidin and reject water decreased about 8 and 12 % (Fig 4.4B(a)(c)), respectively. This result indicated that the aromatic compounds were removed under aerobic condition. The transformation of small MW substances (fraction I) to large MW substances (fraction II) was also found in both reject water and melanoidins (Fig 4.4B(a)(c)). Notably, the transformation of small MW substances to large MW substances was observed during both aerobic and anaerobic digestion, suggesting humification process happened under both conditions. This finding is consistent with the studies of Shao et al. (2013) and Wang et al. (2021), which demonstrated humification process happened during both aerobic and anaerobic digestion and large MW humic substances were finally formed. The formation of larger MW humic substances was attributed to the two types of polymerization process (Stevenson, 1994): i) the oxidation and polyphenolic condensation process; ii) Maillard reactions. The oxidation and polyphenolic condensation process is an enzymatic polymerization process, which contains three steps. Firstly, the polyphenols were derived through depolymerization of biopolymers or synthesized by microorganisms. Then, polyphenols were enzymatically oxidized to quinones. Finally, quinones undergo self-condensation or combine with amino compounds to form humic polymers (Stevenson, 1994). The oxidation and polyphenolic condensation process is considered as the most likely pathway for humic substances formation (Veeken et al., 2000), but the Maillard reaction is also possible. The Maillard reaction is a non-enzymatic

polymerization process. Generally, the Maillard reactions occurs extensively at higher temperature (>100 ºC). However, several studies reported the maillard reaction could proceed slowly at low temperature. Stevenson (1994) reported humic substances were formed slowly through Maillard reaction at normal soil temperature. Pereyra et al. (2010) also reported that maillard reaction happened in milk at 37ºC with a relatively lower kinetic rate compared to higher temperature (60 ºC and 120 ºC). Although the kinetic rate of maillard reaction was low at low temperature, the duration of aerobic and anaerobic digestion was long (30 days). Therefore, maillard reaction may also happen during aerobic and anaerobic digestion process and formed humic substances.

In addition, the variation in the total area at 254nm differed between aerobic and anaerobic digestion. Compared with almost no change in the total area at 254nm during the anaerobic digestion, there was a about 8 and 12 % decrease in the total area at 254nm for reject water and melanoidins in the aerobic digestion (Fig 4.4). This finding suggested the degradation ability of aromatic substances of aerobic biodegradation was greater than that of anaerobic digestion.

4.1.1.2 Evolution of humic substances assessed by SUVA

Fig 4.5(a)(b)(c) shows the variation in SUVA of three types of humic substances (melanoidins, reject water and humic acid) during aerobic and anaerobic digestion. SUVA was used to measure the aromaticity and humification degree of humic substances (Uyguner et al., 2005). According to the studies of White et al. (1997) and Karanfil et al. (2002), samples with high SUVA values (≥4 L/mg/m) have shown to have higher aromaticity and higher content of high-MW dissolved organic material fraction. As could be seen from Fig 4.5(a)(b)(c), humic acid had a much higher SUVA value (~10 L/mg/m) than reject water (~2 L/mg/m) and melanoidins (~3 L/mg/m), which indicated humic acid had a more complex and condensed aromatic structure than compounds in reject water and melanoidins. According to the study of Cabral et al. (2021), several aromatic compounds in humic substances, such as syringol, phenol and

guaiacol, are toxic and cause growth inhibition of biomass. And this growth inhibition is positively correlated with aromaticity (Meinelt et al., 2007). Therefore, the higher aromaticity of humic acid means it may cause stronger growth inhibition of AOO than other two types of humic substances, which is in accordance with the results of acute toxicity test (Fig 4.7).

During aerobic and anaerobic digestion, a general increasing trend of SUVA of all three types of humic substances was observed (shown in Fig 4.5(a)(b)(c)), demonstrating humification degree increased during both aerobic and anaerobic digestion. Notably, the trend of SUVA variation of reject water and humic acid during start-up period was quite similar in both aerobic and anaerobic digestion (Fig 4.5(a)(c)), decreased during the first several days. During aerobic process, SUVA of reject water and humic acid decreased during the first 4 days (Fig 4.5(a)(c)). Similarly, during anaerobic digestion, SUVA of reject water and humic acid decreased during the first 7 days. The decrease in the SUVA observed in both reject water and humic acid during start-up period corresponded with the greater decrease in UV_{254} and slighter decrease in TOC, suggesting aromatic compounds were degraded during this period (shown in Fig 4.5(d)(g)(i)). In contrast, a opposite trend of SUVA variation was observed in melanoidins during start-up period under both aerobic and anaerobic conditions (Fig 4.5 (b)). SUVA values of humic acid increased during start-up period under both aerobic and anaerobic conditions.

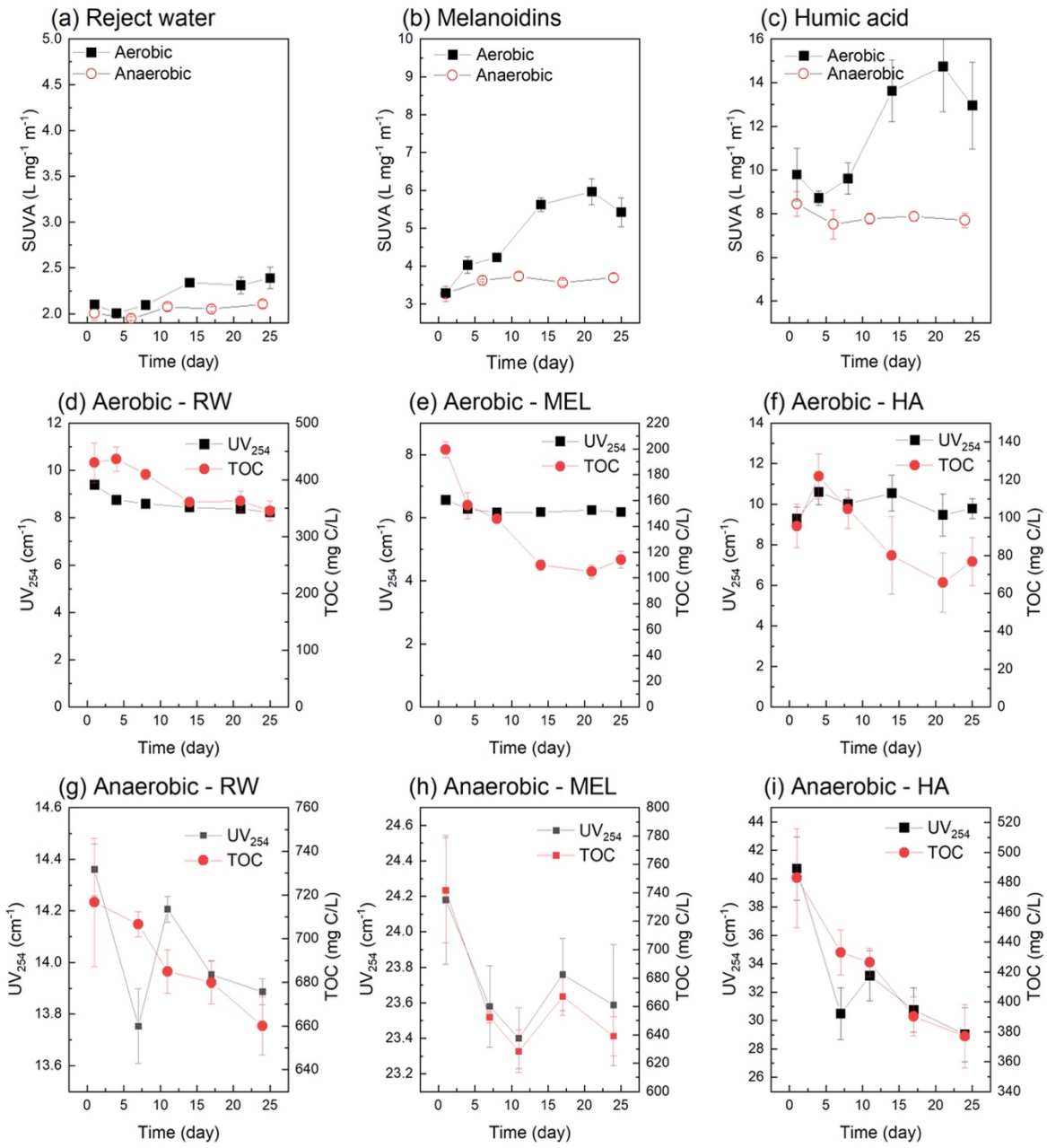


Figure 4.5 The variation of SUVA of reject water (a), melanoidins (b), and humic acid (c) during aerobic and anaerobic digestion. The variation in UV_{254} and TOC of reject water (d)(g), melanoidins (e)(h), and humic acid (f)(i) during aerobic (d)(e)(f) and anaerobic digestion (g)(h)(i).

The increase in SUVA values of melanoidins corresponded with the more decrease in TOC concentration and slighter decrease in UV_{254} (shown in Fig 4.5 (e)(h)), suggesting the easily biodegradable TOC with lower aromaticity in melanoidins was biodegraded during the start-up period. This finding was consistent with the result shown in

biodegradability tests (Fig 4.1), demonstrating melanoidins had more easily biodegradable substrates. Also, Tang et al. (2018) and Wang et al. (2021) observed an increase in SUVA during the start-up period and attributed it to the degradation of easily biodegradable substances with lower aromaticity during AD. In summary, the melanoidins had more labile biodegradable fraction and the degradation of this fraction caused the increase in SUVA during the start-up period. However, reject water and humic acid had less easily biodegradable substances, so the aromatic compounds were degraded, which caused the decreased in SUVA during start-up period.

The trend of SUVA variation of all three types of humic substances was similar after start-up period. SUVA value of all humic substances had a general increase trend (Fig 4.5(a)(b)(c)), which was attributed to the formation of humic substances since more complex and condensed aromatic compounds were formed through the polymerization process. This finding once again demonstrated the formation of humic substances that happened under both aerobic and anaerobic conditions.

In addition, the increase of SUVA value of all three types of humic substances during aerobic process were much higher than that during the anaerobic digestion (shown in 4.4(a)(b)(c)). After the aerobic digestion, the SUVA value of reject water, melanoidins and humic acid increased ~14%, ~65%, and ~32%, respectively. Whereas, after anaerobic digestion, the SUVA value of reject water and melanoidins only increased ~5% and ~13%, respectively. These findings are consistent with an early study by Du et al. (2017), which reported that the SUVA value of DOM after aerobic digestion was much higher than those after anaerobic digestion. Shao et al. (2013) also observed the humification degree in aerobic digestion was much higher than that in anerobic digestion. The higher SUVA after aerobic digestion could be caused by two phenomena. On the one side, more consumable was biodegraded during aerobic digestion, which has been shown in biodegradability assays. On the other side, the higher SUVA after aerobic digestion could be attributed to more complex and condensed aromatic structures formed during aerobic digestion. Because oxidation and polyphenolic

condensation process is easier occurred under aerobic condition than anaerobic condition (Veeken et al., 2000). As mentioned in section 4.1.2.1, the formation of the polyphenols is the first step of oxidation and polyphenolic condensation process. Polyphenols were possibly formed through depolymerization of biopolymers or synthesized by microorganisms (Stevenson, 1994). The depolymerization of biopolymers was accomplished through the oxidation of biopolymers by microorganisms. Therefore, aerobic condition would promote the formation of polyphenols, and thus promote the oxidation and polyphenolic condensation process. More humic substances would be formed during aerobic digestion.

4.1.1.3 Evolution of humic substances assessed by fluorescence EEM

Fig 4.6 shows the variation of normalized volumetric fluorescence of each fluorescence component during aerobic and anaerobic digestion. As can be seen in Fig 4.6 (a)(b)(e)(f), the variation trend of fulvic acid-like volumetric fluorescence of reject water and humic acid was quite similar in both aerobic and anaerobic digestion, fulvic acid-like volumetric fluorescence decreased during the first eight days, then increased and remained stable. The decrease of fulvic acid-like volumetric fluorescence in the first eight days could be caused by the adsorption or biodegradation (Wang et al., 2020). The increasing trend after the first eight days clearly suggested fulvic acid-like substances were formed during both aerobic and anaerobic digestion. The variation of the content of fulvic-acid like substances in the reject water and humic acid were consistent with the aforementioned evolution of humic substances during aerobic and anaerobic digestion. In contrast, the fulvic acid-like volumetric fluorescence of melanoidins showed an opposite trend during the first eight days, which kept increasing during this period (Fig 4.6 (c)(d)). This finding suggested the degradation or adsorption rate was lower than the formation rate of humic substances during melanoidins digestion. The higher humic substances formation rate could be attributed to that melanoidins had more labile degradable substances, which could be degraded and then used to form humic substances through polymerization.

In addition, the increase of the fulvic acid-like volumetric fluorescence of all three types of humic substances was much higher than the humic acid-like volumetric fluorescence during both aerobic and anaerobic digestion (Fig 4.6). Especially, after the aerobic digestion, the increase of fulvic acid-like volumetric fluorescence in melanoidins was ~50% (Fig 4.6(d)). Whereas the humic acid-like volumetric fluorescence in melanoidins only increased ~20% after aerobic digestion. According to the study of Stevenson (1994), not all humic components were formed by the same mechanism. Stevenson (1994) reported the humic acid was mainly formed through the oxidation and polyphenolic condensation process, while fulvic acid was mainly formed from Maillard reactions. Therefore, in this study, Maillard reactions seems like the main pathway for the formation of humic substances during both aerobic and anaerobic digestion.

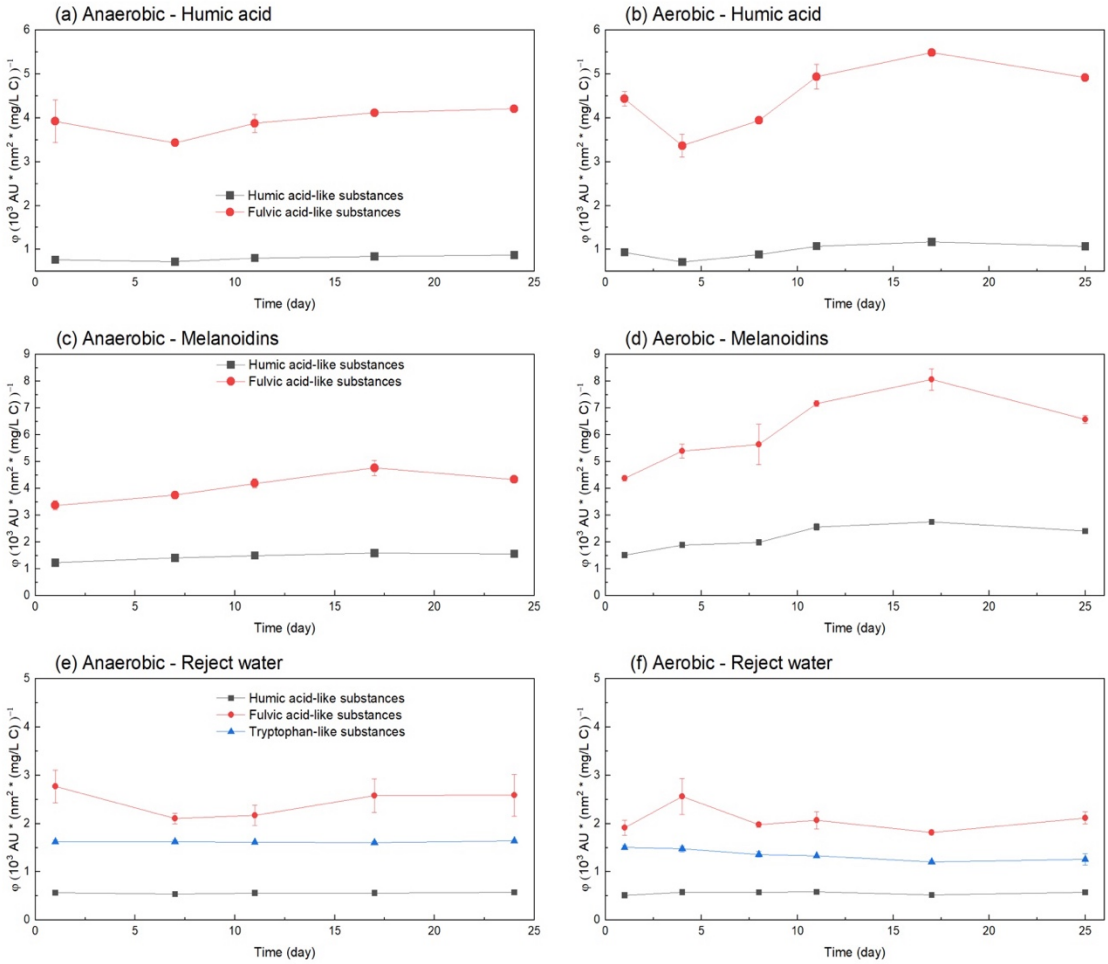


Figure 4.6 Evolution of normalized fluorescence volume of humic acid (a, b), melanoidins (c, d), and reject water (e, f) during anaerobic (a, c, e) and aerobic digestion (b, e, f).

4.2 Acute toxicity tests

Fig 4.7 showed the relative acute inhibition effect of three types of humic substances (humic acid, melanoidins and reject water) on AOO. It was evident from Figure 4.6 that the presence of all three types of HSs caused inhibition on AOO biomass, and the inhibition became stronger with the incremental HSs concentration.

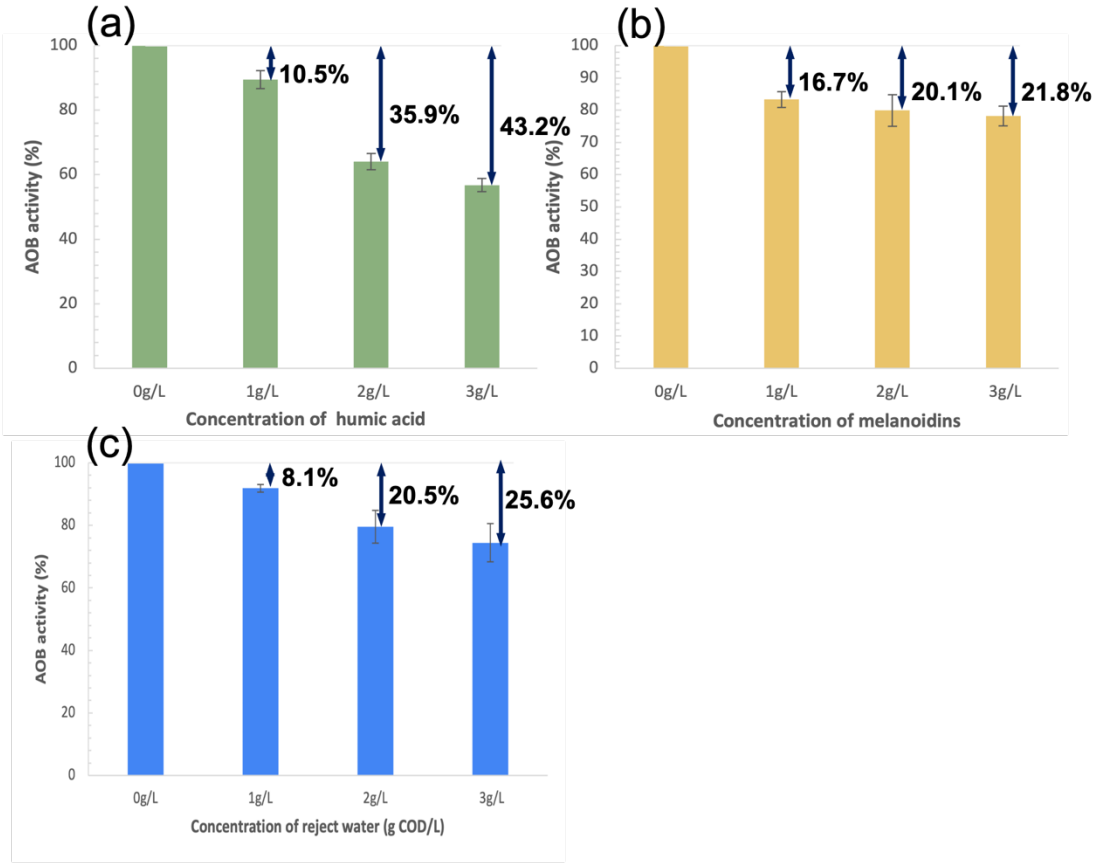


Figure 4.7. Effect of different types of HSs (a) Commercial humic acid; b) Melanoidins; c) reject water on the AOO activity. The AOO activity in the controls was 2.03, 2.75, and 2.99 g N/day/g VS, in the case of humic acid, melanoidins, and reject water, respectively.

Commercial humic acid showed a stronger inhibition effect than other two types of humic substances as seen in figure 4.7(a), AOO biomass decreased 10.5% activity after 1g COD/L humic acid was added, and a significant decrease ($p<0.001$, ANOVA) in AOO activity was observed when AOO is exposed to 2g/L COD of humic acid, 35.9% activity is lost. AOO significantly decreased ($p<0.001$) its activity by 43.2% after 3g

COD/L humic acid added. Compared to commercial humic acid, weak inhibition was observed in adding melanoidins and reject water as seen in Figure 4.7(b)(c). The loss of AOO activity remained stable when AOO was exposed to melanoidins and reject water from 1g COD/L to 3g COD/L. AOO only lost 21.8% and 25.6 % activity when 3g COD/L melanoidins and reject water was added. The inhibition effect of reject water on AOO biomass measured in this study are different to the result reported by Cao et al (2020) and Zhang et al (2018). Cao et al (2020) reported that AOO lost 62.7% activity when exposed to around 2 gCOD/L reject water. Zhang et al (2018) reported that AOO lost more than 40% activity when exposed to 2 gCOD/L reject water. In this study, AOO only lost 25.6% activity when exposed to 3 gCOD/L reject water, which is much lower than these previous studies. The difference in the inhibition effect could be attributed to the composition of reject water. Different THP operating conditions will result in different amounts of melanoidins in the reject water, which will affect the property of reject water.

These results clearly suggested that humic substances inhibited AOO biomass. Humic substances could directly inhibit the biomass growth through the DNA damage and the inactivation of enzymatic system (Ghasimi et al., 2016). Steinberg et al. (2008) also reported humic substances could directly inhibit microorganism activity via ditect toxicity. Also, Bittner (2006) reported that although humic substances have a higher content of high-MW substances, part of them could penetrate into the cells, and inhibit microorganism activity via dircect toxicity.

The inhibition could also be caused by the scarcity of trace elements for AOO due to the complexation between humic substances and trace elements. Humic substances have the ability to form complexes with metal ions, e.g., Cu^{2+}, Fe^{2+}, $Ca2^{+}$, Mn^{2+},. (Hirata, 1981; Gao et al., 1999). Less metals are available for AOO after complexation, whereas metal ions have many important functions in the microbial cells, such as being a functional proportion for more than one-third of known enzymes, formation of charge and concentration gradients across membranes which may be used in transport

processes, intracellular compartmentation, osmotic responses, and sensing (Gadd, 1992). Shafiee et al. (2021) observed tricarboxylic acid cycle and biosynthesis process were significantly affected when Cu and Fe are limited, which indicate Fe and Cu play important roles in the biochemistry of AOO. Therefore, the scarcity of metal ions may be another reason that affect the activity of AOO.

4.5 Long-tern operation of PN reactor

Fig 4.8 showed the evolution of nitrogen in the PN reactor. After 18 days operation, both two reactors had stable performance, the NO_2^--N in the two reactors was 403.1 ± 31.19 mg N/L and 429.6 ± 59.40 mg N/L, respectively. After the reactor had stable performance, 1g COD/L humic substances (melanoidins and reject water) was added into the reactor through the feeding media. However, the expected inhibition effect was not observed, an increase of NO_2^--N was observed in both reactors. After adding melanoidins, NO_2^--N concentration increased from 469.1 mg N/L to 838.3 mg N/L. After adding reject water, NO_2^--N concentration increased from 484.9 mg N/L to 672.5 mg N/L. The increase in NO_2^--N concentration was attributed to the formation of granular sludge in the reactor and the growth of AOO biomass in the tubing and culture medium (shown in Fig 4.9). Therefore, the planned experiments were not performed to investigate whether the inhibitory effect of HS on AOO biomass is due to the scarcity of trace elements.

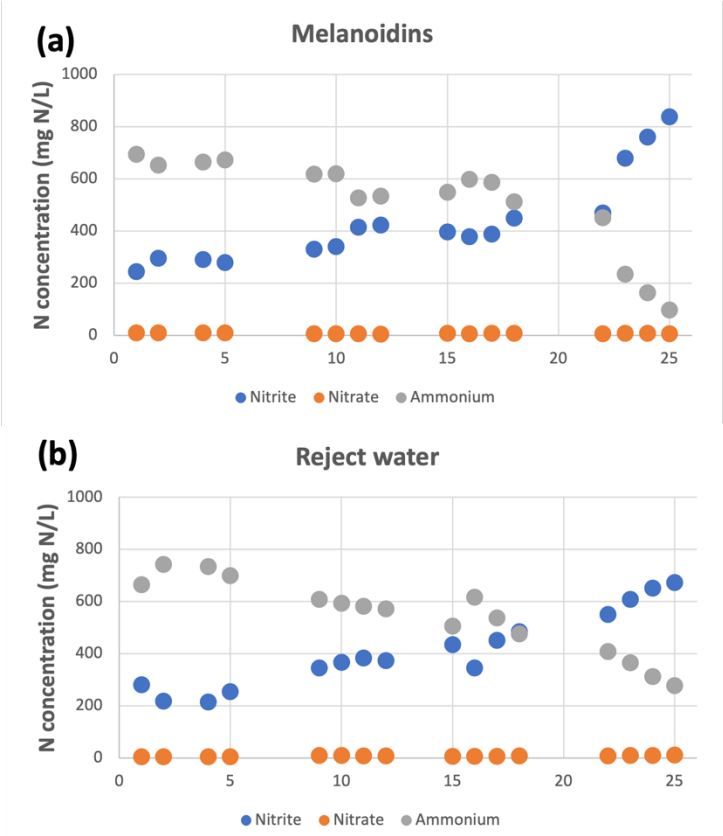


Fig 4.8. Evolution of nitrogen in two PN reactors. (a) melanoidins was added at day 21 (b) reject water was added at day 21

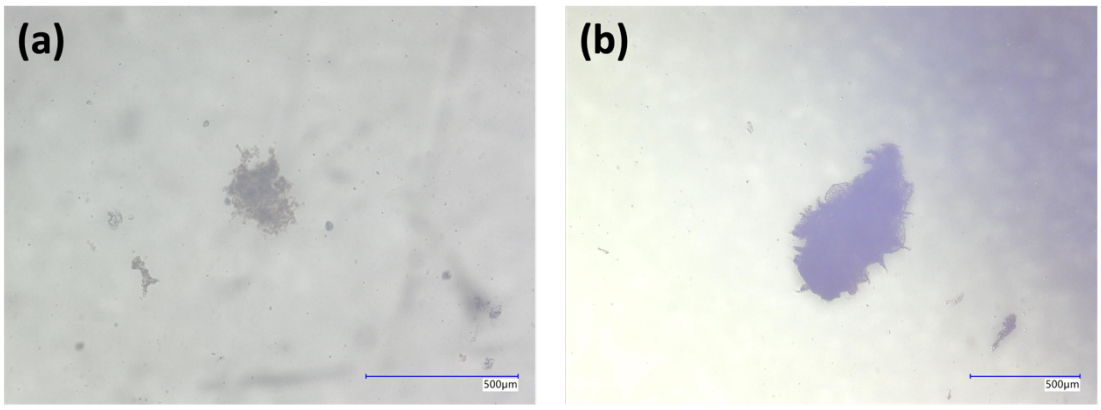


Fig 4.9 Digital microscopic images of culture medium for two reactors (a) reject water added (b) melanoidins added.

5. Conclusions and recommendations

5.1 Conclusions

The conclusions drawn from this study are:

1. **What is the biodegradability of humic substances under anaerobic and aerobic/anoxic conditions?**

   - **Anaerobic biodegradability**

   During the AD process, the cumulative methane production of all three types of humic substances in the end was lower than the blank, which was attributed to humic substances use as electron acceptors during the AD. Very likely, the reduction of active functional groups in humic substances became the preferred pathway over methanogenesis. The rapid methane production of melanoidins during start-up period indicated melanoidins had more easily biodegradable compounds than reject water and humic acid.

   - **Aerobic biodegradability**

   All three types of humic substances had higher biodegradability under aerobic condition, suggesting part of humic substances in the THP-AD centrate could be removed in the partial nitritation process.

2. **How do the structure and molar weight of humic substances change during the process? How do humic substances transform during the biodegradation process?**

   Humic substances were produced during both aerobic and anaerobic digestion. The transformation from small MW fractions to large MW fractions was observed in all three types of humic substances (reject water, humic acid and melanoidins) during both aerobic and anaerobic digestion, demonstrating humification process happed under both conditions. The aromaticity of all three types of humic substances increased after aerobic and anaerobic digestion, and the increase in aromaticity of humic substances during aerobic digestion was much higher than

that during the anaerobic digestion. The content of both fulvic acid-like substances and humic acid-like substances increased after aerobic and anaerobic digestion, clearly demonstrated humic substances were formed during digestion. The possible humic substances formation mechanism could be polyphenolic condensation process and Maillard reactions, but Maillard reactions seems like the main pathway for the formation of humic substance in this study.

3. **Do humic substances affect the oxygen uptake rate of AOO biomass?**

   All three types of humic substances (reject water, humic acid and melanoidins) affect the oxygen uptake rate of AOO biomass. Commercial humic acids had a stronger inhibitory effect than reject water and melanoidins.

5.2 Recommendation

In this study, the results showed that humic substances were formed during both aerobic and anaerobic digestion. According to the previous study, humic substances were formed through two types of polymerization process (Stevenson, 1994): i) the oxidation and polyphenolic condensation process; ii) Maillard reactions. However, the exact process happened during aerobic and anaerobic assays were not investigated. To further elucidate the mechanism of humic substances formation, anaerobic and aerobic experiments could be conducted without the addition of inoculum in subsequent studies to investigate whether humic substances are mainly produced by enzymatic polymerization or non-polymerization reactions.

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