Investigation of stable nitrification in a membrane bioreactor treating reject water

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SINGAPORE

**INVESTIGATION OF STABLE NITRITATION
IN A MEMBRANE BIOREACTOR TREATING REJECT
WATER**

JIANG YISHUAI

SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING

2019

**INVESTIGATION OF STABLE NITRITATION
IN A MEMBRANE BIOREACTOR TREATING REJECT
WATER**

JIANG YISHUAI

School of Civil and Environmental Engineering

A thesis submitted to the
Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

22 July 2019

Date

Jiang YS

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Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

12/07/2019

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Date

.....

Prof Ng Wun Jern

Authorship Attribution Statement

This thesis contains material from two papers published in the following peer-reviewed journals in which I am listed as an author.

Chapter 4 is published as Y. Jiang, L.S. Poh, C.P. Lim, C. Pan, W.J. Ng. Effect of free ammonia inhibition on process recovery of partial nitrification in a membrane bioreactor. *Bioresource Technology Reports* 6, 152-158 (2019). DOI: 10.1016/j.biteb.2019.02.014.

The contributions of the co-authors are as follows:

- Prof Ng provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by Dr Poh and Dr Pan.
- I co-designed the study with Prof Ng Wun Jern and performed all the laboratory work at the School of Civil and Environmental Engineering and Advanced Environmental Biotechnology Centre, Nanyang Environment and Water Research Institute. I also analysed the data.
- Dr Lim assisted in the design of experimental reactor and carried out DGGE interpretation.

Chapter 5 is published as Y. Jiang, L.S. Poh, C.P. Lim, W.J. Ng. Impact of free nitrous acid shock and dissolved oxygen limitation on nitrification maintenance and nitrous oxide emission in a membrane bioreactor. *Science of The Total Environment* 660, 11-17 (2019). doi.org/10.1016/j.scitotenv.2019.01.024.

The contributions of the co-authors are as follows:

- Prof Ng suggested the initial project direction and edited the manuscript drafts.
- I wrote the drafts of the manuscript.
- Dr Poh revised the manuscript and assisted in the N₂O data analysis.
- I performed all the sample measurement, extracted DNA and EPS, collected N₂O data, and conducted data analysis.
- Dr Lim assisted in the design of experimental reactor and conducted part of the qPCR tests.

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Summary

Employment of the nitrification process in membrane bioreactors (MBRs) has attracted much research and practical application interest. The MBR technology combines biological treatment with membrane filtration, and so provides better effluent quality with a lower physical footprint. Additionally, nitrification based nitrogen removal can save up to 60% of the aeration energy requirement and have lower requirement for the electron donor as well as reduced sludge production, compared with the conventional biological nitrogen removal process. Stable nitrification needs not only to favour the growth of ammonia oxidizing bacteria (AOB), but also to inhibit that of nitrite oxidizing bacteria (NOB) in long-term operations. However, both AOBs and NOBs are retained in the MBR due to the membrane separation and particularly so when operated with long solids retention time (SRT). NOBs could then proliferate and disrupt the nitrification process. Membrane fouling is a significant challenge in MBR. The unique characteristic of the nitrification MBR is that nitrite acclimation in the reactor can lead to the increase in free nitrous acid (FNA) concentration. The latter would impact on extracellular polymeric substances (EPS) production and microbial population. Thus, this project had aimed to investigate feasibility of long-term stable nitrification in a MBR with long SRT, and the consequent membrane fouling under the influence of elevated FNA level. Such membrane fouling would then require a fouling mitigation strategy.

In this project, the strategy of applying high free ammonia (FA) in the MBR did not maintain partial nitrification but was found to have had an inhibitory effect on the catabolic and anabolic activities of the enriched *Nitrosomonas* culture. This study indicated that long-term FA inhibition had adverse impact on process recoverability and subsequent failure threshold of partial nitrification. On the other hand, the strategy of *in-situ* generated FNA shock successfully maintained nitrification in a MBR with 43.8 days SRT. Quantitative polymerase chain reaction (qPCR) results showed that NOB cell abundances decreased following the FNA shocks, while AOB population was maintained. The applied strategy also slightly decreased the N₂O emission. The strategy developed in this study verified that spiked FNA shock together with DO limitation can be used for maintaining nitrification in MBRs with long SRTs at

relatively low capital and operating expenditure (CAPEX and OPEX) when treating ammonium rich wastewater.

This study conducted long-term analyses on changes in the trans-membrane pressure (TMP), EPS composition and microbial community change to understand the membrane fouling mechanism of a nitrification MBR. Three-stage membrane fouling development was noted in terms of TMP changes. Previous researches found bound EPS productions are dependent on FNA concentrations, Protein (PN) and polysaccharide (PS) were found to increase with higher FNA concentrations. However, in this study, PN and PS concentrations were reduced by 23.4% and 19.0%, respectively when FNA concentration reached 1.47×10^{-2} mg $\text{HNO}_2\text{-N/L}$. Reduction of EPS consequently led to reduced fouling rate. This suggested that FNA could be regulated to mitigate membrane fouling in the nitrification MBR. High throughput sequencing analysis showed that *Nitrosomonas* was enriched at high FNA concentrations in the nitrification MBR.

This project further systematically investigated the effectiveness of FNA on the membrane fouling mitigation in the nitrification MBR. It was found that FNA up to 2.7×10^{-2} mg $\text{HNO}_2\text{-N/L}$ reduced LB-EPS and TB-EPS in both suspended sludge and biofilm, and thus maintained the TMP in the low range compared to the control nitrification MBRs. Moreover, EPS sub-fraction analysis by LC-OCD-OND explicated that FNA caused the significant reduction in hydrophobic DOC and LMW neutrals, which played a critical role in the PVDF membrane fouling. The EEM-FRI technique illustrated the oxidative effect of FNA triggered slightly more tyrosine like protein but much less SMP and humic acid like substances in the TB-EPS. The XPS analysis also revealed the inhibitory effect of FNA to the composition ratio of functionalities among the bound EPS. As a result, the *in-situ* generated FNA inhibited the biofilm formation in terms of thickness and mass, in a way to mitigate the membrane fouling in the nitrification MBRs. The information obtained in this project was of fundamental significance for understanding membrane fouling mechanism in the nitrification MBR and provided valid evidence for membrane fouling mitigation by FNA when treating high ammonium concentration wastewater.

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List of Abbreviations

Ammonia oxidizing bacteria	AOB
Ammonia oxidizing rate	AOR
Ammonium loading rate (kg N/ (m ³ day))	ALR
Ammonium removal ratio	ARR
Anaerobic ammonium oxidation	Anammox
Anaerobic digestion	AD
Autotrophic nitrogen removal	ANR
Biochemical oxygen demand	BOD
Capital expenditure	CAPEX
Chemical oxygen demand	COD
Completely autotrophic nitrogen removal over nitrite	CANON
Deammonification	DEMON
Denaturing Gradient Gel Electrophoresis	DGGE
Dissolved organic carbon	DOC
Dissolved oxygen (mg/L)	DO
Excitation emission matrix	EEM
Extracellular polymeric substances	EPS
Free ammonia (mg NH ₃ -N/L)	FA
Field emission scanning electron microscopy	FESEM
Fluorescence regional integration	FRI

Free nitrous acid (mg HNO ₂ -N/L)	FNA
Fourier transform infra-red	FTIR
Hydraulic retention time (h)	HRT
Membrane bioreactor	MBR
Mixed liquor suspended solids (mg/L)	MLSS
(Mixed liquor) volatile suspended solids (mg/L)	(ML)VSS
Molecular weight	MW
Loosely bound extracellular polymeric substances	LB-EPS
Liquid chromatography	LC
Nitrite accumulation ratio	NAR
Nitrite oxidizing bacteria	NOB
Organic carbon detector	OCD
Organic nitrogen detector	OND
Operating expenditure	OPEX
Operational taxonomic units	OTU
Oxygen-limited autotrophic nitrification/ denitrification	OLAND
Oxygen uptake rate	OUR
Parts per billion	ppb
Phosphate buffer solution	PBS
Polymerase chain reactions	PCR
Polysaccharide (mg PS/ g VSS)	PS
Polyvinylidene fluoride	PVDF

Protein (mg PN/ g VSS)	PN
quantitative polymerase chain reaction	qPCR
Reverse osmosis	RO
Revolutions per minute	rpm
Specific ammonium conversion rate (kg N/kg VSS.d)	SACR
Sequencing batch reactor	SBR
Single-stage nitrogen removal using anammox and partial nitrification	SNAP
Standard deviation	SD
Sodium hydrogen carbonate concentration	NaHCO ₃
Single-reactor high ammonia removal over nitrite	SHARON
Solids retention time (day)	SRT
Soluble microbial products	SMP
Total ammonia nitrogen (mg N/L)	TAN
Tightly bound extracellular polymeric substances	TB-EPS
Trans-membrane pressure (kPa)	TMP
Total <i>Kjeldahl</i> nitrogen	TKN
Total nitrite nitrogen (mg N/L)	TNN
Volatile fatty acids	VFAs
Waste activated sludge	WAS
Wastewater treatment plant	WWTP
X-ray photoelectron spectroscopy	XPS

List of Symbols

b	endogenous respiration rate (day^{-1}) or decay rate (day^{-1})
$b_{\max}$	maximum decay rate
f_d	fraction of the active biomass that is biodegradable
J_W	water flux through the membrane ($\text{L}/\text{m}^2\cdot\text{h}$)
K_a	ionization constants of nitrous acid
K_b	ionization constants of ammonia
K_W	ionization constants of water
$K_{\text{DO}}^{\text{NOB}}$	affinity for DO of NOB
$K_{\text{FNA}}^{\text{NOB}}$	affinity for FNA of NOB
$K_{\text{I,FA}}^{\text{NOB}}$	inhibition by FA of NOB
$K_{\text{I,FNA}}^{\text{NOB}}$	inhibition by FNA of NOB
n	mole amount of N_2O
P	absolute gas pressure in the N_2O analyser (atm)
R	ideal gas constant ($0.082 \text{ L atm/mol. K}$)
R^2	coefficient of determination
$\text{SRT}_{\min, \text{NOB}}$	minimum SRT for NOB (day)
T	operating temperature ($^{\circ}\text{C}$)
V	measured volume of N_2O (L)
X_{AOB}	biomass of AOB (mg/L)
Y	yield ($\text{kg VSS}/\text{kg NH}_4^+\text{-N}$)
θ	hydraulic retention time (h)
θ_x	solids retention time (day)
μ^{NOB}	growth rate of NOB (day^{-1})
$\mu_{\max}^{\text{NOB}}$	maximum growth rate of NOB (day^{-1})

List of Publications

1. List of Papers

- (1) **Jiang, Y.**, Poh, L.S., Lim, C.P. and Ng, W.J. (2019), " Impact of free nitrous acid shock and dissolved oxygen limitation on nitrification maintenance and nitrous oxide emission in a membrane bioreactor", *Science of the Total Environment* 660: 11-17.
- (2) **Jiang, Y.**, Poh, L.S., Lim, C.P., Pan, C. and Ng, W.J. (2019), " Effect of free ammonia inhibition on process recovery of partial nitrification in a membrane bioreactor", *Bioresource Technology Reports* 6: 152-158.

2. List of Presentations in Conferences

- (1) **Jiang, Y.**, Lim, C.P., and Ng, W.J., “Feasibility of using inherent inhibition to achieve reduced sludge production in a membrane bioreactor (MBR) for treating high ammonium wastewater.” **Oral Presentation**, The Seventh Asian Conference on Sustainability, Energy and the Environment, 8-11 June 2017, Kobe, Japan.
- (2) **Jiang, Y.**, Poh, L.S., and Ng, W.J., “Strategies for maintaining nitrification in membrane bioreactors operated under long solids retention time.” **Oral Presentation**, The Ninth International Conference on Environmental Science and Technology, 25-29 June 2018, Houston, Texas, USA.

Chapter 1 Introduction

1.1 Background and problem statement

Biological nitrogen removal process via the nitrite pathway, i.e. nitrification-denitrification process, has gained attention over the last two decades. Ammonium is first oxidized to nitrite by ammonia oxidizing bacteria (AOB) and subsequently, nitrite is reduced to nitrogen gas by denitrifiers, bypassing nitrate formation and reduction. Compared to conventional nitrification-denitrification process, nitrification-denitrification can reduce by 25% oxygen consumption of the nitrogen oxidation, 30% biomass production and 20% carbon dioxide emission, and save about 40% on the organic substrate demand (Jenicek et al., 2004; Turk and Mavinic, 1986).

Stable nitrification is the critical step for the nitrification-denitrification process. *Nitrosomonas*, which is the most common AOB in WWTP, belongs to a group of chemolithotrophic AOBs that derives energy from ammonia oxidation for biomass growth and maintenance (Bagchi et al., 2012). As autotrophic bacteria, AOB is known to have a slow growth rate (Peng and Zhu, 2006b). Besides, the ammonium concentration in the reject water generated during sludge anaerobic digestion and dewatering varies greatly from 750 (Berends et al., 2005; Perez et al., 2007) to 2500 mg N/L (Meyer and Wilderer, 2004). Notwithstanding the flow of reject water is around 2% of the total flow of raw sewage (Janus and vanderRoest, 1997), if recycled to the headworks of WWTP, reject water would contribute 10-30% of the total nitrogen load in municipal WWTP (Guo et al., 2010). Therefore, side-stream treatment of reject water should be a sophisticated option prior to its final disposal. This requires a large AOB population to manage the high ammonium loads.

The membrane bioreactor (MBR) technology combines membrane filtration with biological treatment to provide better effluent quality. MBR could decouple long solids retention times (SRTs) from hydraulic retention times (HRTs). Retaining biomass in the reactor via membrane filtration, MBRs have been used to enrich bacteria with slow growth rate (Shen et al., 2014; van der Star et al., 2008). MBRs are usually operated with long SRT to increase mixed liquor suspended solids (MLSS)

concentrations, and thus can accommodate the high ammonium load in reject streams. MBRs have been reported to enrich AOB and start-up of nitrification (Shen et al., 2014; Xue et al., 2009).

Stable nitrification needs not only to favour AOB growth, but also inhibit nitrite oxidizing bacteria (NOB) in long-term operations. Nitrite produced by AOB could be oxidized to nitrate by NOB and thus disrupting the nitrification-denitrification process. It has been documented growth of AOB can be favoured and NOB suppressed by manipulating dissolved oxygen (DO) concentration (Munch et al., 1996), pH (Villaverde et al., 1997), SRT (Pollice et al., 2002), free ammonia (FA) (Vadivelu et al., 2007b), free nitrous acid (FNA) (Vadivelu et al., 2006b), and temperature (Hellenga et al., 1998). Nonetheless, NOB would still proliferate in long term operations (Picioreanu et al., 2016). As both AOB and NOB are retained in the MBR, NOB could proliferate with long SRT and when operating conditions fluctuate. Additionally, there are two main NOB genera in WWTPs, *Nitrospira* and *Nitrobacter*, which exhibit different growth strategies (Daims et al., 2001a). *Nitrospira*, representing *K*-strategists, has higher affinity for nitrite and oxygen than *Nitrobacter* which are *r*-strategists (Gilbert et al., 2014). These differences in growth strategies increase the difficulty to inhibit both NOB genera with a single suppression approach. Study is therefore needed to elucidate the method for long term NOB suppression.

Membrane fouling is also an inevitable problem in MBR operation. Membrane separation leads to accumulation of solutes, colloids and biomass on the membrane surfaces. This then leads to decline in the flux and increase in cleaning requirements. It has been reported that biofouling accounts for about 45% of the overall fouling (Komlenic, 2010). Membrane biofouling is a complex process involving microorganism adhesion, and extracellular polymeric substances (EPS) facilitated interaction between biomass and membrane surface (Lu et al., 2016). EPS is considered an important causative agent as it is involved in all identified membrane biofouling mechanisms (Lin et al., 2014).

However, the membrane fouling development process in the nitrification MBR is still unclear. One unique characteristic of the nitrification MBR is that accumulation of nitrite in the reactor leads to increase in FNA concentration, and this can impact EPS

production, biomass concentration and the microbial community. It has been reported FNA could break down the EPS in waste activated sludge (WAS), targeting the protein-like substances (Zhang et al., 2015). To date, the published literature shows few studies about the effect of FNA on EPS production in a *Nitrosomonas* enriched culture. Understanding of the biofouling mechanisms of a nitrifying population under the FNA stress could potentially lead to fouling mitigation strategies for the nitritation MBR.

At the same time, the emission of nitrous oxide (N_2O), a greenhouse gas, during biological nitrogen removal process is of growing concern (Kampschreur et al., 2009). N_2O has 298 times the effect of carbon dioxide in terms of global warming potential (IPCC, 2013) and it can be generated during biological nitrogen removal processes (Kampschreur et al., 2009; Tallec et al., 2006). Studies on N_2O emissions in the nitritation process show large variations among systems. It has been reported that low DO of 0.55 mg/L and high nitrite concentrations up to 500 mg N/L led to higher N_2O emission (Kampschreur et al., 2009; Law et al., 2013). The impact of applied long-term NOB suppression method on the N_2O emission in the nitritation MBR is still unclear.

1.2 Purpose and scope

This project aims to investigate and develop feasible operation strategies for long-term maintenance of stable nitritation in long SRT MBRs treating reject water and to investigate the membrane fouling mechanisms under FNA stress.

The scope of work of this project is as follows:

- 1) To investigate the operating strategies for long-term maintenance of stable nitritation in the long SRT MBR. The effect of high FA and FNA on nitritation maintenance would be investigated, respectively. Variation of key functional bacteria in the population, such as *Nitrosomonas*, *Nitrobacter* and *Nitrospira* would be monitored. N_2O emission from nitritation under the applied strategies would be evaluated. This work may provide important insights into practical operation of nitritation MBRs for high ammonium concentration wastewater treatment.

- 2) To explore the mechanism of membrane fouling in the nitrification MBR by examining the role of EPS in membrane fouling and the effect of FNA on EPS production in a *Nitrosomonas* enriched culture. The results may provide insights into membrane fouling control in the nitrification MBR.
- 3) To examine the application of FNA on membrane fouling mitigation in the nitrification MBR. The effects of FNA on LB-EPS and TB-EPS production by the biofilm and suspended sludge were studied. The EPS component and structure with and without FNA stress were compared. This work may reveal the effectiveness of FNA on membrane fouling mitigation.

1.3 Dissertation organization and overview

This thesis includes eight chapters. Chapter 1, 2, and 3 are the introduction to the project, literature review, and methodology, respectively. Chapter 4 and 5 evaluate the application of FA and FNA on the nitrification maintenance, respectively. Chapter 6 investigates membrane fouling of the nitrification MBR under the stress of increasing FNA concentrations. Subsequently, Chapter 7 explores the application of FNA on the membrane fouling mitigation in the nitrification MBR. Finally, the outcomes of the thesis and future recommendations are presented in Chapter 8.

Chapter 2 Literature Review

2.1 Biological nitrogen removal processes

2.1.1 Conventional nitrification and denitrification

In conventional biological nitrogen removal, nitrogen is removed with a two-step process, nitrification followed by denitrification, as shown in Figure 2.1.a. Nitrification occurs in two sequential oxidation stages: ammonium to nitrite and nitrite to nitrate under aerobic conditions. The most widely recognized genus of AOB is *Nitrosomonas*, whereas *Nitrospira* and *Nitrobacter* are the two most common genus of NOB. Based on measurement of yield and oxygen consumption by *Nitrosomonas* and *Nitrobacter*, the synthesis-oxidation reactions by AOB and NOB are as shown in Eqs. 2.1 and 2.2, respectively (Ahn, 2006).

Eq. 2.1

Eq. 2.2

Based on Eqs. 2.1 and 2.2, the overall synthesis-oxidation reaction of nitrification can be represented as Eq. 2.3:

Eq. 2.3

As shown in the equations, AOB and NOB are autotrophic bacteria. Ammonium is the nitrogen source and carbon dioxide serves as carbon source. The *Nitrosomonas* and *Nitrobacter* cell yields are 0.15 mg cells/mg $\text{NH}_4\text{-N}$ and 0.02 mg cells/mg $\text{NO}_2\text{-N}$, respectively. Therefore, oxygen consumption for ammonia oxidation and nitrite oxidation are 3.16 mg $\text{O}_2\text{/mg NH}_4\text{-N}$ and 1.11 mg $\text{O}_2\text{/mg NO}_2\text{-N}$, respectively. If neglecting cell assimilation, oxygen consumption for nitrogen oxidation from ammonia to nitrate is 4.57 mg $\text{O}_2\text{/mg N}$.

Figure 2.1: Nitrogen cycle: a. nitrification-denitrification, b. nitritation-denitrification, c. *Nitrosomonas* aerobic denitrification or aerobic deammonification, d. *Nitrosomonas* denitrification, e. anaerobic ammonium oxidation and f. overall nitrogen web (Ahn, 2006; Van Loosdrecht, 2004)

The denitrification process is mainly performed by heterotrophic denitrifiers under anoxic conditions. The denitrifiers use nitrate as the electron acceptor and

organic matter as carbon and electron source to reduce nitrate to nitrogen gas, as shown in Eq. 2.4.

Eq. 2.4

Heterotrophic denitrification consists of four consecutive reduction steps from nitrate to nitrite, nitric oxide (NO), N_2O , and finally N_2 . Generally, the conventional nitrification - denitrification process is used for low strength wastewater (total $\text{NH}_4\text{-N}$ concentration less than 100 mg N/L) (Ahn, 2006; Van Hulle et al., 2010) as it is a simple process for nitrogen removal.

2.1.2 Nitritation process

For the past two decades, biological nitrogen removal with nitritation and denitrification has been of growing interest for wastewaters with high ammonium concentration (Peng and Zhu, 2006b), and have been successfully applied at full scale. for example, the single-reactor high ammonia removal over nitrite process (SHARON) is a successful nitritation-denitrification process achieved at full scale (Hellings et al., 1998; Mulder et al., 2001).

Unlike the nitrification-denitrification process, the nitritation-denitrification process uses nitrite, instead of nitrate, as the intermediary compound (Figure 2.1.b). Ammonia is oxidized to nitrite and then the nitrite is reduced as shown in Eq. 2.5, skipping the nitrate steps.

Eq. 2.5

Compared to the conventional process, the nitritation and denitrification process holds advantages as identified below (Beccari et al., 1983; Peng and Zhu, 2006b; Turk and Mavinic, 1987; van Kempen et al., 2001):

- 1) Saving 25% oxygen consumption during nitritation, and hence implying energy savings in the aerobic stage (operational expenditure, OPEX);

- 2) Saving up to 40% of organic carbon source during the anoxic denitrification stage (OPEX);
- 3) Denitrification rates are 1.5 to 2 times faster than nitrification, shorter HRT and smaller reactor footprints (capital expenditure, CAPEX);
- 4) 33-35% less sludge production during nitrification and 55% less during denitrification, smaller volume of sludge to be disposed (OPEX);
- 5) 20% less CO₂ emission.

Therefore, the application of nitrification and denitrification process for high ammonium concentration wastewater would save both OPEX and CAPEX in WWTPs compared to the traditional process.

However, there are also some drawbacks in the nitrification process:

- 1) As a rate-limiting step in the nitrification-denitrification process, the start-up and stable operation of nitrification play an important role in the overall performance. Nitrite produced by nitrification process would be easily and fast oxidized by NOB (Wang et al., 2020). Therefore, growth of AOB should be favoured and that of NOB should be selectively suppressed via different operation parameters. The start-up duration had been reported to extend to 40 days in different studies (Blackburne et al., 2008b; Yang et al., 2007)
- 2) NOB gradually acclimates the operation conditions in the long term operation, and thus requires combined operation strategies or higher inhibition concentration (Ma et al., 2017).
- 3) N₂O emission during nitrification process. It has been reported N₂O emission is higher in the nitrification process than a full nitrification reactor (Wei et al., 2014). This is probably due to the high nitrite concentration but DO limited condition in the bulk liquid (Castro-Barros et al., 2015; Pijuan et al., 2014; Rathnayake et al., 2015; Wei et al., 2014).

2.2 Operation strategies for nitrification start-up and long-term AOB out-competition over NOB

Suppression of NOB is essential for stable partial nitrification. As shown in Table 2.1, different control strategies have been employed to start-up and maintain partial nitrification. DO control is an effective method for nitrification start-up (Laurenzi et al.,

2016), and competition for oxygen between AOB and NOB is the key mechanism (Picioreanu et al., 2016) with the AOB having a lower oxygen half saturation constant (0.2-1.5 mg/L) than the NOB (1.2-1.5 mg/L) (Ge et al., 2015; Regmi et al., 2014).

However, successful nitrification have been reported both at low DO concentration (0.3-0.7 mg/L) (Ma et al., 2009) and at high DO (>1.5 mg/L) (Ge et al., 2014; Regmi et al., 2014). Regmi et al. (2014) had even reported a higher AOB oxygen half saturation coefficient (1.14 mg O₂/L) than NOB's (0.2 mg O₂/L).

The contradiction in the literature implies the complexity of long-term nitrification maintenance, which needs a better understanding of the microbial community. A possible explanation is there are two major types of NOB present in WWTPs, i.e. *Nitrospira* sp. and *Nitrobacter* sp., and these NOBs employ different growth kinetics (Blackburne et al., 2007; Regmi et al., 2014). *Nitrobacter* sp. function as *r*-strategists with higher specific growth, higher nitrite half-saturation constant, but lower oxygen affinity, whereas the *Nitrospira* sp. are *K*-strategists with lower specific growth rates and higher oxygen affinity. Hence, the *Nitrobacter* sp. would be abundant in conditions where nitrite accumulated in the bulk liquid (Isanta et al., 2015), whereas *Nitrospira* sp. favoured conditions with limited nitrite, such as in a one-stage partial nitrification anammox reactor (Winkler et al., 2011) or nitrifying reactor with ammonium to NO_x ratio of 1 (Regmi et al., 2014). Therefore, different nitrification start-up strategies could be applied: with low DO favouring AOB growth over *Nitrobacter* sp., while high DO provided competitive advantage for AOB over *Nitrospira* sp (Wang et al., 2016b).

With the above consideration, there is then the situation when both *Nitrospira* sp. and *Nitrobacter* sp. are present in the reactor, manipulating only DO would not be sufficient to suppress both groups. As shown in Table 2.1, various strategies are applied to start up and maintain nitrification.

Alternating anoxic and aerobic aeration operation could also start-up nitrification. There is activity lag time for NOB behind AOB during transition from anoxic or anaerobic to aerobic conditions (Kornaros et al., 2010). Kornaros et al. (2010) proposed a kinetic model and verified the hypothesis that NOB enzyme is deactivated

under anoxic conditions and activated under aerobic conditions. Thus NOB would have lower growth rates during anoxic disturbance while the AOB is hardly affected. Bournazou et al. (2013) verified the possible mechanism based on a mathematical model that the NOB would be inhibited under periodic aerobic anoxic operation in sequencing batch reactors (SBRs). Ge et al. (2014) adopted this strategy to achieve $81.5 \pm 9.2\%$ nitrite accumulation in a step feed plug-flow process, treating municipal wastewater with average ammonium concentration of 48.8 mg N/L and DO concentration between 0.3 to 2 mg/L.

SRT is another parameter to manipulate to achieve partial nitrification by setting the operating SRT to be lower than the NOB minimum SRT but higher than the AOB minimum SRT (de Graaff et al., 2010). It had been reported the minimum doubling time of AOB is 7-8 hours, which is shorter than that of NOB (10-13 hours) (Peng and Zhu, 2006b). Thus, this would then wash out the NOBs from the reactor (Blackburne et al., 2008a). By adopting a shorter SRT, Yuan and Oleszkiewicz (2011) achieved around 84% nitrite accumulation at high DO concentration (>3 mg/L) and low temperature (13-15°C).

Besides these operation parameters, inhibition by FA or FNA are also widely applied to start up nitrification (Peng and Zhu, 2006a). Concentrations of FA and FNA are functions of pH, temperature, total ammonium or nitrite concentration (Anthonisen et al., 1976). FA is considered the main nitrogen substrate for AOB instead of ammonium (Suzuki et al., 1974). NOB is much more sensitive to FA than AOB. Anthonisen et al. (1976) reported NOB inhibition by FA at 0.1 to 1 mg $\text{NH}_3\text{-N/L}$, while the threshold inhibition concentration on AOB was 10-150 mg $\text{NH}_3\text{-N/L}$. Vadivelu et al. (2007b) found 6 mg $\text{NH}_3\text{-N/L}$ of FA completely stopped NOB biosynthesis. Chung et al. (2007) had reported nitrite accumulation occurred at FA concentration of 20 mg $\text{NH}_3\text{-N/L}$, with NAR over 0.9.

Table 2.1 Process configurations, performance and control strategies for nitrification.

System	Configuration	Control strategy	Scale (L)	Operation conditions	NAR (%)	Reference
Activated sludge	SBR	Real time control based on pH	54000	DO>2.5 mg/L, pH 6.9-7.6, T 11.9-26.5, SRT 13 d	>95	Yang et al. (2007)
	SBR	Intermittent aeration	2000	DO<0.2mg/L, pH 7.2-7.6, T 12.6-24 °C, SRT 16 d	>95	Kornaros et al. (2008)
	SBR	Aerobic duration control based on OUR	10	pH 7.1±0.3, T 18-25°C, SRT 8 d	80	Blackburne et al. (2008a)
	SBR	Real-time control	10	DO 2.5 mg/L, pH 7.2-7.6, T 12-25 °C, SRT 30 d, HRT 8-12 h	>90	Guo et al. (2009)
	A/O	Low DO	300	DO 0.4-0.7 mg/L, pH 7.5, T 21±1 °C, SRT 15 d, HRT 9-10 h	>95	Ma et al. (2009)
	SBR	SRT	60	DO > 3mg/L, pH 7.5, T 13-15 °C, SRT 8d, HRT 12 h	~84	Yuan and Oleszkiewicz (2011)
	MUCT	Low DO, HRT	70	DO 0.3-0.5 mg/L, pH 7.1-7.4, T 23-28 °C, SRT 30±5 d, HRT 6h	>90	Zeng et al. (2013)
	MBfR	Real time control based on pH	0.97	pH 7.1-7.6, T 20°C, HRT 4.5 h	94	Lee et al. (2013)
	MBR	Reducing HRT	5.5	DO 0.15-0.2, pH 7.2-7.6, T 25°C, SRT 100 d, HRT 8 to 2.4 h	~100	Zhang et al. (2013)

	UCT	Intermittent aeration	340	DO 0.3-2.0 mg/L, pH 7.0-7.8, T 16-28°C, SRT 10 d, HRT 8 h	~85	Ge et al. (2014)
	CSTR	Online control by maintaining NH ₄ -N equal to NO _x -N, high DO, minimum SRT	340	DO>1.5 mg/L, pH 7.5, T 25°C, SRT 4-8 d, HRT 3 h	~80	Regmi et al. (2014)
	SBR	Volumetric nitrogen loading rate, DO	26	DO 0.7 mg/L, pH 7.3-8.0, T 22 ± 3°C, SRT 18 d, HRT 8 h	~100	Katsou et al. (2015)
	SBR	SRT		DO >5 mg/L, pH 7.7, T 30±1°C	>95	Wang et al. (2016a)
	SBR	FNA sludge treatment, DO	11	DO 0.3-0.8 mg/L, pH 7.5, T 22±1 °C, SRT 12 d, HRT 13.2 h	~80	Wang et al. (2016b)
Aerobic granules	Continuous flow	Aerobic granule with low NOB	2.3	DO 7 mg/L, T 28±1 °C, HRT 12 h	85-90	Wan et al. (2013)
	Airlift reactor	Low DO/TAN ratio	2.5	DO 1-5 mg/L, pH 8.0±0.1, T 30-12.5°C	-	Isanta et al. (2015)
	Airlift reactor	Low DO/TAN ratio of 0.04±0.02 mg O ₂ /mg N	5.2	DO 0.5-2.5 mg/L, pH 8.0±0.1, T 10°C, HRT 2.5±0.3	~98	Reino et al. (2016)

Notes: A/O: anaerobic/oxic process, A/B: adsorption/bio-oxidation process, MUCT: modified University of Cape Town process, MBfR:

Membrane biofilm reactor, UCT: University of Cape Town process, CSTR: continuous stirred-tank reactor

As for FNA, Anthonisen et al. (1976) reported the threshold inhibition concentration for AOB to be at 0.22 - 2.8 mg $\text{HNO}_2\text{-N/L}$ while Vadivelu et al. (2006a) reported 0.10 mg $\text{HNO}_2\text{-N/L}$ of FNA inhibited *Nitrosomonas* growth although the *Nitrosomonas* enriched culture still oxidized ammonia at half of the maximum rate even after exposure to 0.5 - 0.63 mg $\text{HNO}_2\text{-N/L}$. Wang et al. (2016b) employed FNA sludge treatment with low DO to restore nitrification in municipal wastewater. After treating $\frac{1}{4}$ of the sludge with 1.82 mg N/L FNA for 24 h daily for around 126 days *Nitrobacter* sp. abundance dropped from $7.3 \pm 2.6\%$ to $4.9 \pm 1.2\%$ of total bacteria while *Nitrospira* sp. decreased from $15.7 \pm 3.9\%$ to $0.4 \pm 0.1\%$, and nitrite accumulation was around 60%. Further lowering DO from 2.5-3.0 to 0.3-0.8 mg/L, reduced *Nitrobacter* sp. abundance to $1.8 \pm 0.8\%$ and nitrite accumulation reached 80%.

With high ammonium concentration in the side stream application, FA concentration would exceed the NOB inhibition range of 0.02 to 0.7 mg $\text{NH}_3\text{-N/L}$ and inhibit NOB. Nitrification would lead to pH drop as it generates H^+ . With nitrite accumulating and pH dropping, FNA would then start to inhibit NOB activity. The FNA inhibition levels on a 50% reduction in AOB activity have been reported to be in the range of 0.42–1.72 mg $\text{HNO}_2\text{-N/L}$. Lower FNA concentrations of 0.011–0.07 mg $\text{HNO}_2\text{-N/L}$ would inhibit NOB and 0.026–0.22 mg $\text{HNO}_2\text{-N/L}$ could completely inhibit NOB (Zhou et al., 2011). Wang et al. (2014b) found at 0.24 -1.35 mg $\text{HNO}_2\text{-N/L}$ (nitrite concentration of 100-550 mg N/L , pH 6.0 ± 0.1 , and $T=22^\circ\text{C}$), FNA was more biocidal to NOB than to AOB.

Another property to be taken into consideration is the morphology of AOB. i.e. in the form of suspension cells, flocs, granular or biofilm. Stehr et al. (1995) reported that *Nitrosomonas* could secrete EPS and self-form flocs. Picioreanu et al. (2016) had reported although the intrinsic half-saturation coefficient of AOB (which is not affected by diffusion) is lower than that of NOB, values measured in flocs indicated NOB had better affinity for oxygen than AOB. This seemed to imply high DO concentration favoured nitrification maintenance in the nitrifying flocs. However, based on a three-dimensional (3-D) diffusion-reaction model, Picioreanu et al. (2016) had verified that growing in the cell clusters (micro colonies) of activated sludge flocs,

the oxygen yields of AOB were larger and the average AOB colony sizes were also larger than those of NOB. These two differences led to a larger DO consumption rate and a quicker DO decrease in the AOB colonies, due to stronger concentration gradients (Picioreanu et al., 2016). It was then highlighted that the largest increase in the apparent half-saturation coefficients was linked to the colony size, rather than to the floc size in activated sludge flocs (Picioreanu et al., 2016). This explained why high DO concentration also maintained stable nitrification in the literature (Han et al., 2016).

The same holds true for the granular sludge or biofilm. More stable nitrification and efficient NOB repression could be achieved by oxygen limitation caused by stratified population with AOB at outer shell and NOB in the inner layers (Picioreanu et al., 2016; Poot et al., 2016). Therefore, given that high DO concentration is applied in the mixed liquor of granular sludge or biofilm reactor, the microbial community stratification creates the DO gradients to suppress NOB activity. Another approach for nitrification start-up in the granular reactors is shortening of the settling time for selection of quick settling granules. Wang et al. (2016a) reported around 95% nitrite accumulation achieved in domestic sewage by shortening the settling time gradually from 15 minutes to 4 minutes to washout NOB and produced partial nitrification granular sludge.

In SBR system, real time aeration control strategy has been successfully employed to manipulate aeration durations to achieve nitrification (Lee et al., 2013; Yang et al., 2007). As ammonia oxidation causes the pH to decrease, pH profiles can be used to determine end of aeration when $\text{dpH}/\text{dt} \geq 0$ (Guo et al., 2009). Besides pH profiles, *in-situ* oxygen uptake rate (OUR) can be used to control aeration duration with the threshold point set from 15 to 25 mg $\text{O}_2/(\text{L h})$ (Blackburne et al., 2008a). But the effectiveness of real time control is dependent on the accuracy of pH or OUR sensing and hence probes need regular maintenance.

2.3 Nitrous oxide emission

N_2O emission is a key problem in biological nitrogen removal at WWTPs (Kampschreur et al., 2009). N_2O is an important greenhouse gas, about 300 times stronger than CO_2 with regards to the global warming potential (IPCC, 2013),

(Desloover et al., 2012). The N_2O emission factor is defined as the fraction of influent nitrogen load emitted as N_2O . However, there is large variation in this N_2O emission factor based on the data collected from WWTPs. While typical values range from 0.01% to 1.8%, these can be even higher than 10% (Kampschreur et al., 2009; Wang et al., 2011). This would have been caused by the different water qualities, treatment technologies, operating conditions as well as environmental factors (Wang et al., 2016d).

As shown in Figure 2.1.b, c and d, N_2O can be produced in biological nitrogen removal processes via three pathways - namely incomplete hydroxylamine oxidation, nitrifier denitrification, and heterotrophic denitrification (Ni and Yuan, 2015; Wunderlin et al., 2013; Wunderlin et al., 2012).

2.3.1 N_2O generated in nitrification process

During nitrification, ammonia is oxidized to nitrite by AOB via hydroxylamine (NH_2OH) in its primary energy generating metabolic pathway (Arp and Stein, 2003). If there is incomplete oxidation of NH_2OH to nitrite under low DO conditions, nitrosyl radical (NOH) would be generated as an intermediate (Igarashi et al., 1997) and this is then biologically transformed to NO and then N_2O , or chemically broken down to N_2O ($2\text{NOH} \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O}$) (Poughon et al., 2001). This pathway is the NH_2OH oxidation pathway as shown in Figure 2.2.a. It is also possible for the AOB to convert nitrite to NO with the NirK enzyme, followed by reduction of NO to N_2O by the Nor enzyme (Chandran et al., 2011; Kim et al., 2010), and this known as the nitrifier denitrification pathway.

To date, it is generally accepted that nitrifier denitrification increases with nitrite accumulation under low DO concentration ($\text{DO} < 1 \text{ mg/L}$) (Wunderlin et al., 2013), while NH_2OH oxidation's contribution to N_2O emission increases with DO (Peng et al., 2014). Increased N_2O emission was observed during rapidly changing environmental conditions, such as oxygen limitation (Kampschreur et al., 2008) and ammonia shock loads (Burgess et al., 2002). N_2O emission is not expected but should be down-regulated during nitrification process as it would release considerable amount of N_2O to the atmosphere with regards to the loading of WWTPs.

Figure 2.2 Simplified representation of the three N₂O production pathways by ammonia oxidizing bacteria (A) and heterotrophic denitrifiers (B): nitrifier denitrification, NH₂OH oxidation and heterotrophic denitrification pathways (Ni and Yuan, 2015)

2.3.2 Abiotic N₂O generation

Recently, abiotic production of N₂O has been re-examined (Zhu-Barker et al., 2015)., abiotic N₂O production was identified at high nitrite concentrations (Stuven et al., 1992). Nitrite can participate in chemical reactions either as an oxidizer or as a reducer. Under nitrite accumulation conditions, nitrite could react with other nitrogen intermediates, i.e. NH₂OH. As an intermediate in the biological oxidation of ammonia to nitrite by AOB, NH₂OH could also be detected in the bulk liquid, in the range of 0.01-1 mg N/L in nitrification processes (Soler-Jofra et al., 2016). As discussed in the previous two sections, NH₂OH and nitrite are precursors for N₂O production. Recent work had suggested possibility of abiotic N₂O production at high nitrite concentrations, where the enzymes related to biotic N₂O production are inhibited by high FNA concentrations (Harper et al., 2015; Soler-Jofra et al., 2016; Wang et al., 2016c). Using isotopic analysis, Terada et al. (2017) elucidated the direct participation of NH₂OH and nitrite: $\text{NH}_2\text{OH} + \text{HNO}_2 \rightarrow \text{N}_2\text{O} + 2 \text{H}_2\text{O}$. In a SHARON reactor treating high ammonium concentration wastewater, one third of the total measured N₂O emission was estimated to be linked to this abiotic pathway (Soler-Jofra et al., 2016).

2.4 FNA toxicity and Tolerance of *Nitrosomonas*

FNA, the protonated form of nitrite, exists in equilibrium with nitrite. As a cytotoxin, FNA has biocidal effects to a broad range of microbes involved in WWTP down to parts per billion (ppb) levels (Zhou et al., 2011). As the freely diffusing uncharged lipophilic species, FNA can pass through the cytoplasmic membrane and then disproportionately form cytotoxic reactive nitrogen and reactive oxygen species. FNA affects both catabolic and anabolic processes, and inhibits microorganism growth. Jiang et al. (2011) achieved long-lasting reduction of anaerobic sewer biofilm with FNA, decreasing viable microorganisms from 80% to 5-15%. Huang et al. (2019) found that low levels (<0.02 mgN/L) of FNA remarkably inhibited (over 90%) the conjugative transfer antibiotic resistance by altering intracellular ions and disabling transfer apparatus.

A study on the tolerance of denitrifying bacteria to FNA revealed the following mechanisms of how FNA acts as a cytotoxin (Hartop et al., 2017), as shown in Figure 2.3:

Figure 2.3 Scheme for possible cytotoxic processes of nitrite and FNA in aerobic cultures of *Paracoccus denitrificans* (Hartop et al., 2017).

- 1) FNA leads to the formation of reactive nitrogen and oxygen species in the cytoplasm including nitric oxide (NO), nitrogen dioxide (NO₂), peroxyxynitrite (ONOO⁻), hydroxide ion (OH⁻), and hydrogen peroxide (H₂O₂). These decomposed compounds from FNA lead to cellular function

damage, interruption of metal centres in protein active sites, disruption of biofilm attachment, and finally cell death.

- 2) FNA acts as an uncoupler of energy generation and growth processes (Zhou et al., 2011). FNA forms a short-circuit by transporting protons across the inner membrane and back into the cell by increasing conductance of the cytoplasmic membrane. As a result, ATP synthesis is by-passed.
- 3) FNA directly inhibits electron carriers (Bodenmiller and Spiro, 2006).

Despite the biocidal effects of FNA, susceptibility to it varies considerably among microorganisms. For example, as discussed in Section 2.2.3, NOB is more sensitive to FNA compared to AOB, thus FNA is a selected approach to start-up the nitrification process (Law et al., 2015). Laloo et al. (2018) combined metagenomics and a quantitative sequential windowed acquisition of all theoretical fragment ion mass spectra metaproteomic approach and revealed the mechanisms of persistence of AOB *Nitrosomonas* to the biocide effect with FNA concentration of 1.82 mg HNO₂-N/L.

As described in Figure 2.4, the decomposition of FNA into reactive nitrogen and oxygen species in the *Nitrosomonas* cell mediates oxidative stress, lead to decreased cellular iron bioavailability and phage activation, and cause DNA/protein damage. It is found that *Nitrosomonas* upregulate the following mechanisms to resist the biocide effect of FNA (Laloo et al., 2018):

- 1) Secretion of oxidative stress enzymes. Proteins involved in oxidative stress were detected upon exposure to FNA, including cytochrome *c551* peroxidase, pteridine reductase, *S*-adenosylmethionine synthase as well as antioxidant protein thioredoxin and its associated domain proteins.
- 2) Denitrification. Denitrifying enzyme nitrite reductase was found at an increased expression level upon exposure to FNA (Laloo et al., 2018). By reducing toxic nitrite, denitrifying enzymes contribute to protecting the AOB by lowering nitrite concentration (Poth and Focht, 1985; Zhou et al., 2011)
- 3) Expression of enzymes for energy conversion. Ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO), the key nitrifying and energy generation enzymes in *Nitrosomonas* were found to be of high

abundance on exposure to FNA (Laloo et al., 2018). As FNA is reported to uncouple energy generation (Hartop et al., 2017), the need to maintain internal homeostasis led to the adoption of energy-dependent mechanisms of *Nitrosomonas* (Laloo et al., 2018).

Figure 2.4 Overview model of the responses of *Nitrosomonas* on exposure to FNA. The red arrows and boxes represent metabolic pathways that are upregulated. (Laloo et al., 2018).

- 4) DNA/protein repair. Single-stranded DNA-binding protein, the histone-like DNA binding HU-beta protein was found related to DNA repair enzymes under FNA stress. Peptide methionine sulfoxide reductase and chaperone protein ClpB, important repair enzyme for protein, were upregulated strongly to help in the refolding of denatured stress-damaged protein (Laloo et al., 2018).
- 5) Other stress responses. Phosphomannomutase/phosphoglucomutase which is associated with alginate and lipopolysaccharide biosynthesis was found to be severely inhibited under FNA stress (Laloo et al., 2018). The alginate and lipopolysaccharide, as model EPS polysaccharides, play important roles in biofilm formation. Therefore, it has been postulated that EPS secretion and subsequent biofilm formation would be inhibited under FNA stress.

Biosynthesis of various amino acids was also down regulated on exposure to FNA. In addition, cellular iron bioavailability was altered by FNA, leading to greater expression of enzymes associated with iron transport (Laloo et al., 2018).

2.5 Membrane bioreactors and membrane fouling

2.5.1 Membrane bioreactors for nitrification

By combining membrane separation and biological treatment, MBR technology has been considered a well-established and mature bioprocess with many full scale applications all over the world for the treatment of municipal and industrial wastewater. MBRs have inherited both advantages from membrane separation and conventional biological treatment process, such as small footprint, stable and high effluent quality, high volumetric loading, and lower excess sludge production (Bagheri and Mirbagheri, 2018; Lu et al., 2016). Due to membrane filtration with full biomass retention, MBRs have been used to enrich bacteria with slow growth rate. For example, Wang et al. (2009a) employed a lab-scale MBR to start up Anammox process from the conventional activated sludge. Compared to SBR, MBR had been proved to be more effective for Anammox start-up (Tao et al., 2012), with better biodiversity and higher ecological stability (Wang et al., 2012), due to high sludge retention property of MBR configuration.

So far, nitrification process has also been employed to start up or maintain nitrification of high ammonium streams (Huang et al., 2016; Shen et al., 2014). Nittami et al. (2011) started up nitrification by controlling the influent ammonium concentration (161 mg N/L) and DO concentration (0.6 mg/L) in a submerged MBR. Satisfying nitrification was obtained after a start-up period of 50 days, and achieved ammonium conversion rates of 0.8 kg N/m³/d (Shen et al., 2014). A shorter start-up duration of 26 days was reported by Huang et al. (2016) with high NAR of 94.6%.

Despite the advantages mentioned above, membrane fouling is still a major problem that hinders the widespread and large-scale applications. Membrane fouling increases TMP and decrease permeate flux, which increases operating costs (Bagheri

and Mirbagheri, 2018; Meng et al., 2017). Detailed mechanisms of membrane fouling and fouling mitigation approaches are reviewed in the next sections.

2.5.2 Membrane fouling mechanisms

Membrane fouling is a major issue in the application of the MBR. The international union of pure and applied chemistry working party on membrane nomenclature had defined membrane fouling as “the process resulting in loss of performance of a membrane due to deposition of suspended or dissolved substances on its external surfaces, at its pore openings, or within its pores” (Koros et al., 1996). This then leads to increase in TMP and decrease in permeate flux and productivity. As a result, OPEX increased due to increased energy and membrane cleaning costs.

Much effort has been made to investigate the factors affecting membrane fouling (Komlenic, 2010; Lin et al., 2014; Lin et al., 2010; Meng et al., 2009; Meng et al., 2007; Wang et al., 2009b). HRT, SRT, DO and, biomass characteristics, i.e. MLSS/MLVSS, EPS, soluble microbial products (SMP), particle size, floc structure and microbial community are some of the main factors contributing to membrane fouling (Shao et al., 2019).

Typically, there are three stages in membrane fouling development (Zhang et al., 2006), as shown in Figure 2.5. In stage 1, a short-term rise in TMP occurs due to the pore blocking caused by attachment of bio-flocs and formation of an adsorptive “conditioning” biofilm. In stage 2, TMP increases slowly, either linearly or weakly exponentially. During this stage, pore blocking, biopolymer deposition, biofilm formation and its further growth all contribute to the increasing fouling layer coverage. In stage 3, there is sudden rise in TMP caused by sudden changes in the biofilm or cake layer structure.

2.5.3 Role of EPS in the membrane biofouling

Biofouling results from interactions between the sludge suspension and membrane module, and accounts for about 45% of the total fouling (Komlenic, 2010). EPS is believed to play critical roles in the biofouling process, involving pore blocking by colloidal particles, adhesion of foulants, formation of gel layer and cake

layer, cake layer consolidation, and spatial and temporal changes of biofilm during the long-term operation (Chen et al., 2019; Lin et al., 2014; Zhang and Jiang, 2019).

Generally, polysaccharides (PS) and proteins (PN) are regarded as the main components in the EPS (Meng et al., 2017; Shen et al., 2014). Due to their high molecular weights and gelatinous behaviour, PS are reported to have higher membrane rejection compared to PN and humic acid substances (Seviour et al., 2009), and are thus deemed the primary membrane foulants in MBR (Meng et al., 2017; Teng et al., 2019).

Figure 2.5 Mechanism of membrane fouling for a bioreactor operating at constant flux (Bagheri and Mirbagheri, 2018; Zhang et al., 2006)

The involvement of EPS PN and humic substances complicates membrane biofouling. PS and PN are reported to participate in non-covalent interaction, which

leads to high membrane fouling potential (Neemann et al., 2013). Besides, humic substances also incorporate with PS and PN via hydrophobic and electrostatic interactions (Pivokonsky et al., 2015; Wilen et al., 2003).

2.5.4 Strategies of membrane fouling mitigation

To maintain sustainable application of MBR, different membrane fouling mitigation strategies have been proposed (Bagheri and Mirbagheri, 2018; Lin et al., 2014; Meng et al., 2017).

- 1) Modification of membrane materials. Fan et al. (2001) modified the hydrophobic Polyvinylidene fluoride (PVDF) membrane to hydrophilic and found low fouling potential on the modified PVDF membrane. Membrane fouling could be controlled by restricting the membrane and foulants interaction and subsequent foulant to foulant interaction. Hydrophobic foulants tend to adsorb onto the hydrophobic membrane and then change the surface properties, which facilitates further conjugation of other foulants (Kimura et al., 2012). By modifying membrane materials, membrane fouling could be controlled at the initial contact of foulants to membrane surface.
- 2) Hydrodynamic shear stress on the membrane fouling mitigation. Hydrodynamic shear stresses affect the deposition of bio-flocs onto the membrane surface, in a way to control membrane fouling. Hydrodynamic shear stresses can be generated via the cross-flow velocity along the membrane surface of feed, coarse bubble aeration, and vibration (Li et al., 2016).
- 3) Chemical cleaning of membranes. Chemical cleaning is usually conducted on severely fouled membranes to recover permeability. The membrane permeability recovery mainly depends on the chemical reactions between chemical reagents and the deposited foulants. Usually, there are five types of chemical reagents, i.e. acids, base, disinfectants or oxidants, chelates and surfactants. Commonly used chemical are citric acid, sodium chlorite, hydrogen peroxide, and ozone (Sun et al., 2018). Recently, FNA is explored for reverse osmosis membrane chemical washing (Filloux et al., 2015).

- 4) Biological control of membrane fouling. Biological control methods focus on the inhibition of biofilm formation on the membrane surface (Xiong and Liu, 2010), to prevent cell to cell or cell to membrane interactions. Currently, biological control of membrane fouling involves quorum quenching for inhibition and structure optimization of bio-cake (Weerasekara et al., 2016; Yeon et al., 2009), and enzymatic and bacterial degradation of SMP and EPS (Singh et al., 2015).

Chapter 3 Materials and Methods

This chapter describes the materials and methods employed in this study. More detailed descriptions on specific experimental conditions can be found in the following chapters.

3.1 Reactor setup and membrane module

3.1.1 Laboratory scale membrane bioreactor

The laboratory-scale MBR had a working volume of 2.5 L, as shown in Figure 3.1, with the membrane module submerged in it. The MBR was equipped with a heating tape to maintain temperature at $34 \pm 1^\circ\text{C}$. Aeration was provided through fine bubble air diffusers. DO could be manipulated by adjusting flow rates and the aeration cycle. The reactor was sealed except for the outlets so that gas samples can be extracted from the headspace for N_2O measurements.

Figure 3.1: Schematic diagram of laboratory-scale experimental setup.

The MBR was seeded with an enriched AOB culture drawn from a chemostat. Sludge samples were collected from the sludge discharge valve during agitation while liquid samples were taken from the membrane module permeate.

3.1.2 Membrane module and membrane cleaning

A hollow fibre membrane module (pore size < 0.1 μm , PVDF, Memstar, Singapore) was submerged inside the MBR. The submerged membrane module is shown in Figure 3.2, consisted of 80 pieces of hollow fibres. The membrane module had a surface area of 588 cm^2 . One end of the hollow fibres was sealed with epoxy glue while the other end was fixed to a suction chamber connected to a pump. The discharge pump was operated at 7-minute-on-1-minute-off mode, while feed was introduced continuously.

Figure 3.2: Hollow fibre membrane module in the MBR.

When the trans-membrane pressure (TMP) reached 30 kPa, the membrane module was removed from the reactor to be washed and this may include chemical cleaning. The detailed chemical cleaning protocol is as follows: (i) membrane module was physically cleaned with tap water to remove sludge trapped on and between the hollow fibres. The dislodged sludge was returned to the reactor to minimise sludge loss; (ii) The module was then soaked in 0.5% citric acid for 2 h to remove inorganic materials; and (iii) after washing with tap water, the module was soaked in 0.5% sodium hypochlorite (NaClO) for 2 h before finally rinsing with tap water again.

3.2 Materials

3.2.1 Synthetic wastewater composition

The MBR was fed with synthetic wastewater with the composition as shown in Table 3.1. Ammonium chloride (NH_4Cl) and sodium hydrogen carbonate concentration (NaHCO_3) were changed as appropriate in different phases of the project. The modified trace elements supplement used is as shown in Table 3.2 and was based on van de Graaf et al. (1996).

Table 3.1 Composition of synthetic wastewater used in this project

Chemicals	Synthetic wastewater							
Ammonium (mg N /L)	50	100	200	240	300	400	500	650
NH_4Cl (g/L)	0.191	0.382	0.764	0.917	1.146	1.528	1.91	2.48
NaHCO_3 (mg/L Alk (CaCO ₃))	407	814	1628	1628	1628	2442	2442	2849
NaHCO_3 (g/L)	0.69	1.38	2.76	2.76	2.76	4.14	4.14	4.83
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (mg/L)	200	200	200	240	300	300	300	300
CaCl_2 (mg/L)	56	56	56	67.2	84	84	84	84
KH_2PO_4 (mg P/L)	88	88	88	88	88	88	88	88
Trace Ele. Sol. I &II (mL/L)	0.5	0.5	1	1	1	1	1	1

3.2.2 Reagents and chemicals

All chemicals were of analytical grade purchased from VWR, Singapore. Ultrapure water (18 M Ω cm resistivity) was obtained from a MilliQ water dispenser (Millipore Advantage A10). A 1 mg/mL stock solution of glucose was purchased from Sigma-Aldrich and stored at 4 °C before PS measurement.

Table 3.2 Composition of trace elements solutions

	Compound	Concentration (g/L)
Trace element sol. I	EDTA	6
	FeSO ₄ ·7H ₂ O	9
Trace element sol. II	EDTA	15
	ZnSO ₄ ·7H ₂ O	0.43
	CoCl ₂ ·6H ₂ O	0.24
	MnCl ₂ ·4H ₂ O	0.99
	CuSO ₄ ·5H ₂ O	0.25
	NaMoO ₄ ·2H ₂ O	0.22
	NiCl ₂ ·6H ₂ O	0.19
	Na ₂ SeO ₄ ·10H ₂ O	0.21
	H ₃ BO ₃	0.014

3.3 Experiment protocols

3.3.1 Extraction of bound EPS, SMP, LB-EPS and TB-EPS

The bound EPS was extracted by the heat-extraction method (Yin et al., 2015). Sludge samples taken from the MBR were centrifuged at 4000 revolutions per minute (rpm) for 10 minutes. The resulting sludge pellets were then re-suspended with 0.05% NaCl solution to their original volumes. The mixture was put into a 60 °C water bath for 30 minutes and then centrifuged again at 12000 rpm for 15 minutes. The EPS was finally obtained by filtering the supernatant using 0.45 µm membrane filter.

A modified heat extraction method was used to extract SMP, LB-EPS and TB-EPS from batch tests (Li and Yang, 2007; Yin et al., 2015). Sludge samples taken from the batch test were first centrifuged at 4000 rpm for 5 minutes and the supernatant was filtered through a 0.45µm membrane and collected as SMP. The sludge pellet was then resuspended with 0.05% NaCl solution (preheated to 70 °C) and heated to 50 °C. After 1-minute vortex resuspension and centrifuging at 4000 rpm at 4 °C for 10 minutes, the supernatant was collected after 0.45 µm membrane

filtration as LB-EPS. The residual sludge pellet was re-suspended to the original volume with 0.05% NaCl solution, incubated in a 60 °C water bath for 30 minutes, and centrifuged at 12000 rpm at 4 °C for 15 minutes. The supernatant was collected following 0.45µm membrane filtration as TB-EPS.

3.3.2 Genomic deoxyribonucleic acid extraction

50 mL sludge samples were collected twice per week and centrifuged at 4000 rpm for 10 minutes. 40 mL supernatant was decanted while the remaining 10 mL biomass sludge was suspended and divided into six 2 mL micro tubes with 1.5 mL each. These micro tubes were centrifuged at 14000 g for 10 minutes at 4°C into biomass pellets. The biomass pellets were then washed three times with sterilized water and stored at -80°C before DNA extraction. Genomic deoxyribonucleic acid (DNA) was extracted using FastDNA™ SPIN kit for soil (MP Biomedicals, Singapore) in accordance with the manufacturer's instructions. The DNA concentrations and purities were measured with NanoDrop One (Thermo Scientific, Singapore) and further quantified with Quant-iT PicoGreen dsDNA reagent (Thermo Fisher Scientific, Singapore).

3.3.3 Microbial community analysis by DGGE

Universal primers targeting the bacterial 16S rDNA, 341F (5'-CCTACGGGAGGCAGCAG-3') and 907R (5'-CCGTCAATTCMTTTGAGTTT-3') (Yan et al., 2008), and ammonia monooxygenase (*amoA*, functional gene for AOB), *amoA*-1F-deg6 (5'-GGGGHTTYTACTGGTGGT-3') and *amoA*-i (5'-CCCCTCIGIAAAICCTTCTTC-3') (Winkler et al., 2013), were used. The GC-clamp was appended to the 5' end of the forward primer for differentiation in denaturing gradient gel electrophoresis (DGGE). All polymerase chain reaction (PCR) amplifications were performed using a Thermal Cycler C1000 (Biorad). Prokaryotic 16S rDNA was PCR amplified using the following protocol: thermal cycling was performed using an initial denaturation step at 95°C for 5 minutes, followed by 10 cycles of two identical touch-down PCR amplifications (denaturation at 95°C for 15 seconds, annealing at 70°C for 30 seconds with a 1°C/cycle decrement, and extension at 72°C for 45 seconds), 10 cycles of regular PCR (denaturation at 95°C for 15 seconds, annealing

at 60°C for 30 seconds, and elongation at 72°C for 45 seconds) and a final extension step for 10 minutes at 72°C. PCR reactions for the *amoA* gene were performed as follows. After an initial denaturation step at 95°C for 5 minutes, 30 cycles of regular PCR (denaturation at 95°C for 30 seconds, annealing at 50°C for 40 seconds, and elongation at 72°C for 40 seconds) and a final extension step for 10 minutes at 72°C. The PCR products were resolved with a denaturing gradient of 30% to 60% urea-formamide. Prominent bands were excised. DNA was extracted, purified and cloned as described by Lim et al. (2011). For each excised band, about 8 insert harbouring clones were selected for DNA sequencing. The sequences obtained in this study have been deposited in the GenBank under accession numbers MH333195 to MH333206.

3.3.4 Microbial community analysis by qPCR

The abundance of target microbial communities was determined by quantitative PCR (qPCR) (Light Cycler 480, Roche, Mannheim, Germany), using the primers listed in Table 3.3 targeting the 16S rRNA. For one reaction, 1µl of DNA template was added to 5µl Light Cycler SYBR Green Master Mix, 0.5µl 10µM Forward primer, 0.5µl 10µM Reverse primer and 3µl water.

Table 3.3 Primers used for qPCR analysis of AOB, *Nitrobacter*, *Nitrospira* and Universal bacteria

Target	Primer	Sequence (5'-3')	Reference
<i>β</i> - Subdivision AOB	CTO189f [#]		Araki et al. (2004), Kowalchuk et al. (1997)
	CTO654r	CTAGCYTTGTAGTTTCAAACGC	
<i>Nitrobacter</i>	FGPS872	CTAAAACTCAAAGGAATTGA	Degrange and Bardin (1995)
	FGPS1269	TTTTTTGAGATTTGCTAG	
<i>Nitrospira</i>	NSR1113f	CCTGCTTTCAGTTGCTACCG	Dionisi et al. (2002)
	NSR1264r	GTTTGCAGCGCTTTGTACCG	
Universal	341f	CCTACGGGAGGCAGCAG	Muyzer et al. (1993)
	518r	ATTACCGCGGCTGCTGG	

[#] Mix CTO189fA-B (5'- GGAGRAAAGCAGGGGATCG-3') and CTO189fC (5'- GGAGGAAAGTAGGGGATCG-3') at ratio 2:1.

The AOB and NOB populations in percentage were corrected for 16S rRNA gene copy number per cell by assuming that one copy exists per cell of AOB,

Nitrospira and *Nitrobacter*, and an average of 3.6 copies per cell for the general bacterial population (Dionisi et al., 2002; Stoddard et al., 2015). The NOB population is the summation of *Nitrospira* and *Nitrobacter*.

3.3.5 Microbial community analysis by next generation sequencing

Seven sludge samples in Chapter 5 on days 2, 68, 93, 106, 126, 138 and 198, and one biofilm sample on the hollow fibre surface on day 198 were collected for microbial community analysis. V3 region of 16S rRNA gene was amplified by PCR, using primer pair 338F (5'-CTCCTACGGGAGGCAGCAG-3') and 519R (5'-TTACCGCGGCTGCTGGCAC-3'). Barcodes were added to the 5' end of 519R. All PCR analysis was carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Singapore). The quality of PCR products was determined by electrophoresis on 2% agarose gel. Samples with bright main strip between 400-450bp were chosen for further purification with Qiagen Gel Extraction Kit (Qiagen, Germany). Then the libraries were generated with NEBNext® Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs, Singapore), and quantified via Qubit and qPCR. The 16S rRNA gene sequences were processed by Illumina platform based on paired-end sequencing (Magoc and Salzberg, 2011).

Sequences analysis was performed with Uparse software (Uparse v7.0.1001 <http://drive5.com/uparse/>). Operational taxonomic units (OTU) were acquired from the sequences at a 97% similarity level. Both Alpha diversities including Observed-species, Chao1, Shannon, Simpson, ACE, Good-coverage, and Beta diversity on both weighted unifracs, were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3).

3.3.6 Field emission scanning electron microscopy

Membrane hollow fibres before and after physical wash as well as the biofilm samples were investigated using field emission scanning electron microscopy (FESEM, JSM-7600F) under standard high-vacuum conditions at magnification of 100, 2000 and 20000X. Before FESEM analysis, samples were fixed with 3% of glutaraldehyde in 0.1M phosphate buffer solution (PBS) at pH7.2 for 2 hours, washed with PBS, dehydrated in a series of ethanol concentrations (50%, 60%, 70%, 80%,

90% and 100%) in distilled water for 15 minutes each, and freeze-dried. Subsequently, specimens were coated with platinum and glued to metallic bases with carbon tape.

3.4 Analytical methods

3.4.1 Mixed liquor suspended solids and mixed liquor volatile suspended solids

MLSS and mixed liquor volatile suspended solids (MLVSS) were determined in accordance with the Standard Methods for the Examination of Water and Wastewater (APHA, 2017). For MLSS and MLVSS analysis, 20 mL of sludge was filtered through 1.5 μm filter paper (Whatman, USA), and residual sludge was dried in a 105 °C oven overnight. The dried samples were weighted after cooling to ambient temperature in a desiccator and recorded for MLSS measurement. The dried samples were then heated in a furnace at 550 °C for 0.5 hours, followed by cooling in a desiccator. The difference in sample mass was recorded as MLVSS. All samples were measured in duplicate, and the standard deviation (SD) was within $\pm 5\%$.

3.4.2 Determination of nitrogen compounds in the effluent

Ammonium- ($\text{NH}_4^+\text{-N}$) and nitrite- ($\text{NO}_2^-\text{-N}$) were determined in accordance with Standard Methods (APHA, 2017). Nitrate- ($\text{NO}_3^-\text{-N}$) was measured with test kit TNT 835 (Hach, USA). Before measurement of $\text{NO}_3^-\text{-N}$, samples were pre-treated with 50 mg/L sulfamic acid for 10 minutes to remove nitrite interference.

3.4.3 Determination of polysaccharide and protein in EPS contents

PSs were measured using the phenol-sulfuric acid method (Dubois et al., 1956). PNs were determined using the modified Lowry-Folin method with the protein assay kit (ThermoFisher, USA). Glucose and bovine serum albumin were used as the standards, respectively. Each measurement was repeated twice. The impact of nitrite and nitrate on PS measurements was corrected as shown in Eq. 3.1 according to Appendix. A:

$$C_{PS} = r C_{PS,meas} - 0.067 C_{NO_3-N} \quad \text{Eq. 3.1}$$

where r is the dilution ratio by the addition of sulfamic acid for the nitrite elimination, $C_{PS, meas}$ is the measured concentration of the PS concentration, and C_{NO_3-N} is the nitrate concentration of the sample.

3.4.4 Liquid chromatography-organic carbon-organic nitrogen detection

For analysis of the EPS composition, the Liquid chromatography-organic carbon - organic nitrogen detection system (LC-OCD-OND), equipped with three online detectors, namely organic carbon detector (OCD), UV detector, and organic nitrogen detector (OND), was used to continuously analyse the relative signal response of organic carbon, UV absorbance and organic nitrogen. Details of the measurement procedure can be found elsewhere (Huber et al., 2011). Injection volume of samples was set at 1000 μ L. The identified organic fractions were divided into two parts, i.e. hydrophobic and hydrophilic dissolved organic carbon (DOC). The hydrophilic DOC was subdivided into biopolymers (>10 kDa), humic acid like substances (approx. 500 Da), building blocks (300-500 Da), low molecular weight neutrals (LMW neutral, <350 Da), and LMW acids (<350 Da). The concentration of these fractions was quantified with a software (Chrom CALC, DOC-LABOR, Karlsruhe, Germany), and the unit was mg equivalent carbon per litre (mg-C/L).

3.4.5 Three-dimensional excitation emission matrix spectra with fluorescence regional integration technique

A fluorescence spectrophotometer (LS55, Perkin Elmer Company, USA) was employed to determine three-dimensional excitation emission matrix (EEM) spectra. Excitation scans were performed from 200 to 400 nm at a 10 nm interval. For each excitation wavelength, emission wavelengths were scanned from 280 nm to 550 nm at a step of 2 nm. A 10 nm band-pass of excitation and emission slits and 1200 nm/minute of scanning speed were set in this study, with a threshold of fluorescence emission intensity of 600 a.u.

The EEM spectrum was delineated into five regions as shown in Figure 3.3, i.e. tyrosine-like proteins (Region I, E_x/E_m wavelengths: 200-240/280-330 nm), tryptophan-like proteins (Region II, E_x/E_m wavelengths: 200-240/330-380 nm), fluvic acid-like substances (Region III, E_x/E_m wavelengths: 200-240/380-550 nm),

soluble microbial byproduct-like substances (Region IV, E_x/E_m wavelengths: 250-280/280-380 nm), and humic acid-like substances (Region V, E_x/E_m wavelengths: 280-400/380-550 nm) (Chen et al., 2003). Fluorescence regional integration (FRI) technique was employed to further analyse and quantitatively assess the changes of componential structures of the EPS according to previous study (Sun et al., 2018)

Figure 3.3: Location of EEM peaks (symbols) based on literature reports and operationally defined excitation and emission wavelength boundaries (dashed lines) for five EEM regions (Chen et al., 2003).

3.4.6 X-ray photoelectron spectroscopy analysis

To measure the local functionality of the freeze-dried EPS, X-ray photoelectron spectroscopy (XPS) was conducted with a Kratos Axis Supra spectrophotometer (Shimadzu, Japan) equipped with a dual anode monochromatic K_{α} excitation source ($h\nu = 1486.7$ eV). All binding energies for elements of interest were corrected against an adventitious carbon C 1s core level at 284.8 eV. All XPS peaks were fitted using Shirley background together with Gaussian-Lorentzian function.

3.4.7 Fourier transform infra-red spectroscopy

To characterize the major functional groups in the EPS, the freeze-dried EPS powder was analysed using Fourier transform infra-red (FTIR) spectra (FT-IR/NIR spectrometer Frontier, Perkin Elmer, USA). The FTIR spectra were collected

between 4000 to 400 cm^{-1} with the interval of 1 cm^{-1} and the total number of scans was 16 with the resolution of 4 cm^{-1} .

3.4.8 Nitrous oxide measurement

N_2O concentration (ppmV) in the off-gas was measured on-line with a N_2O analyzer (Model 46i, Thermo Scientific). Headspace of the MBR was sealed and off-gas exited the reactor from a single port at the top of the MBR, which was connected to the N_2O analyzer with a T-joint. During this period, the N_2O analyzer drew the off-gas at flow rate of 0.5 L/minute and N_2O concentrations were measured every 3 seconds and logged every minute. Assuming all dissolved N_2O was stripped into the gas phase by aeration, the cumulative amount of N_2O emitted per day was calculated by summing the product of the N_2O concentration logged every minute with the average off-gas flowrate. The amount of N_2O emitted from MBR was then calculated with the ideal gas law equation:

$$PV = nRT \quad \text{Eq. 3.2}$$

where P is the measured absolute gas pressure in the N_2O analyzer (1 atm), V is measured volume of N_2O (L), n is amount of N_2O (moles), R is the universal gas constant (0.082 L atm/mol. K), and T is the absolute temperature of the detector (276 K). The amount of N_2O in moles was then multiplied by 0.028 to convert to mass of nitrogen (mg N). The N_2O emission factor was calculated by N_2O emission rate divided by ammonium removal rate.

3.4.9 Particle size analysis

Particle size distribution was measured by Shimadzu SALD-3101 laser diffraction particle size analyzer (Shimadzu Co., Ltd, Kyoto, Japan).

3.5 Calculations

3.5.1 FA and FNA

FA and FNA are functions associated with total ammonia nitrogen (TAN, mg N/L), total nitrite nitrogen (TNN, mg N/L), pH, and temperature. Concentrations of

FA and FNA were calculated as shown in equations Eqs. 3.3-3.6 (Anthonisen et al., 1976):

$$FA = \frac{TAN \cdot 10^{pH}}{(K_b/K_w + 10^{pH})} \quad \text{Eq. 3.3}$$

$$\frac{K_b}{K_w} = \exp\left(\frac{6334}{273+T}\right) \quad \text{Eq. 3.4}$$

$$FNA = \frac{TNN}{(K_a \cdot 10^{pH} + 1)} \quad \text{Eq. 3.5}$$

$$K_a = \exp\left(-\frac{2300}{273+T}\right) \quad \text{Eq. 3.6}$$

where K_a , K_b , and K_w are the ionization constants of nitrous acid, ammonia and water, respectively.

3.5.2 Nitrite accumulation ratio

The nitrite accumulation ratio (NAR) was calculated based on nitrite and nitrate concentration in the effluent according to Eq. 3.7 (Yang et al., 2007):

$$NAR = \frac{NO_2^- - N}{NO_2^- - N + NO_3^- - N} \quad \text{Eq. 3.7}$$

3.5.3 Specific ammonium conversion rate and cell-specific ammonium oxidizing rate

Specific ammonium conversion rate (SACR) was calculated as shown in Eq. 3.8 (Khan and Bae, 2016):

$$SACR = \frac{\text{ammonium loading rate} \times \text{ammonium oxidation ratio}}{VSS} \times 100 \quad \text{Eq. 3.8}$$

where ammonium oxidation ratio = $(NO_2^- - N_{\text{effluent}} + NO_3^- - N_{\text{effluent}}) / NH_4^+ - N_{\text{influent}}$.

The cell-specific AOR were derived from the method of Daims et al. (2001b) and calculated according to the following equation:

$$\text{cell specific AOR} = \frac{(\Delta NH_4^+ \times 10^{-3} \text{ mol} \div 14 \text{ mg}) \times r \times 0.9}{\text{total cells in the reactor}} \times 10^{15} \text{ fmol/mol} \quad \text{Eq. 3.9}$$

where cell-specific AOR is expressed as fmol N per cell per h, ΔNH_4^+ is the ammonium removed from the reactor in mg N/L, r is the reactor flow rate (0.208 L/h), 0.9 is the correction factor to account for ammonium removed not only by AOB but also by assimilation (Gujer et al., 1999) and adsorption (Nielsen, 1996), and the estimated total cells in the reactor is calculated by multiplying the AOB cell number per mL obtained from qPCR with the effective reactor volume.

3.5.4 Yield of AOB

The yield of AOB was calculated based on the following Eq. 3.10 as described by Rittmann et al. (1999):

$$X_{AOB} = \frac{\theta_x}{\theta} \times \left[Y_{AOB} \times \frac{1+(1-f_d) \times b_{AOB} \times \theta_x}{1+b_{AOB} \times \theta_x} \times \Delta\text{ammonia} \right] \quad \text{Eq. 3.10}$$

where θ is HRT (0.5 day), θ_x is the SRT (58.5 days), b_{AOB} is the endogenous respiration rate (0.15 day^{-1}), f_d is the fraction of the active biomass that is biodegradable (0.8). X_{AOB} is the biomass of AOB in mg/L, calculated in two models: 1) assuming the diameter and the total density of an AOB cell to be $1 \mu\text{m}$ and 0.636 g/cm^3 (Coskuner et al., 2005), respectively, X_{AOB} was converted by multiplying AOB cell numbers (cells/mL) obtained by qPCR and cell mass (Bellucci et al., 2011); 2) assuming that the VSS consisted of bacteria and EPS, as no organic carbon source was added in the feed, X_{AOB}' was calculated by Eq. 3.11, which was further used to calculate Y_{AOB}' .

$$X_{AOB}' = (\text{VSS} - \text{EPS}) \times \text{AOB\%} \quad \text{Eq. 3.11}$$

where AOB% is the AOB percentage measured by qPCR.

3.5.5 Theoretical minimum NOB SRT

A kinetic model was used to estimate the growth rate and minimum SRT for NOB without consideration of *in-situ* generated FNA shock according to de Graaff et al. (2010). The inhibition of FA and FNA on the growth rate of NOB are included in Eq. 3.12 (Hellings et al., 1999), while the dependence of the decay rate on DO was calculated with a Monod model as shown in Eq. 3.13 (Jubany et al., 2009). The minimum SRT for NOB was calculated with Eq. 3.14 (Ahn et al., 2008).

$$\mu^{\text{NOB}} = \mu_{\text{max}}^{\text{NOB}} \cdot \frac{\text{FNA}}{K_{\text{FNA}}^{\text{NOB}} + \text{FNA} + \text{FNA}^2 / K_{\text{I,FNA}}^{\text{NOB}}} \cdot \frac{K_{\text{I,FA}}^{\text{NOB}}}{K_{\text{I,FA}}^{\text{NOB}} + \text{FA}} \cdot \frac{\text{DO}}{K_{\text{DO}}^{\text{NOB}} + \text{DO}} \quad \text{Eq. 3.12}$$

$$b = b_{\text{max}} \cdot \frac{\text{DO}}{K_{\text{DO}}^{\text{NOB}} + \text{DO}} \quad \text{Eq. 3.13}$$

$$\text{SRT}_{\text{min,NOB}} = 1 / (\mu^{\text{NOB}} - b) \quad \text{Eq. 3.14}$$

where $\mu_{\text{max}}^{\text{NOB}}$ is the maximum growth rate of NOB (1.02 d⁻¹), $K_{\text{FNA}}^{\text{NOB}}$ is the affinity for FNA of NOB (0.0014 mg N/L), $K_{\text{I,FNA}}^{\text{NOB}}$ is the inhibition by FNA of NOB (2.8 mg N/L), $K_{\text{I,FA}}^{\text{NOB}}$ is the inhibition by FA of NOB (1.0 mg N/L), $K_{\text{DO}}^{\text{NOB}}$ is the affinity for DO of NOB (0.5 mg/L) and b_{max} is the maximum decay rate (0.17 d⁻¹). All these parameter values were obtained from Sin et al. (2008).

Chapter 4 Effect of Free Ammonia on an Enriched *Nitrosomonas* Culture and Process Recovery of Partial Nitrification in a Membrane Bioreactor

4.1 Introduction

Nitrification based nitrogen removal processes such as nitrification-denitrification and partial nitrification - anammox can save up to 60% of the aeration energy and have lower electron donor requirements as well as sludge production, compared with the conventional biological nitrogen removal process. A stable nitrification process relies on promoting activities of AOB and/or restraining activities of NOB by manipulating operational parameters, such as low DO (de Graaff et al., 2010), high pH (Villaverde et al., 1997), high FA (Vadivelu et al., 2007a), high FNA (Vadivelu et al., 2006b), and shorter SRT (Regmi et al., 2014).

Among these, FA is an effective approach for nitrification start-up, especially for wastewater with high ammonium concentration (Vadivelu et al., 2006a). NOB is much more sensitive to FA than AOB and is reported to be inhibited by FA at about 0.1 to 1 mg $\text{NH}_3\text{-N/L}$ (Anthonisen et al., 1976). Vadivelu et al. (2007b) found that 6 mg $\text{NH}_3\text{-N/L}$ of FA could completely stop the biosynthetic process of NOB. Chung et al. (2007) found that nitrification was achieved at FA concentration of 20 mg $\text{NH}_3\text{-N/L}$, with nitrite accumulation ratio (NAR) over 0.9.

FA is regarded as the main nitrogen substrate for AOB rather than ammonium or TAN (Suzuki et al., 1974). However, overly high FA has inhibitory effect on AOB activities. The threshold concentration for inhibition on AOB was found to be 10-150 mg $\text{NH}_3\text{-N/L}$ (Anthonisen et al., 1976). FA concentration of 25 mg $\text{NH}_3\text{-N/L}$ was reported to cause a 40% decrease in ammonia oxidation rate for *Nitrosomonas* enriched culture (Balmelle et al., 1992) although Hellinga et al. (1999) found *Nitrosomonas* enriched biomass first showed inhibition at 70 mg $\text{NH}_3\text{-N/L}$. As these *Nitrosomonas* enriched biomass was taken from a reactor with high ammonia removal (over nitrite) while treating high ammonium concentration of 500 mg $\text{NH}_4^+\text{-N/L}$, it had likely been acclimated to the higher FA concentrations. Reject water

generated after sludge digestion units in WWTPs contains ammonium concentration in the range of 750 to 1500 mg N/L (Perez et al., 2007) which leads to the FA reaching 25 to 50 mg $\text{NH}_3\text{-N/L}$ at pH 7.5 in warm temperature above 30 °C. Therefore, it is necessary to elucidate the FA inhibitory concentration of the AOB enriched culture especially for treating high ammonium concentration wastewaters.

Although these studies estimated the FA inhibition on the energy generation process based on ammonium/oxygen consumption rates under different FA concentrations, the anabolic and catabolic activities of AOB under FA inhibition are still not clear. In a trial to assess threshold inhibitory concentration of FA on the anabolic and catabolic activities of enriched AOB culture, Vadivelu et al. (2006a) failed to detect inhibitory effect of FA up to 16 mg $\text{NH}_3\text{-N/L}$. Most of these FA inhibition studies were conducted with relatively short-term batch tests and few studies have reported on process recoverability after FA inhibition.

Therefore, this study aimed to examine FA inhibition on the anabolic activities of an enriched *Nitrosomonas* culture in a 2.5 L reactor operated over the longer term of 126 days. *Nitrosomonas* belongs to a group of chemolithotrophic AOB that derives energy from ammonia oxidation for biomass growth and maintenance processes (Bagchi et al., 2012). It is hypothesized that AOB catabolic and anabolic activities are inhibited under relatively high FA concentrations. Cell-specific AOR was used to estimate the relative catabolic activity. EPS production is associated with the anabolic cell synthesis mechanism and which is supported by the non-growth associated energy consumption i.e. maintenance energy (Robinson et al., 1984). The anabolic activities were investigated and compared using AOB cell abundance, cell yield and EPS production. The recovery of AOB activities after reduction of FA concentration was also tracked.

4.2 Experimental

4.2.1 Experiment setup

A laboratory-scale MBR used in this study is described in Section 3.1.1. The TMP was measured with a pressure gauge. Aeration was provided via fine bubble air diffusers with different flow rates and aeration cycles to control the DO. pH was

maintained by dosing 0.2 mol/L hydrochloric acid (HCl) or 0.2 mol/L sodium hydroxide (NaOH). The pH probe can measure 0.01 difference and the instrument was calibrated every 2 weeks. HRT was fixed at 12 hrs throughout the study. Sludge samples were collected twice a week for determination of MLSS and EPS, and for DNA extraction. No excess sludge was discharged. The MBR was operated with a long SRT of 58.5 days. The composition of synthetic wastewater is described in Section 3.2.1.

4.2.2 Operation conditions

In this study, the experiment was conducted in four phases (Table 4.1). In phase A (days 1-18), the MBR was initiated to achieve partial nitrification with feed of 100 mg $\text{NH}_4^+\text{-N/L}$. After successful start-up, feed concentration was increased to 200 mg $\text{NH}_4^+\text{-N/L}$ in phase B (days 19-58). Subsequently, pH was reduced from 8.3-8.5 in phase B to 8.1-8.3 in phase C (days 59-88), and finally to 7.8-7.9 in phase D (days 89-126). It need be noted that although the differences in pH were small, the differences in FA had been significant. For example, FA concentration for 150 mg $\text{NH}_4^+\text{-N/L}$ was 25.45 mg $\text{NH}_3\text{-N/L}$ at pH 8.3 but 17.13 mg $\text{NH}_3\text{-N/L}$ at pH 8.1.

Table 4.1: Operation conditions of MBR in this study.

Phase (days)	Feed $\text{NH}_4^+\text{-N}$ (mg N/L)	DO range (mg/L)	pH range
A (1-18)	100	<0.1	8.3-8.5
B (19-58)	200	0.1-0.2	8.3-8.5
C (59-88)	200	0.1-0.3	8.1-8.3
D (89-126)	200	0.1-0.3	7.8-7.9

4.3 Results

4.3.1 Reactor performance

In this study, the impact of FA on partial nitrification in the MBR was investigated under different combination of pH and feed ammonium concentration (Table 4.1). MBR performance is described in Figure 4.1a. Figure 4.1b shows the FA and FNA values over the whole study.

Partial nitritation started from day 13 onwards in phase A, with nitrite to ammonium ratio of 0.94. When ammonium concentration in the feed increased to 200 mg N/L in phase B, the nitrite concentration increased to 68.22 mg N/L on day 26.

Figure 4.1: The reactor performance. a Profile of nitrogenous compounds in the effluent as well as NAR in this study, b FA and FNA concentrations

However, the nitrate concentration simultaneously increased to 16.1 mg N/L, with the NAR decreasing from 0.85 to 0.72. DO was then decreased from 0.33 mg/L to 0.14 mg/L and pH was adjusted from 8.25 to 8.43. The corresponding FA was in the range of 29.44 - 42.84 mg NH₃-N/L. Although NAR was then restored to 0.95, nitrite concentration in the effluent remained at 34.55 ± 10.05 mg N/L in phase B. pH was then decreased from 8.42 to 8.27 in phase C, and FA concentration was in the range of 16.43 to 30.23 mg NH₃-N/L. Nitrite concentration increased to 41.86 ± 7.66 mg N/L, while NAR remained at 0.97 ± 0.01 . In phase D, pH was further decreased to 7.86 leading to FA dropping to 8.89 ± 0.95 mg NH₃-N/L. As a result, AOB activity recovered and almost doubled compared to that in phase B. However, nitrite concentration had remained at 62.27 ± 9.47 mg N/L, and partial nitrification was not achieved at the end of this study (nitrite / ammonium ratio of 0.54).

4.3.2 Microbial community structure

Evolution of the overall MBR bacterial population was elucidated by DGGE based on 16S rRNA gene (Figure 4.2a). Considering the long SRT in this study, sludge samples of day 1, day 54, and day 125 were taken for analysis. As phase B ended on day 58, the sample on day 54 represented biomass under high FA concentration while the day 125 sample represented biomass with FA inhibition removed. Sequence and phylogenetic affiliation of the bacteria based on prominent bands in the bacterial community DGGE gel are shown in Table 4.2. Throughout the study, the predominant AOB in the enriched biomass were phylogenetically affiliated with *Nitrosomonas* sp belonging to β -proteobacteria (Table 4.2). DNA sequences closely-related to AOB were recovered from among the prominent bands 1 and 4 in the MBR (Figure 4.2a), shifting from *Nitrosomonas* sp. Clone NSEU-1 (99% similarity) on day 1 to *Nitrosomonas* sp. HP8 (99% similarity) from day 54 onwards.

Figure 4.2: DGGE profile at day 1, day 54 and day 125; a evolution of bacterial communities: the number corresponds to the prominent band for further cloning; b evolution of AOB communities

Evolution of the MBR AOB population was further elucidated by using the *amoA* gene (Figure 4.2b). Resolution of the PCR-amplified *amoA* fragment by DGGE showed that majority of the AOB population persisted throughout this study.

Table 4.2 Phylogenetic affiliations of prominent bands in the bacterial community DGGE profile of MBR.

Bands and clones ^a	Phylogenetic relationship		
	Closest relative	Similarity (%)	Accession No.
1-1, 4	Uncultured <i>Nitrosomonas</i> sp. Clone NSEU-1	99	AB500057
1-6, 7, 8	Uncultured <i>Nitrosomonas</i> sp. Clone NSEU-1	99	AB500057
2-2	<i>Nitrosomonas</i> sp. HP8 partial 1	100	HF678378
4-1, 3, 4, 6, 7, 8	<i>Nitrosomonas</i> sp. HP8	99	HF678378

^a The number before the dash corresponds to the bands in Figure 4.2a; the second numerals distinguish different clones recovered from the same band.

4.3.3 FA inhibition on the catabolic activities of *Nitrosomonas*

SACR reflected AOB activity at entire biomass population level, while cell-specific AOR showed AOB activity at the AOB cell level. Overall, SACR and cell-specific AOR exhibited similar trends in the study (Figure 4.3).

Figure 4.3: SACR and cell-specific AOR during the whole study

Both SACR and cell-specific AOR remained at low level in phase B, varying from 0.19 kg N/ (kg VSS d) and 0.9 ± 0.03 fmol/ (cell h) to 0.79 kg N/ (kg VSS d) and 3.05 ± 0.14 fmol/ (cell h), respectively. As FA decreased to 23.64 ± 4.51 mg $\text{NH}_3\text{-N/L}$, SACR and cell-specific AOR recovered to 1.32 kg N/ (kg VSS d) and 4.34 ± 0.21 fmol/ (cell h), respectively.

Compared to SACR, cell-specific AOR exhibited AOB activity more precisely (Figure 4.3), as it defined the metabolic rate of the AOB cell (Bellucci et al., 2011). It has been suggested that cell-specific AOR between 4 and 10 fmol/ (cell h) could meet the energy needs of AOB (Pickering, 2008). This suggested that the energy generation from ammonia oxidation in phase B was inhibited by FA.

4.3.4 FA inhibition on the anabolic activities of *Nitrosomonas*

Figure 4.4 shows the evolution of AOB and NOB percentages, as well as AOB cell numbers per dry biomass in the study. Overall, AOB was the dominant group in

the biomass, varying from 46.7% to 78.0% (Figure 4.4a). At FA concentration of 32.47 ± 6.59 mg $\text{NH}_3\text{-N/L}$, AOB percentage decreased from 57.2% to 50.2% in phase B, and further decreased to 46.7% on day 69 in phase C. AOB biomass decreased from 8.11×10^{11} cells/g VSS on day 5 to 4.57×10^{11} cells/g VSS on day 22, and further to 3.437×10^{11} cells/g VSS on day 40 (Figure 4.4b). The abundance of AOB population doubled from 5.04×10^{11} cells/g VSS on day 56 to 1.07×10^{12} cells/g VSS on day 82. This was probably due to the decrease in FA to 23.64 ± 4.51 mg $\text{NH}_3\text{-N/L}$. As FA decreased subsequently in phase C, AOB percentage increased to 53.0%, and further increased to 78.0% in phase D after FA dropped to 8.89 ± 0.95 mg $\text{NH}_3\text{-N/L}$.

Figure 4.4: Abundance of 16S rRNA gene of key functional species in the MBR by qPCR; a evolution of AOB and NOB percentage; b AOB cell abundance.

AOB yield was also calculated based on the model described by Rittmann et al. (1999), as shown in Eq. 3.10, and results are shown in Figure 4.5. In this study, AOB yield calculated by both models gave much lower values than the theoretical value of 0.34 kg VSS/ kg $\text{NH}_4^+\text{-N}$ (Rittmann et al., 1999). This would mean the MBR had reduced sludge production under high FA. The yield calculated with model 2 (Y_{AOB}') was larger than that with model 1 (Y_{AOB}) throughout the study, and this was probably because the cell diameter and density selected in model 1 was under non-FA inhibition conditions (Coskuner et al., 2005).

To estimate the cell yield under the high FA concentration, yield per cell was calculated and expressed by Y_{AOB}' divided by the AOB abundance in the reactor. Positive correlation was found between yield per cell and FA concentration from day 22 to day 69 in phases B and C, with the coefficient of determination (R^2) of 0.8988 (Figure 4.6).

Figure 4.5: AOB yield calculated based on two models - Model 1 (Y_{AOB}) assumes the diameter and the total density of an AOB cell to be 1 μm and 0.636 g/cm^3 , respectively; Model 2 (Y_{AOB}') assumes biomass equals to the difference of VSS and EPS.

Figure 4.6: Correlation between FA concentration and AOB yield per cell calculated with Model 2 in phase B and C ($R^2=0.8988$)

4.3.5 EPS secretion

Figure 4.7 shows the evolution profile of EPS composition in terms of PN and PS, as well as the PN/PS ratio in the various phases. Overall, there was more PN than PS, with PN/PS ratio varying from 1.12 to 2.36. The PN had varied from 16.4 ± 0.05 to 33.24 ± 0.12 mg/g VSS, while PS varied from 7.29 ± 0.59 to 23.34 ± 1.44 mg/g VSS.

EPS production decreased from 14.74 ± 4.75 mg PS/g VSS and 22.74 ± 5.13 mg PN/g VSS in phase A to 11.58 ± 2.75 mg PS/g VSS and 21.4 ± 3.73 mg PN/g VSS in phase B and then increased to 16.25 ± 4.93 mg PS/g VSS and 30.78 ± 8.98 mg PN/g VSS in phase C. After FA decreased to 9.33 ± 1.06 mg NH₃-N/L early in phase D, EPS further increased to 22.63 ± 1.01 mg PS/g VSS and 33.16 ± 0.12 mg PN/g VSS. However, from day 106 onwards, EPS decreased to 13.56 ± 3.18 mg PS/g VSS and 21.59 ± 3.40 mg PN/g VSS, probably due to the increase in FNA.

To better estimate the inhibitory effect of FA on the EPS production, EPS content versus FA concentration in phases A, B and C was plotted (Figure 4.8). Both PS and PN was found to decrease after FA reached 25.98 mg NH₃-N/L.

Figure 4.7: Evolution profile of EPS content and PN/PS ratio in the MBR

Figure 4.8: Effect of FA concentration on the EPS content (PS and PN) in phases A, B and C

4.3.6 AOB activity recovery after FA inhibition removal

AOB recovered activity 12 days after FA was reduced to 8.89 ± 0.95 mg $\text{NH}_3\text{-N/L}$ in phase D. The highest SACR of 2.29 kg N/ (kg VSS d) was then achieved, while cell-specific AOR reached 10.34 ± 0.8 fmol/ (cell h) (Figure 4.3). PS and PN productions in the EPS increased to 22.63 ± 1.01 mg PS/g VSS and 33.16 ± 0.12 mg PN/g VSS, respectively (Figure 4.7). As pH was reduced to alleviate FA toxicity, FNA concentration increased from 7.6×10^{-4} mg $\text{HNO}_2\text{-N/L}$ on day 87 to 1.56×10^{-3} mg $\text{HNO}_2\text{-N/L}$ on day 100 (Figure 4.1b). The FNA concentration increase in phase D was matched with AOB cell abundance decrease from 1.07×10^{12} cells/g VSS on day 82 to 8.80×10^{11} cells/g VSS on day 99 (Figure 4.4b). After exposure to FNA of 1.58×10^{-3} mg $\text{HNO}_2\text{-N/L}$ for 27 days, PS decreased to 10.15 ± 0.20 mg PS/g VSS while PN decreased to 18.23 ± 0.04 mg PN/g VSS. This resulted in sharp declines in both the AOB percentage and AOB cell number, from 78.0% and 8.80×10^{11} cells/g VSS to 48.4% and 1.64×10^{11} cells/g VSS, respectively. The decrease in EPS production and AOB abundance indicated the enriched biomass' lower tolerance to FNA after an extended period of exposure to high FA concentrations.

4.3.7 NOB activity

NOB population accounted for 2.2% to 16.9% of overall biomass during this study (Figure 4.4a). The abundance of NOB varied from 6.10×10^9 cells/g VSS to 1.32×10^{11} cells/g VSS. Although NOB percentage reached 16.9% on day 40, NOB activity remained inhibited by the high FA concentrations in phases B and C, and the nitrate concentration had remained at 0.97 ± 0.42 mg N/L (Figure 4.1a). However, after FA decreased to 8.89 ± 0.95 mg $\text{NH}_3\text{-N/L}$ in phase D, NOB population increased to from 2.3% to 10.7%. Correspondingly, nitrate concentration in the effluent increased from 0.85 to 5.30 mg N/L (Figure 4.1a), which led to the NAR decreasing to 0.91 at the end of phase D.

4.4 Discussion

Microbial community analysis (Figure 4.2b) revealed that *Nitrosomonas* was the dominant bacteria throughout the study. *Nitrosomonas*, which are autotrophic ammonia oxidizers, belong to a group of chemolithotrophic AOB that derive energy

from ammonia oxidation for biomass growth and maintenance processes (Bagchi et al., 2012). As substrate of *Nitrosomonas*, FA can diffuse across the cell membrane while ammonium is rejected by the cell lipid membrane (Kadam and Boone, 1996). Figures 4.1 and 4.3 showed that by decreasing FA from 34.34 ± 6.85 mg NH₃-N/L (phase B) to 8.89 ± 0.95 mg NH₃-N/L (phase D), AOB activity increased by 47.7%, while cell-specific AOR increased by 80.2%. This suggested there might have been FA inhibition in phases B and C and that the threshold FA concentration for inhibition might have been 23.64 ± 4.51 mg NH₃-N/L in phase C, below which effluent nitrite concentration, AOB cell numbers, SACR, and cell-specific AOR increased (Figures 4.1, 4.3, and 4.4b).

This is in accordance with previous studies as summarized in Table 4.3. Balmelle et al. (1992) found 40% decrease in ammonia oxidation rate at 25 mg NH₃-N/L for a *Nitrosomonas* enriched culture, while Vadivelu et al. (2006a) reported that FA up to 16 mg NH₃-N/L did not have any inhibitory effect on either the catabolic or anabolic processes of *Nitrosomonas* enriched culture. The FA inhibition on the catabolic and anabolic activities as well as the process recoverability after FA inhibition removal in this study are discussed in this section.

4.4.1 FA inhibition on the catabolic and anabolic activities of *Nitrosomonas*

Since FA has high permeability through the cell membrane, it is believed FA changed the intracellular pH, interfered with the trans-membrane pH gradient required for ATP synthesis, and thus inhibited the energy generation from ammonium oxidation (Kadam and Boone, 1996; Wittmann et al., 1995). Thus, the cell-specific AOR in phase B was lower than the suggested AOB energy needs. The decrease in energy generation led to the decrease of AOB abundance as well as the low yield. Due to the increase in intracellular pH caused by FA, AOB would need to spend more energy on maintaining homeostasis or cell osmotic pressure, prior to spending energy on biomass production under energy generation inhibited conditions (Dawes and Ribbons, 1964). Vadivelu et al. (2007b) also speculated that nitrifiers might have to spend all the energy produced for cell maintenance in the scenario that energy production of nitrifiers was inhibited by FA.

However, as shown in Figure 4.6, higher yield per AOB cell seemed to have occurred under high FA concentration ($R^2 = 0.8988$). It was hypothesized that under the FA inhibition, AOB tended to increase cell mass instead of propagation. Figure 4.6 shows higher yield per AOB cell ($R^2=0.8988$) occurred at higher FA concentration but cell abundance had decreased as shown in Figure 4.4b. This was in accordance with previous studies which had noted under inhibition or stress, cells struggled for survival leading to changes in cell morphology and density (Boulon et al., 2010; Malek and Izumo, 1996).

Table 4.3 Comparison of FA inhibition concentrations obtained in this study with the literature reports

Reference	FA inhibition	Remarks
Anthonisen et al. (1976)	10-150mg NH ₃ -N/L found to be the threshold concentration for inhibition on AOB	Activated sludge treating municipal wastewater
Balmelle et al. (1992)	40% decrease in ammonia oxidation rate at 25 mg NH ₃ -N/L	Enriched <i>Nitrosomonas</i> culture in an SBR system treating synthetic wastewater containing 400 mg NH ₄ -N/L
Hellinga et al. (1999)	Initiated inhibition at 70 mg NH ₃ -N/L	Biomass from a SHARON reactor with ammonium around 500 mg NH ₄ -N/L in the reactor
Vadivelu et al. (2006a)	FA up to 16 mg NH ₃ -N/L did not have any inhibitory effect on either the catabolic or anabolic processes	Enriched <i>Nitrosomonas</i> culture in an SBR system
This study	Threshold FA concentration for inhibition might have been 23.64 ± 4.51 mg NH ₃ -N/L on the relative catabolic and anabolic processes	Enriched <i>Nitrosomonas</i> culture in an MBR treating synthetic wastewater containing 200 mg NH ₄ -N/L

This could explain why the yield estimated by Model 1 gave a lower value than Model 2 (Figure 4.5). The difference between the two models was influenced by how the AOB biomass was calculated. The cell diameter and density used in model 1 was selected from a suspended cell culture under non-FA inhibited situation (Coskuner et al., 2005). Whereas in this study, AOB cells might activate mitigation mechanisms to relieve the FA toxicity and consequently increased the cell density or size. It has also been reported that AOB cells produced N-acyl homoserine lactones (AHL) during starvation and thus had a higher cell density (Batchelor et al., 1997).

4.4.2 Implication of EPS production on maintenance energy consumption under FA inhibition

EPS is associated with the anabolic cell synthesis machinery and supported by the non-growth-associated energy consumption i.e. maintenance energy (Robinson et al., 1984). Stehr et al. (1995) reported that *Nitrosomonas* could secrete EPS and self-form flocs. The EPS functioned as a buffer against toxic compounds as well as against changing environmental conditions (Batchelor et al., 1997; Stehr et al., 1995). As bacteria accords priority to maintenance energy, most of which is distributed to EPS formation (Robinson et al., 1984), it is a natural response of microbes to increase EPS production upon exposure to stress such as a toxic environment. However, when the toxin reaches to the inhibitory level, catabolic activities are inhibited and thus EPS production decreases (Sheng et al., 2005). Similar trend was also shown in this study (Figure 4.8): both PS and PN production decreased after FA reached 25.98 mg NH₃-N/L, which implied this was the threshold concentration of FA inhibition in this study.

PN and PS in this study were less than those reported in an AOB-enriched nitrifying SBR (Wei et al., 2015) and far less than those reported in a nitrification MBR (Lu et al., 2016). The PN/PS ratio was in the range of 1.77 to 2.24 (Figure 4.6). Similar values had also been found in a nitrifying SBR where nitrification was completely inhibited at FA higher than 10 mg NH₃-N/L (Yang et al., 2004). In their study, the PN/PS ratios at high FA concentration of 23.5 and 39.6 mg NH₃-N/L were 1.61 and 1.72, respectively. This was comparable with values in this study. The synthesis of cell PS would be unlikely under high ammonia concentration (Harris and Mitchell, 1973; Yang et al., 2004). Sheng et al. (2005) had confirmed that under

inhibition, more PN was secreted than PS to protect the cell by diffusion limitation and/or by chemical binding. All these contributed to the high PN/PS ratios in this study.

4.4.3 Process recovery after FA inhibition removal

After FA was reduced to 8.89 ± 0.95 mg $\text{NH}_3\text{-N/L}$, the cell-specific AOR increased from 5.15 ± 0.54 to 13.6 ± 1.61 fmol/ (cell h) (Figure 4.3). Despite the increased AOB activity, nitrite concentration in the effluent remained at 62.61 ± 10.15 mg N/L over the following 27 days (Figure 4.1a). As discussed before, the energy generated should meet the needs of AOB growth. After exposure to FNA concentration of 1.58×10^{-3} mg $\text{HNO}_2\text{-N/L}$, the decreased AOB abundance (Figure 4.4b) indicated the sensitivity of AOB biomass to FNA. It has been reported the cell-specific AOR could indicate risk of nitrification failure, and high cell-specific AOR but low AOB cell number in this study suggested the partial nitritation in the MBR would likely fail (Bellucci et al., 2011; Eyice et al., 2015).

The tolerance of *Nitrosomonas* to FNA in this study was two orders of magnitude lower than those reported in the literature. Anthonisen et al. (1976) found the threshold concentration for AOB to be 0.22 - 2.8 mg $\text{HNO}_2\text{-N/L}$. Vadivelu et al. (2006a) reported 0.10 mg $\text{HNO}_2\text{-N/L}$ of FNA inhibited growth of *Nitrosomonas*, and these enriched *Nitrosomonas* culture still oxidized ammonia at half of the maximum rate even exposure to 0.5 - 0.63 mg $\text{HNO}_2\text{-N/L}$ (Vadivelu et al., 2006a). One possible reason could be that EPS production in this study was not enough to protect the AOB bacteria. The highest PS and PN concentration in this study were 22.63 ± 1.01 mg PS/g VSS and 33.16 ± 0.12 mg PN/g VSS, respectively, which were much less than those in a nitritation MBR treating high ammonium concentration wastewater (estimated 44.44 mg PS/g VSS and 57.78 mg PN/ g VSS, respectively based on the figures in Shen et al. (2014)). Although EPS production increased after FA decreased in early phase D (Figure 4.7), EPS production decreased sharply with the increase of FNA in late phase D. Another hypothesis would be that the cell density and cell morphology might be changed under FA inhibition and thus FNA could pass through the cell membrane more easily.

On the other hand, both NOB population and nitrate in the effluent exhibited an increasing trend in phase D. Although NOB was reported to cease biosynthesis at FA of 7.5 mg NH₃-N/L (Vadivelu et al., 2007a), in this study, NOB was acclimated at FA of 34.34 ± 6.85 mg NH₃-N/L, and proliferated when FA decreased to 8.89 ± 0.95 mg NH₃-N/L in phase D (Figs. 4.1b and 4.4a). Since nitrite concentration increased, this could also have provided substrate for NOB in phase D, which stimulated NOB growth and induced NOB activity.

4.5 Concluding remarks

This study described the long-term inhibitory effect of FA on the anabolic and catabolic activities of enriched *Nitrosomonas* culture. FA concentration of 34.34 ± 6.85 mg NH₃-N/L clearly inhibited AOB activities. The threshold concentration of FA inhibition on *Nitrosomonas* was likely 23.64 ± 4.51 mg NH₃-N/L. The yield was noted to be lower than the theoretical yield value of 0.34 kg VSS/kg NH₄⁺-N, and the differences in the yield estimated by the two models suggested AOB cell density or size might be higher due to the cell maintenance activity against FA inhibition. Although AOB activity recovered within 12 days after decreasing FA to 8.89 ± 0.95 mg NH₃-N/L, a sharp decline in AOB cell number by 62.73% occurred at FNA concentration of 1.58×10^{-3} mg HNO₂-N/L. This suggested long-term inhibition by FA influenced recoverability of AOB.

It should therefore be taken care to ensure FA below the inhibitory concentration for AOB when using FA in start-ups or long term maintaining (partial) nitrification, especially for treatment of high ammonium concentration wastewater. Further studies on evolution of cell structure and cell morphology under FA inhibition could be undertaken for deeper understanding of the inhibition phenomenon.

Chapter 5 Impact of Free Nitrous Acid Shock on Nitrification Maintenance and Nitrous Oxide Emission in a Membrane Bioreactor

5.1 Introduction

FNA, the protonated form of nitrite, has gained interest since its biocidal effects to a broad range of microbes found in WWTPs was reported (Zhou et al., 2011). FNA treatment at 2.0 mg $\text{HNO}_2\text{-N/L}$ for 24 - 42 h could reduce excess sludge production by 28% (Wang et al., 2013b). FNA treatment also enhanced the biodegradability of WAS by improving volatile fatty acid production (Wang et al., 2014a; Wang and Yuan, 2015), and is effective at exposure time shorter than 8 hours (Zahedi et al., 2017). Recent studies have demonstrated FNA could serve as a pre-treatment method for sludge fermentation to enhance methane (Wang et al., 2013a) or short-chain fatty acids production (Zhao et al., 2015) from WAS, to better achieve nutrient removal and energy recovery at WWTPs.

FNA can be generated in the WWTPs through nitrification of the reject stream from sludge dewatering units (Law et al., 2015). Ammonium concentration in the reject stream had been reported to vary from 750 to 1500 mg N/L (Berends et al., 2005) and up to 2500 mg N/L in sludge liquor (Meyer and Wilderer, 2004). A large AOB population is required to manage the high ammonium loads. However, the AOB's slow growth rate prolongs the nitrification start-up period. The MBR, which decouples HRT from SRT, can be employed to start up or maintain nitrification of high ammonium streams (Huang et al., 2016; Shen et al., 2014). MBRs are usually operated with high MLSS and long SRTs, and thus can accommodate the high loading of ammonium in reject streams.

However, the retention of NOB in the MBRs disrupts the nitrification process. It is well documented that NOB activities could be suppressed by low DO concentrations, temperature, pH, FA as well as FNA (Ge et al., 2015; Sinha and Annachhatre, 2007). However, NOB could still acclimate and proliferate in long term operations (Picioreanu et al., 2016). Besides, there are two main NOB genera in

WWTPs, *Nitrospira* and *Nitrobacter*, which exhibit different growth strategies (Daims et al., 2001a). *Nitrospira*, representing *K*-strategists, has higher affinity for nitrite and oxygen than *Nitrobacter* which are *r*-strategists (Gilbert et al., 2014). These different strategies increase the difficulty to inhibit NOB with DO limitation. Wang et al. (2016b) recycled 25% of sludge to a side stream with FNA treatment at 1.82 mg HNO₂-N/L for 24 hours and successfully reduced both *Nitrospira* and *Nitrobacter* in a DO limited environment. But high FNA concentration at 2.0 mg HNO₂-N/L over long exposure time could break down the EPS into smaller molecules (Zhang et al., 2015) and severe membrane fouling have been reported due to increase in EPS under high FNA concentrations (Shen et al., 2014).

Of growing concern is the emission of N₂O during nitrification. N₂O is a strong greenhouse gas with global warming potential which is about 300 times that of carbon dioxide. Its emission should, therefore, be minimized. Nitrifier denitrification and NH₂OH oxidation, are two biological pathways identified for N₂O production by AOBs (Kampschreur et al., 2009). It has been reported that low DO of 0.55 mg/L and high nitrite concentration up to 500 mg N/L led to higher N₂O emission, compared to nitrite concentration of 200-250 mg N/L at DO above 1.3 mg/L (Kampschreur et al., 2009; Law et al., 2013). High FNA and low DO has been reported to lead to higher N₂O emission via aerobic denitrification to detoxify FNA (Kampschreur et al., 2009; Zhou et al., 2011).

It should be noted that the hydrogen ion is generated during nitrification (Sinha and Annachatre, 2007) and thus pH would progressively decrease if base buffer dosing is stopped. Based on these previous observations, this study proposed a novel *in-situ* generated FNA shock strategy combined with DO limitation to maintain nitrification in MBRs operated under long SRTs. The principle of *in-situ* generated FNA shock is that the FNA concentration increased following accumulation of nitrite and decreasing pH to the concentration which inhibited NOB. The main aim of this study was to investigate the strategy of DO limitation and *in-situ* generated FNA shock to maintain stable nitrification in MBRs operated under long SRT. A second objective was to evaluate the impact of such strategy on N₂O emission.

5.2 Experimental

5.2.1 Reactor setup and operation conditions

The laboratory scale aerobic MBR used in this study is described in Section 3.1.1 as shown in Figure 3.1. The initial mixed liquor volatile suspended solids (MLVSS) was 592 mg/L. The reactor was fed with synthetic wastewater as described in Table 3.1. The MBR was operated at SRT of 43.8 days. HRT was fixed at 9 ± 1 hrs throughout the experiment. Sludge samples were collected twice a week for the measurement of MLSS, and for DNA extraction. From day 165 onwards, gas samples were collected for N₂O measurement. The MBR operation details are summarized in Table 5.1.

Table 5.1: Operation conditions and strategies applied in this experiment

Operation period	NH ₄ ⁺ -N (mg N/L)	DO (mg/L)	<i>in-situ</i> generated FNA shock	pH	T (°C)
Day 1-82	200-300	<0.5	No	7.80-8.15	32±1
Day 83-106	400-500	0.5-0.8	No	7.81±0.05	32±1
Day 107-156	500	<0.5	Yes	7.81±0.05	32±1
Day 157-204	650	0.5-0.8	Yes	7.81±0.05	32±1

5.2.2 Nitrification maintenance strategies

Feed ammonium concentration was increased stepwise from 200 mg NH₄⁺-N/L to 650 mg NH₄⁺-N/L, when nitrification had been stably maintained for at least 10 days with nitrite accumulation ratio (NAR) over 0.90 following each step change. pH was maintained at around 7.8 by dosing 0.5 mol/L NaHCO₃ solution. Temperature (T) was kept at 32 ± 1 °C with heating tape.

DO limitation (<0.5 mg/L) was applied for the start-up and maintenance of (partial) nitrification. The *in-situ* generated FNA shock method was first applied on day 134 and then implemented when NOB percentage was higher than 10.0% as detected by qPCR analysis on day 149 and day 195. Dosing of NaHCO₃ would then be stopped. pH in the reactor progressively decreased from 7.8 to 6.1-6.3 within the 12-hour

treatment period. Concentrations of FNA were calculated according to Anthonisen et al. (1976). The highest FNA concentrations reached were 0.39, 0.51 and 0.61 mg $\text{HNO}_2\text{-N/L}$ in the three FNA shocks, respectively. Figure 5.1 depicts the variation of pH and FNA concentrations during the *in-situ* generated FNA shock on day 195.

Figure 5.1: Variation of pH and calculated FNA concentrations during *in-situ* generated FNA shock conducted on Day 195.

5.3 Results and Discussion

5.3.1 Nitrification performance in the MBR

The variations of ammonium, nitrite and nitrate concentrations in the effluent of the nitrification MBR are shown in Figure 5.2a. Ammonium concentration had been increased stepwise from 200 to 650 mg $\text{NH}_4^+\text{-N/L}$, and maximum ammonium loading rate (ALR) reached 1.81 kg $\text{N}/(\text{m}^3 \text{ day})$ with HRT fixed at 9 ± 1 hours (Figure 5.2b). Influent ammonium was converted to nitrite with ammonium removal ratio (ARR) of 0.965 (Figure 5.2b) and NAR of 0.960 at the end of this experiment, indicating high nitrification performance in the MBR operated at long SRT of 43.8 days. Correspondingly, FNA increased to 1.47×10^{-2} mg $\text{HNO}_2\text{-N/L}$ at the end of this study.

The operation conditions are summarized in Table 5.1. DO and pH profiles are depicted in Figure 5.2c. Partial nitrification was first initiated with feed ammonium concentration of 200 to 240 mg $\text{NH}_4^+\text{N/L}$ from day 1 to day 48. There was failure in the air delivery line following moisture condensation in the airflow meter from day 49 to day 59, resulting in low DO in the MBR. Imbalance of nitrogen mass between the influent and effluent was found during this period. Nitrification was achieved with feed 240 mg $\text{NH}_4^+\text{N/L}$ after fixing the air flow meter on day 60. DO fluctuated from day 85 to day 106, which resulted in nitrate build-up that deteriorated the nitrification. To suppress the NOB activity, DO limitation was applied from day 107. After nitrification was restored, the strategy of *in-situ* generated FNA shock was first applied on day 134 and then applied again when NOB percentage was higher than 10.0% as measured by qPCR on day 149 and day 195. The nitrate concentration in the effluent remained at 26.74 ± 4.46 mg N/L from day 136 to day 204 (Figure 5.2a).

MLVSS had increased from 573 mg/L to 2383 mg/L, with MLVSS to MLSS ratio remaining at 0.72 ± 0.02 (Figure 5.2d), indicating good sludge activity and hence potential to treat high ammonium concentration wastewater (Wentzel et al., 2002).

Figure 5.2: Reactor performance of nitrification MBR under long SRT; a profiles of $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, and $\text{NO}_3^-\text{-N}$ in the effluent as well as NAR; b profiles of ammonium loading rate and ARR; c profiles of DO and pH; d profiles of MLSS, MLVSS and MLVSS/MLSS ratio during the whole experiment; the arrow indicates the *in-situ* generated FNA shock.

5.3.2 DO limitation and *in-situ* generated FNA shock strategies for maintenance of nitrification under long SRT

DO of lower than 0.5 mg/L successfully started up the nitrification process in 65 days (Figure 5.2a). AOB out competed NOB at low DO conditions because of higher oxygen affinity (Ge et al., 2015). However, as NOB acclimated, and NOB population increased (Figure 5.3), DO fluctuation from day 72 to day 117 led to accumulation of nitrate, and this caused failure of nitrification with NAR decreasing to 0.54 on day 116 (Figure 5.2a). *Nitrobacter*, which functioned as *r*-strategists, had higher specific growth rates and lower substrate affinity (Regmi et al., 2014). With the achievement of nitrification from day 65 onwards, *Nitrobacter* cell abundance increased by 242 times from 6.49×10^7 copies/g VSS on day 72 to 1.57×10^{10} copies/g VSS on day 82, and further increased to 9.99×10^{10} copies/g VSS on day 117 (Figure 5.3). Compared to *Nitrobacter*, *Nitrospira* functioned as *K*-strategists with lower specific growth rates and higher substrate affinity and was the dominant NOB in this study. *Nitrospira* adapted to the hypoxic environmental niches (Lücker et al., 2010). This increased the difficulty of suppressing *Nitrospira*. Extremely low DO of 0.1 mg/L successfully restored nitrification. The cell abundance of *Nitrospira* was decreased by 67.6% from day 117 to day 128. However, the low DO concentration also suppressed AOB activity during days 167 to 177, and 143.8 to 429.6 mg $\text{NH}_4^+\text{-N/L}$ ammonium remained in the effluent (Figure 5.2a). Therefore, the DO limitation strategy was useful to provide a competitive advantage for AOB during start-up of nitrification but not sufficient for sustaining long-term process stability.

Another approach commonly used was FNA inhibition (Zhou et al., 2011). The threshold FNA inhibition levels on a 50% reduction in AOB activity were in the range of 0.42–1.72 mg $\text{HNO}_2\text{-N/L}$, while lower concentrations of 0.011–0.07 mg $\text{HNO}_2\text{-N/L}$ would start to inhibit NOB and 0.026–0.22 mg $\text{HNO}_2\text{-N L}^{-1}$ could completely inhibit NOB (Zhou et al., 2011). In essence, the nitrification process would lead to pH drop as it generated H^+ . With the nitrite accumulating and pH decreasing, FNA would accumulate to inhibit NOB activity (Figure 5.1).

Model estimations also revealed utility of *in-situ* generated FNA shock on nitrification maintenance (Table 5.2). Generally, AOB has shorter minimum SRT than

NOB (Blackburne et al., 2008b), thus, de Graaff et al. (2010) adopted SRT that was shorter than the minimum SRT for NOB and successfully maintained nitrification for almost 400 days. In this study, without *in-situ* generated FNA shock, the minimum SRTs for the NOB to have impact in the reactor, varied from 3.16 to 24.7 days. These were all much shorter than the SRT used in this study to accumulate the AOB. To illustrate this, the NOB population had increased from day 75 to day 115 when DO fluctuation occurred (Figure 5.3). With the increase in FNA concentration and decrease of DO concentration, minimum SRTs for the NOB had increased to 24.7 days on day 125-144 and 12.3 days on day 185-204, respectively. NOB populations increased from 4.78×10^{10} copies/g VSS on day 159 to 8.68×10^{10} copies/g VSS on day 191 between the second and third *in-situ* generated FNA shock (Figure 5.3). There was still chance that nitrification could be reduced by NOB accumulation under the long SRT. However, implementing the *in-situ* generated FNA shock, NOB activity and cell abundance had remained suppressed from day 135 onwards (Figs. 5.2a and 5.3).

Table 5.2: Minimum SRT for NOB under the conditions without consideration of *in-situ* generated FNA shock

Operation period	Feed (mgNH ₄ ⁺ -N/L)	Average FNA (mg HNO ₂ -N/L)	Average FA (mg NH ₃ -N/L)	Average DO (mg/L)	SRT _{min} , NOB (day)
Day 73-82	300	0.0063	0.2	0.42	4.18
Day 86-100	400	0.0090	0.2	0.64	3.16
Day 125-144	500	0.0117	1.2	0.10	24.7
Day 185-204	650	0.0164	2	0.72	12.3

It was noted the two main NOB genera in the MBR, *Nitrospira* and *Nitrobacter*, exhibited different responses to the combined strategies of DO limitation and *in-situ* generated FNA shock. Compared to *Nitrobacter*, *Nitrospira* was more competitive under low DO concentration, and remained the dominant NOB genus throughout the study. Under the synergistic effect of DO limitation and FNA inhibition, *Nitrobacter*

was suppressed and cell abundance decreased by 60.6%, 96.4% and 90.6% after 3 *in-situ* generated FNA shocks. *Nitrospira* cell abundance decreased by 68.9%, 50.0% and 62.0%, respectively (Figure 5.3). Despite of proliferation of NOB population under the long SRT in this study, DO limitation and the FNA shock maintained nitrification successfully.

Figure 5.3: Variations of key functional bacteria abundance; the arrow indicates the *in-situ* generated FNA shock.

Nitrosomonas had remained the dominant bacteria in the MBR throughout this study. A recent study had shown that *Nitrosomonas* on exposure to high FNA could up-regulate oxidative stress enzymes, denitrification, and DNA and protein repair mechanisms (Laloo et al., 2018). Thus, AOB activity and cell abundance had been barely affected by the *in-situ* generated FNA shock treatment (Figs. 5.2a and 5.3).

5.3.3 Nitrous oxide emission

In this study, the daily average concentration of N₂O in the off-gas varied from 11.3 to 57.3 ppmV, which corresponded to 0.82 to 3.38% (average $2.1 \pm 0.72\%$) of the total ammonium - nitrogen removed in the MBR (Figure 5.4). Overall, the average

N_2O emission factor of $2.1 \pm 0.72\%$ reached in this study was comparable to the $2.0 \pm 1.0\%$ from a full-scale granular sludge reactor (Castro-Barros et al. 2015).

Figure 5.4: Nitrous oxide emission in the nitrification MBR; air flow rate: 1 L/minute from day 165 to 178, 2 L/minute from day 178 to 204; the insert presented the N_2O emission factors at the corresponding nitrite concentration from day 165 to 178.

In this study, the N_2O emission factor at flow rate of 1 L/minute was found to be negatively correlated with the nitrite concentration (Figure 5.4), which corresponded to previous findings at nitrite concentration of 195.6 to 510.0 mg N/L (Law et al., 2013). Generally, it has been supposed that the nitrifier denitrification pathway is inhibited at high nitrite concentrations (Law et al., 2013; Pocquet et al., 2016). Under the average DO concentration of 0.55 mg/L, ammonium in the feed was partially oxidized to nitrite, with 109.3 to 429.7 mg N/L ammonium remaining in the effluent (Figure 5.2a). To improve the nitrification, the air flow rate was increased to 2 L/minute, however, a N_2O emission peak occurred from day 178 to day 185 (Figure 5.4). Increased N_2O emission was reported to be associated with increased DO concentration (Kampschreur et al., 2009). However, doubling the airflow rate just

increased the DO from 0.55 to 0.72 mg/L. The oxygen was consumed by AOB and as a result, the nitrite concentration increased to 554.5 ± 29.41 mg N/L, while the corresponding FNA concentration increased to 1.47×10^{-2} mg HNO₂-N/L from day 180 on (Figure 5.2). It should be noted that the enriched AOB culture used in this study was cultivated at the high nitrite (up to 590.0 mg N/L) and low DO (less than 0.10 mg/L) conditions. Pathways of nitrifier denitrification and NH₂OH oxidation may still contribute to the N₂O emission. N₂O emission has been reported to be one of the mechanisms for AOB to mitigate FNA toxicity (Wang et al., 2016c). High FNA concentration might trigger AOB to increase nitrite reductase, NirK, activities in order to reduce FNA toxicity, and N₂O would be the product of nitrite reduction by AOB. Besides, Law et al. (2012) found that the specific N₂O emission rate was exponentially correlated to the ammonia oxidation rate, and the chemical decomposition of the unstable nitrosyl radical (NOH) during NH₂OH oxidation would be the main N₂O emission pathway under the high nitrite concentration. In this study, after increasing the air flow rate, the ammonia oxidation rate increased as revealed by the increase of nitrite concentration from day 178 to day 180. As nitrite concentration remained relatively stable from day 185 to day 189, the N₂O emission decreased.

Recently, the importance of abiotic NH₂OH conversion on N₂O emission under high nitrite concentration was re-examined (Soler-Jofra et al., 2016; Zhu-Barker et al., 2015). The abiotic pathway involving the NH₂OH and FNA was found to be: $\text{NH}_2\text{OH} + \text{HNO}_2 \rightarrow \text{N}_2\text{O} + 2 \text{H}_2\text{O}$ (Harper et al., 2015). It should be noted that the average nitrite concentration was 554.5 mg N/L after doubling the air flow rate (Figure 5). Although the NH₂OH concentration was not measured in this study, the residue NH₂OH concentration in similar conditions as this MBR was reported to be 0.03 -0.15 mg N/L (Jiang et al., 2015; Soler-Jofra et al., 2016; Yu and Chandran, 2010). It was postulated that the NH₂OH generated via ammonia oxidation reacted with the increased FNA and led to N₂O production during this period. Soler-Jofra et al. (2016) estimated that the abiotic NH₂OH pathway would contribute to one third of the N₂O emission in a nitrification reactor treating reject water.

N₂O emission factor was found to slightly decrease from $2.2 \pm 0.16\%$ (day 188 to 194) to $1.9 \pm 0.37\%$ (day 195 to 199) after the FNA shock on day 195. However, this inhibition was reversible and the N₂O emission factor increased to $2.1 \pm 0.29\%$ on day 200 to 204. It is possible that the enzymes involved in N₂O production was further inhibited during the FNA shock (Wang et al., 2016c). This suggested that the strategy developed in this study at least did not increase the N₂O emission in the nitrification process but might contribute to less N₂O production.

5.3.4 Practical implications for high ammonium concentration wastewater treatment

Table 5.3 summarizes the strategies for maintaining nitrification to treat high ammonium concentration wastewater. In this study, the novel combined strategy of low DO and *in-situ* generated FNA shock was employed. Compared with other studies, the ALR of 1.81 kg N/(m³ d), ARR of 0.97 and NAR of 0.96 achieved in this study were among the higher values, especially under long SRT (Table 5.3).

The findings obtained in this study could have important implications for high ammonium concentration wastewater treatment. Currently, nitrification based biological nitrogen removal processes are operated in more than 100 full-scale side-stream WWTPs worldwide, more than 50% of which are SBR (Lackner et al., 2014). Compared with other systems, MBR has a smaller foot print as the secondary clarification is replaced with membrane separation, and thus may have lower overall capital expenditure (CAPEX) (Meng et al., 2017). Aeration in bioprocesses is reported to account for 11% to 23% of energy demands of a large-scale hollow fibre MBR (Verrecht et al., 2010). In this study, the maintenance strategy could also decrease the operating expenditure (OPEX) of the MBR. Intermittent aeration was employed to control DO at less than 0.8 mg/L, which can contribute to lower aeration cost.

Table 5.3: Comparison of nitrification maintenance strategies for treatment of high ammonium concentration wastewater.

Reactor system	ALR (kg N /(m ³ day))	Maintenance strategy	ARR	NAR	Reference
Air-lift continuous reactor	1.1	DO>2 mg/L, pH 7.5-7.7, SRT 1-17 days,	0.50*	0.98*	(de Graaff et al., 2010)
Complete stirred tank reactor	3.04	DO>1.5mg/L, pH 8.0, SRT 8-12 days, FA 10-20 mg NH ₃ -N/L	0.73	0.96	(Chen et al., 2010)
Complete stirred tank reactor	0.8	DO>1.0 mg/L, pH 7.0-7.5, SRT 14-18 days, FA 1-3 mg NH ₃ -N/L,	0.86	0.85	(Chen et al., 2010)
MBR	0.98*	DO 0.5-1.0 mg/L, pH 7.5-6.6, prolonged SRT	0.82*	0.90*	(Shen et al., 2014)
SBR	0.75	pH set-point of 6.2 with control of bicarbonate dosing, SRT 11 days	0.93	0.97	(Law et al., 2015)
Air-lift reactor	2.1	DO 0.3-2.2, increasing NH ₄ ⁺ -N concentration, SRT>30 days	0.91	0.80	(Chai et al., 2015)
Continuous aeration tank	2.05	DO 3.75 mg/L, FA 10.61 mg NH ₃ /L, SRT 15-18 days	0.91	0.84	(Sui et al., 2016)
MBR	0.27	DO 0.78-1.38 mg/L, FA 1.13 mg/L	0.88	0.95	(Huang et al., 2016)
MBR	1.81	DO <0.8 mg/L, <i>in-situ</i> generated FNA shock, SRT 43.8 days	0.97	0.96	This study

*The data were estimated based on the figures in the corresponding studies.

Different from the FNA side treatment reported in the literature (Wang et al., 2016b), *in-situ* generated FNA shock in this study was a spontaneous process when base buffer dosage is stopped, and did not require extra CAPEX. Compared to 1.82 mg HNO₂-N/L FNA and 24 hours' FNA side treatment (Wang et al., 2016b), the *in-situ* generated FNA shock with 0.39 - 0.61 mg HNO₂-N/L successfully maintained nitrification performance without additional cost. Moreover, the *in-situ* generated FNA shock did not promote, but on the contrary, reduced N₂O emission. Therefore, the strategy developed in this study could not only effectively maintain stable nitrification, but also save the CAPEX and OPEX when treating high concentration ammonium wastewaters.

5.4 Concluding remarks

This chapter reported the MBR operated with long SRT could maintain stable nitrification via *in-situ* generated FNA shock and DO limitation.

- Nitrification was achieved and maintained for 204 days with ammonium concentration from 200 mg NH₄⁺-N/L to 650 mg NH₄⁺-N/L, and with ARR and NAR over 0.97 and 0.96, respectively.
- Activities of *Nitrospira* and *Nitrobacter* were suppressed and populations were reduced by the combined strategy of DO limitation and *in-situ* generated FNA shock.
- N₂O emission was $2.1 \pm 0.72\%$ of the ammonium removed and *in-situ* generated FNA shock did not increase the N₂O emission.
- Maintenance of nitrification in MBRs with the combined strategy investigated in this study would reduce both of CAPEX and OPEX for the treatment of high concentration ammonium wastewater.

Chapter 6 Effect of Free Nitrous Acid on Microbial Extracellular Polymeric Substances Production and Membrane Fouling in a Nitritation Membrane Bioreactor

6.1 Introduction

Stable nitritation needs not only the growth of AOB to be favoured, but also to inhibit that of NOB's in long-term operations. As both AOB and NOB are retained in the MBR due to the membrane separation, NOB could proliferate and disrupt the nitritation process under fluctuating operating conditions (Jiang et al., 2019). Chapter 5 discussed successful maintenance of stable nitritation in the long SRT MBR, via *in-situ* generated FNA shock to suppress NOB activity and population in long-term operation (Jiang et al., 2019).

Membrane fouling is inevitable as a result of the interaction between membrane module and the suspended sludge comprising solutes, colloids and bacteria flocs (Lin et al., 2012; Meng et al., 2009). It is reported biofouling contributes around 45% of the overall fouling (Komlenic, 2010). Therefore, it is crucial to understand the biofouling mechanism so as to develop biological fouling mitigation strategies. EPS has been regarded as the primary foulant in biofouling and due to their complex composition, structure and adhesive properties, EPS could be involved in a number of the fouling mechanisms (Lin et al., 2014).

The membrane biofouling in the nitritation MBR has not yet been extensively reported in the literature. A unique characteristic of the nitritation MBR is the presence of nitrite and hence increases in the FNA concentration. The latter would impact EPS production and the microbial community. Shen et al. (2014) had reported lower fouling rates under lower ammonium loads in a nitritation MBR, but high fouling rates occurred when ammonium loads increased to over 0.6 kg N/m³ d. Although that study did not fully address the reason for this, there was association between fouling rate increase and the increase in FNA concentration.

Previous chapters have discussed the biocidal effect of FNA. More importantly, FNA at 2.0 mg HNO₂-N/L could breakdown EPS in WAS and so disintegrated

activated sludge flocs (Zhang et al., 2015), which had raised concern over broken-down EPS fouling membrane. Although *Nitrosomonas* could tolerate relatively high FNA concentration (Laloo et al., 2018), it is unclear how a *Nitrosomonas* enriched culture would react under high stress of FNA in terms of EPS production and microbial community change, and consequent impact on membrane fouling.

Therefore, this chapter's objective was to investigate the impact of FNA exposure on membrane fouling in a nitrification MBR. The suspended sludge EPS production and microbial community dynamics were investigated in tandem with the increase in FNA concentrations. Batch tests were conducted to illustrate the effect of FNA on the bound EPS composition. The results of the current study may provide insight into membrane fouling and fouling control strategies in the nitrification MBR for practical implementations.

6.2 Experimental

6.2.1 Nitrification MBR

The laboratory scale MBR and the submerged membrane module have been described in Chapter 5. One end of the hollow fibres was sealed with epoxy glue while the other end was fixed to a suction chamber connected to a suction pump. The feed medium contained 200 to 650 mg N/L ammonium and was devoid of organic carbon as described in Chapter 5 (Jiang et al., 2019). The MBR was operated at $34 \pm 1^\circ\text{C}$ with HRT of 9 ± 1 h and SRT of 43.8 days throughout the study. The discharge pump was operated at 7-minute-on-1-minute-off mode, while feed was introduced continuously. Sludge samples were collected twice a week for MLSS and particle size measurement and for EPS and DNA extraction. TMP was measured with a pressure gauge and recorded online. During this study, permeate flux was maintained at 5.76 ± 0.49 L/m²h to maintain constant HRT. Intermittent aeration was supplied with aeration flow rate of 2 L/minute to maintain DO in the range of 0.3 to 0.8 mg/L. The membrane module was taken out for physical washes when TMP exceeded 30 kPa as described in Chapter 3. The washed sludge and biofilm were returned to the reactor to avoid excessive sludge loss.

6.2.2 Batch experiments

In the batch tests, the bound EPS were classified into LB-EPS and TB-EPS. Six sets of batch tests were conducted with the *Nitrosomonas* enriched culture freshly taken from the nitrification MBR. In each test, 100 mL of sludge was washed twice with the feed solution (but without NH_4Cl), to remove the nitrite in the bulk liquid, and then re-suspended with nitrite stock solution to the targeted FNA value at pH 7.8 in an Erlenmeyer flask. Before and after each batch test, 10 mL of sludge was taken to measure MLSS and MLVSS. pH was adjusted with 1 M HCl. Then the flask was placed in the water bath shaker at 70 rpm and $34\pm 0.1^\circ\text{C}$ for 4 hours. To maintain the AOB activity, ammonium chloride stock solution was added at hourly intervals to maintain the bulk ammonium concentration at about 20 mg N/L. Prior to each NH_4Cl addition, 2 mL of bulk liquid was filtered through $0.45\mu\text{m}$ membranes for the measurements of ammonium, nitrite and nitrate. At $t = 1$ h and the end of the batch test, 20 mL of sludge sample was collected from the flask to extract SMP, LB-EPS and TB-EPS.

6.3 Results and Discussion

6.3.1 Membrane fouling development

Partial nitrification initiated in 38 days and nitrification from Day 65. Thereafter, stable nitrification was maintained with ARR and NAR of 0.97 and 0.96, respectively. The maximum ammonium loading rate achieved was $1.81 \text{ kg N}/(\text{m}^3 \text{ day})$ with feed ammonium concentration increasing from 200 to 650 mg N/L. MLVSS increased from 573 mg/L to 2383 mg/L. Table 6.1 shows the effluent concentration of different nitrogen compounds and average FNA concentration at steady-state when NAR was stably maintained at over 0.90 for at least 10 days. Detailed results have been described elsewhere (Jiang et al., 2019).

Figure 6.1 depicts the TMP development. Increase in TMP indicated the typical three-stage fouling development (Zhang et al., 2006). An example may be seen in the TMP evolution profile from day 95 to day 135 in Figure 6.2. In stage 1, TMP increased rapidly due to pore blockage and initial deposition of biomass (approximately 5 days). In stage 2, a weakly exponential rise of TMP indicated slow

fouling of the membrane (approximately 30 days). This was not withstanding the operation flux in this study was below the critical flux. EPS had facilitated biomass adhesion to the membrane surface, and biofilm was observed as shown in Figure 6.3a. The accumulated bacteria on the hollow fibre surface formed a cake layer. Further increasing magnification to 20,000X (Figure 6.3b), it could be seen the biofilm's bacteria in the biofilm were covered with EPS. A sudden rise in TMP was then noted in stage 3 (approximately 5 days) (Figure 6.2).

Table 6.1: Reactor performance and average FNA concentration at steady state in each operation period.

Operation Period	Feed	Average	Effluent concentration at steady state ^a		
	NH ₄ ⁺ -N (mg N/L)	FNA (mg HNO ₂ - N/L)	(mg N/L)		
			NH ₄ ⁺ -N	NO ₂ ⁻ -N	NO ₃ ⁻ -N
Day 1-38	200	1.47×10 ⁻³	80.82±4.03	72.13±4.55	8.45 ±0.65
Day 39-73	240	4.93×10 ⁻³	1.038±0.83	185.3±9.09	12.09±0.71
Day 74-83	300	6.82×10 ⁻³	2.074±0.67	253.8±6.70	17.91±3.61
Day 84-101	400	9.10×10 ⁻³	2.934±1.00	324.1±17.2	35.18±3.41
Day 102-156	500	1.34×10 ⁻²	24.42±17.0	460.0±31.4	28.58±4.29
Day 157-204	650	1.47×10 ⁻²	27.37±15.9	538.0±29.9	25.66±4.97

^a Steady state at each operation period was considered when NAR was stably maintained over 0.90 for at least 10 days.

After removal of the biofilm from the hollow fibre surface by physical washing, TMP decreased from 30 to 5.4 - 8.6 kPa, suggesting membrane fouling was largely contributed by the cake layer. Physical washes successfully restored performance of the MF membrane. Nevertheless, there was residual clogging as the TMP upon restarting was higher than new membranes. This indicated development of irreversible fouling and over the 204 days of operation, this effect accumulated. As shown in Figure 6.3c, small particles remained on the surface of the hollow fibre after

wash. This also led to the faster stage 1 fouling in the later phase of this study (Figure 6.1).

Figure 6.1: Profile of TMP development throughout the experiment; the arrow indicates application of *in situ* FNA shock.

Figure 6.2: Typical three-stage TMP increase from day 95 to day 135

Figure 6.3: FESEM of biofilm on the hollow fibre surface collected on day 198 with different magnifications; a 100X magnification; b 20,000X magnification; c membrane surface after physical wash on day 198; d membrane surface of new hollow fibre.

6.3.2 Effect of biomass EPS on membrane fouling

EPS production and particle size of suspended flocs were examined regularly to investigate their roles in membrane fouling (Figure 6.4). Stable nitrification and the increase of ammonium loading led to the increase of FNA in this study. TMP had also increased more sharply from day 165 (Figure 6.1). With FNA increasing from 5.10×10^{-3} mg $\text{HNO}_2\text{-N/L}$ on day 65 to 1.47×10^{-2} mg $\text{HNO}_2\text{-N/L}$ on day 180, both particle size and EPS production increased. This was probably due to the biomass' resistance to the biocidal effects of FNA. The EPS matrix functioned as a protective layer against the adverse influences of FNA on the biomass. EPS PN and PS increased from 8.20 to 60.28 mg/g VSS and 4.74 to 30.46 mg/g VSS, respectively over the period. The increase in EPS facilitated cell-to-cell interaction, strengthened microbial

structure by forming a polymeric matrix, and thus led to the increased particle size in this study. Particle size increased from $19.0 \pm 0.04 \mu\text{m}$ on day 54 to $65.3 \pm 2.23 \mu\text{m}$ on Day 200.

Figure 6.4 Profile of evolution of PS and PN in the EPS, particle size of sludge flocs and FNA concentration in the MBR; the arrow indicates the *in situ* FNA shock. The error bar represents the standard deviations.

SMP have been reported to be the predominate foulants that determine fouling extent in MBRs (Drews et al., 2007; Zhang et al., 2016). SMP are defined as soluble organic compounds that are released into solution from substrate metabolism with biomass growth and biomass decay (Barker and Stuckey, 1999; Teng et al., 2019). In this study, there were no significant differences in SMP PS and PN concentrations between effluent and the suspended sludge. This meant rejection of SMP by the membrane could be neglected. The SMP PS and PN concentrations were 1.08 to 1.79 mg PS/ g VSS and 3.61 to 5.18 mg PN/ g VSS, respectively. These were low compared to values in the bound EPS, which are in accordance with previous study in a nitrifier enriched activated sludge MBR (Sepehri and Sarrafzadeh, 2018). Given that no organic compounds were added in the feed solution, EPS were the main

membrane foulant in the nitrification MBR with EPS PS and PN corresponding to the fouling rate as shown in Figure 6.5.

Figure 6.5 Relationships between EPS with membrane fouling rate. a Protein; b Polysaccharide.

PS and PN would have been the main foulants in the EPS, contributing to membrane fouling by pore blocking, adhesion of biomass to membrane surface and cake layer formation (Meng et al., 2017). There was more PN in the bound EPS than PS (Figure 6.4). It has been reported *Nitrosomonas* preferentially secreted more PN (Lu et al., 2016). Besides, suspended flocs were complexes of different heterotrophic and autotrophic microorganisms, where hydrolysis and biodegradation of EPS

occurred (Merkey et al., 2009). Compared to PN, PS was utilized faster (Zhang and Bishop, 2003), thus leading to low PS in the EPS matrix.

Of interest to note is that, after FNA concentration reached 1.47×10^{-2} mg HNO₂-N/L from day 180, EPS PN and PS concentration decreased by 23.4% and 19.0%, respectively (Figure 6.4). This was consistent with a previous study showing PN reduction under high FNA concentrations (Shen et al., 2014). This phenomenon would have been enhanced by the *in-situ* FNA shocks generated to maintain stable nitrification on day 134, day 149 and day 195. FNA concentrations had then progressively increased to 0.39, 0.51 and 0.61 mg HNO₂-N/L in 12 hours, respectively (Jiang et al., 2019). EPS concentrations had also decreased after the FNA shocks (Figure 6.4). Although EPS played a role in protecting the microbes against adverse FNA impact, sufficiently high FNA concentrations could inhibit protein synthesis and uncouple energy generation and hence reduce energy for maintenance and growth (Laloo et al., 2018; Zhou et al., 2011).

Table 6.2 summarizes the average concentrations of FNA, PS, PN and fouling rates in term of dTMP/dt for each physical washing cycle.

Table 6.2 Average concentrations of FNA, EPS PS and PN, and fouling rate in different operation periods.

Operation period	Average FNA (mg HNO ₂ -N/L)	Average PS (mg PS/g VSS)	Average PN (mg PN/g VSS)	dTMP/dt (kPa/day)
Day 1-60	0.13×10^{-2}	9.18	13.34	0.36
Day 63-83	0.57×10^{-2}	8.17	14.00	0.41
Day 101-130	0.95×10^{-2}	16.32	31.30	0.55
Day 135-166	1.27×10^{-2}	25.92	54.83	0.76
Day 170-186	1.45×10^{-2}	27.16	49.70	0.98
Day 187-204	1.48×10^{-2}	24.20	43.85	0.73

As shown in Table 6.2, the fouling rate had increased as EPS PS and PN concentrations increase. The increase in bound EPS would have promoted the attachment of microbial cells to the membrane surface. Although the membrane module was exposed to shear stresses induced by aeration and the filtration relaxation, suspended flocs had nonetheless deposited on the membrane surface and formed

biofilm during operation, which is in accordance with previous study (Lin et al., 2014). The increase in bound EPS led to higher fouling rate and shorter development duration of stage 2 fouling (Table 6.2 and Figure 6.1). Most importantly, the reduction in EPS by FNA from Day 187 on contributed to the decrease of the fouling rate. This implies that FNA could be manipulated in situ to mitigate membrane fouling in the MBR.

6.3.3 Effect of FNA on the production of SMP, LB-EPS and TB-EPS

Batch tests were conducted to investigate the effect of FNA on the production of SMP, LB-EPS and TB-EPS of the *Nitrosomonas* enriched culture. Table 6.3 summarizes the ammonium, nitrite and nitrate concentrations measured during the tests. Ammonium was not detected in all the tests, which implied the good ammonium oxidation activity by *Nitrosomonas*. Since the DO was not controlled in the tests, accumulation of nitrate was observed. These were comparable to the nitrate level in the nitrification MBR as shown in Table 6.1.

Table 6.3 Ammonium, nitrite and nitrate concentrations in the batch tests

Batch test		Average FNA concentration (mg HNO ₂ -N/L)					
		control	7.25×10 ⁻³	1.03×10 ⁻²	1.51×10 ⁻²	2.01×10 ⁻²	3.23×10 ⁻²
NH ₄ ⁺ -N (mg N/L)	t=0 - 4 h	N.D. ^a	N.D. ^a	N.D. ^a	N.D. ^a	N.D. ^a	N.D. ^a
NO ₂ ⁻ -N (mg N/L)	t=0	0.6	241.1	357.0	548.6	725.1	1170.0
	t=1h	20.1	253.5	366.2	548.8	724.1	1162.8
	t=2h	39.4	261.9	368.9	541.1	716.9	1148.4
	t=3h	54.6	262.4	362.4	523.0	701.1	1119.6
	t=4h	66.8	257.4	350.3	497.8	670.6	1084.6
NO ₃ ⁻ -N (mg N/L)	t=0	N.D. ^b	N.D. ^b	N.D. ^b	N.D. ^b	N.D. ^b	N.D. ^b
	t=1h	1.60	2.75	2.62	8.84	6.49	4.56
	t=2h	2.71	6.52	9.93	16.79	12.17	10.47
	t=3h	7.80	12.90	17.35	24.49	20.47	19.76
	t=4h	11.91	18.47	23.06	32.18	28.93	27.17

^a not detected, which is under the detection range (< 2 mg N/L).

^b not detected, which is under the detection range (<0.2 mg N/L).

Figure 6.6 shows the SMP, LB-EPS and TB-EPS concentrations with respect with various FNA concentrations.

Figure 6.6 Effect of FNA concentration on the production of SMP, LB-EPS and TP-EPS; a protein concentration; b polysaccharide concentration.

TB-EPS accounted for 84.3% of the bound EPS. PN and PS concentrations in the SMP were low, compared to these in the bound EPS (Figure 6.6). This was because the feed solution was devoid of organic matters. Similar to the bound EPS in the nitrification MBR (Figure 6.4), there was more PN than PS in both LB-EPS and TB-EPS (Figure 6.6).

PN and PS concentrations in both LB-EPS and TB-EPS decreased, upon exposure to FNA concentration up from 1.51×10^{-2} mg $\text{HNO}_2\text{-N/L}$ to 3.23×10^{-2} mg $\text{HNO}_2\text{-N/L}$. FNA at 2.0 mg $\text{HNO}_2\text{-N/L}$ has been verified to break down EPS in activated sludge flocs, leading to the partial release of EPS into soluble phase and cell lysis (Zhang et al., 2015). Zhang et al. (2016) also reported increase in SMP upon exposure to pharmaceutical compounds due to the toxic effect to the biomass.

In this study, however, despite of PN and PS reduction in the LB-EPS and TB-EPS, there was only slight increase in the SMP PN and PS concentrations. This was likely because the applied FNA concentrations in this study's tests were two orders of magnitude lower than the FNA concentration used in sludge digestion pretreatment. *Nitrosomonas* could up-regulate defensive mechanisms on exposure to high FNA, such as oxidative stress enzymes, denitrification, and DNA and protein repair (Arp and Stein, 2003; Laloo et al., 2018). The relatively low SMP and the good ammonium oxidation activity (Table 6.3) implied that the microorganisms in the *Nitrosomonas* enriched culture remained cytoactive under the FNA stress. The batch tests verified that FNA concentrations of 1.51×10^{-2} to 3.23×10^{-2} mg $\text{HNO}_2\text{-N/L}$ reduced production of LB-EPS and TB-EPS but did not induced cell lysis.

6.3.4 Microbial community analysis

High-throughput sequencing of bacterial 16S rRNA gene was performed to better understand changes in the microbial community as the membrane fouling progressed. Table 6.4 summarizes the microbial community richness and diversity data. Species richness estimated by chao1 and ACE and diversity evaluated by Shannon and Simpson indices indicated decreasing trend (Table 6.4). This could be due to the accumulation of bacteria acclimated to the increased ammonium loading and FNA concentration.

Figure 6.7a shows the weighted unifracs distance matrix as well as the relative abundance of top 10 phyla. The clustering similarity tree showed changes in the microbial community. After successful start-up of nitrification from Day 65, the microbial community had remained relatively stable. At genus level, *Nitrosomonas* was the dominant genus (Figure 6.7b), which was expected since only ammonium was fed and *Nitrosomonas* is the most common AOB in the WWTP (Reino et al., 2016). The relative abundance of *Nitrosomonas* varied from 19.9% to 52.3% of the total population, indicating AOB enriched in the sludge with increase in ammonium loading.

Table 6.4 Evolution of microbial community richness and diversity throughout the experiment.

Sample	OTU ²	Observed	Richness		Diversity index		phylogenetic diversity
		species	chao1	ACE	Shannon	Simpson	
Day 2	621	584	612.1	614.7	5.076	0.916	51.27
Day 65	556	513	552.6	554.9	4.335	0.880	50.37
Day 93	524	473	531.5	532.8	3.231	0.772	40.47
Day 106	513	464	504.3	505.9	3.447	0.822	43.36
Day 128	553	498	552.6	554.4	3.494	0.810	51.91
Day 138	524	524	746.1	671.3	3.096	0.714	61.28
Day 198	504	455	491.1	499.9	3.552	0.844	47.40
Biofilm ¹	514	479	501.0	513.8	3.608	0.819	41.45

¹ biofilm sample was collected from membrane surface on day 198.

² OTUs were defined by the 97% identity threshold.

There was no obvious difference in the microbial community in suspended sludge and in the biofilm on Day 198 (Figure 6.7). This was contrary to an earlier report with a nitrification MBR, where microbial succession in the biofilm on the membrane surface was found from autotrophic colonization to heterotrophic domination but the microbial community in suspended sludge remained autotrophic domination (Lu et al., 2016). In this study, similar to AOB enrichment in suspended sludge, exposure to FNA had led to similarity and that almost half of the relative abundance was contributed by *Nitrosomonas* in the biofilm (Figure 6.7b).

A considerable portion of the microbial community in both suspended sludge and biofilm was heterotrophic bacteria (Figure 6.7b). For instance, unidentified *Saprospiraceae* and *Anaerolineaceae* accounted for 15.8% and 8.76% of the relative abundance in the suspended sludge on day 198 and, 18.9% and 8.81% in the biofilm, respectively.

Figure 6.7: Cluster tree based on weighted unifrac distance and micribial community of; a. relative abundace of top 10 phyla in sludge samples collected on day 2, 65, 93, 106, 128, 138, 198 and biofilm on the hollow fibre surface

collected on day 198; b. relative abundance of top 10 genera of the microbial community.

The coexistence of autotrophic AOB and heterotrophic bacteria in the sludge and biofilm in the absence of an applied organic carbon source has been reported to be a synergic system (Chu et al., 2015; Lu et al., 2016). It is believed that the nitrifiers produce organic matters by way of biomass decay or substrate metabolism and released metabolic products, such as SMP and EPS, which then supported the heterotrophs (Ni et al., 2011). *Saprospiraceae* is widely distributed in freshwater lakes, hypersaline mats and activated sludge (Ginige et al., 2005; Ley et al., 2006; McIlroy and Nielsen, 2014), and has been shown to hydrolyse and utilize complex organic compounds in activated sludge wastewater treatment systems (McIlroy and Nielsen, 2014). It has also been associated with protein degradation (Xia et al., 2008). *Anaerolineaceae* is usually involved in anaerobic digestion systems and plays a key role in the initial activation of long chain n-alkane biodegradation and primary fermenters of WAS (Liang et al., 2016; McIlroy et al., 2017).

The low DO in this study and the biofilm structure on the hollow fibre surface also created suitable environment for those heterotrophic bacteria to survive. In mutualistic symbiosis, to resist FNA's biocidal stress, the heterotroph would reside in the inner parts of flocs or deeper layers of the biofilm, and in so doing expanded floc and biofilm size. However, the abundance of unidentified *Saprospiraceae* and *Anaerolineaceae* presented a declining trend, negatively corresponded to the increase of FNA concentrations from day 106 to day 198 (Figure 6.7b). The biocidal effect of FNA decreased the relative abundance of these heterotrophic bacteria. On the other hand, the tolerance of *Nitrosomonas* to FNA led to its enrichment in the nitrification MBR. This suggested that FNA had an impact on the dynamics of the microbial community in this nitrification MBR.

6.3.5 Implications for membrane fouling mitigation in nitrification MBR

EPSs are the primary membrane foulants in the nitrification MBR, while floc deposition, adhesion and subsequent cake layer formation contributed to the second stage of membrane fouling. However, EPS production could be reduced by FNA. It could be therefore be possible to mitigate membrane fouling by adjusting FNA

concentration. In the literature, FNA had been reported as a chemical washing agent for reverse osmosis (RO) membranes (Filloux et al., 2015). Filloux et al. (2015) had shown it feasible to use FNA to clean biofouling and scale at a nitrite concentration of 50 mg NO_2^- -N/L and pH of 3.0. However, FNA cleaning at low pH could deteriorate the membrane (Meng et al., 2017). In this study, FNA was regulated via step-increasing nitrite concentration which was converted by AOB through ammonium in the feed. EPS played an important role in membrane fouling in this study, especially in cake layer formation. Interestingly, increasing FNA to 1.47×10^{-2} mg HNO_2 -N/L (average nitrite concentration of 551.8 mg NO_2^- -N/L at average pH 7.79 and $34 \pm 1^\circ\text{C}$) led to 23.4% in PN and 19.0% in PS reduction, and subsequently led to the decrease in fouling rate. Inhibition on EPS excretion and biofilm disruption have been proposed as emerging biofouling control strategies (Xiong and Liu, 2010). The results obtained in this study suggested that, instead of serving as a post-chemical washing agent, FNA manipulated via conversion of ammonium to nitrite by AOB could be used to *in situ* mitigate membrane fouling in the nitrification MBR.

6.4 Concluding remarks

In this chapter, the membrane biofouling process in a nitrification MBR was characterized in terms of TMP development, EPS composition and microbial community analysis. A three-stage fouling development was described based on the patterns of TMP changes. EPS played a critical role in the membrane fouling development process in the nitrification MBR. TB-EPS accounted for 84.3% of the bound EPS and more PN was found in the bound EPS. EPS PN and PS concentration were found to decrease by 23.4% and 19.0%, respectively when FNA concentration reached 1.47×10^{-2} mg HNO_2 -N/L. Batch tests also verified the FNA stress reduced the production of LB and TB EPS but did not induce cell lysis which would have released PS and PN into SMP. Microbial community analysis showed that FNA also played a role in the dynamics of microbial community composition.

Chapter 7 Mitigation of Membrane Fouling in a Nitritation Membrane Bioreactor by *in-situ* Generated Free Nitrous Acid

7.1 Introduction

The membrane fouling mechanism in the nitritation MBR discussed in Chapter 6 argued cake layer formation was the main cause of biofouling. Biofilm formation is a natural phenomenon of microbial growth. However, due to the permeate drag and back transport generated by membrane filtration, biofilm formation can be accelerated in MBRs, compared to medium-based biofilm reactors (Lu et al., 2016). EPS plays a significant role in the adhesion of microorganisms initially deposited on the membrane surface and the cohesion between subsequent foulants (Li and Elimelech, 2004). In chapter 6, FNA was shown to reduce the PS and PN concentration in the LB-EPS and TB-EPS of sludge. However, there is still limited information on components reduced in the bound EPS by FNA and their corresponding roles in the membrane fouling.

This chapter discusses the feasibility of applying *in-situ* generated FNA to mitigate membrane fouling in a nitritation MBR. The effectiveness of FNA as regulated by pH and nitrite concentration was investigated compared to a control nitritation MBR. Extracted EPS from the suspended sludge and biofilm on the membrane surface was characterized using three-dimensional excitation-emission matrix spectra with the fluorescence regional integration technique (EEM-FRI), size-exclusion chromatography coupled with LC-OCD-OND, and XPS to determine the change in composition in these EPS samples caused by FNA.

7.2 Experimental

7.2.1 Nitritation MBRs for studying the effect of pH regulated FNA on membrane fouling mitigation

Two identical laboratory-scale nitritation MBRs, i.e. MBR 1 and MBR 2, were operated in parallel with working volume of 2.5 L each. A microfiltration membrane module (Memstar, Singapore), consisting 80 pieces of PVDF hollow fibres (pore size less than 0.1 μm), was submerged in each nitritation MBR, and its effective surface

area was 588 cm². The sludge biomass was inoculated from an enriched *Nitrosomonas* culture which was operated to treat 650 mg NH₄⁺-N/L synthetic wastewater for over one year. To investigate the effect of FNA on membrane fouling mitigation, MBR 1 was operated at pH 7.10 while MBR 2 at pH 7.70, maintained with 0.5 M NaHCO₃ solution. Both MBRs were operated at HRT of 8.7 h, SRT of 44 days, and temperature of 34 °C. TMP was measured with a pressure gauge and recorded online.

The operation details are described on Table 7.1. In phase 1, feed medium contained only 200 mg NH₄⁺-N/L with no organic carbon as described previously (Jiang et al., 2019). When the TMP in any one reactor exceeded 30 kPa, both membrane modules were taken out for chemical washing and the feed ammonium concentration increased. Then feed ammonium concentration was increased to 300 mg NH₄⁺-N/L and identified as phase 2. To minimise experimental errors caused by any difference in the membrane modules, the modules were interchanged after phase 1.

Table 7.1 Operation conditions of the nitrification MBRs.

	MBR 1		MBR 2		MBR 2a	MBR 2b
	Phase 1	Phase 2	Phase 1	Phase 2		
pH	7.10±0.03		7.71±0.02		7.71±0.05	7.72±0.02
Initial MLVSS (g/L)	1.860		1.865		1.295	1.365
Feed (mg N/L)	NH ₄ ⁺ 200	NH ₄ ⁺ 300	NH ₄ ⁺ 200	NH ₄ ⁺ 300	NH ₄ ⁺ 300 + NO ₂ ⁻ 650	NH ₄ ⁺ 300

Sludge samples were collected twice a week for MLSS and MLVSS measurement and for SMP, LB-EPS and TB-EPS extraction. Early biofilm on the membrane surface was taken a week after start of the experiment. Late biofilm was taken when the TMP exceeded 30 kPa. The biofilm samples were concentrated to 200 mL, 50 mL of which was used for the VSS measurement and for LB-EPS and TB-EPS extraction, while 10 ml was used for FESEM analysis. The remaining biofilm sample was returned to the corresponding reactor to reduce biomass loss.

Membrane modules were then chemically washed. The chemical cleaning protocol is described in Section 3.1.2.

7.2.2 Nitritation MBRs for studying the effect of nitrite regulated FNA on membrane fouling mitigation

Two identical nitritation MBRs, MBR 2a and MBR 2b, were operated in parallel. The reactors were operated as for MBR 2 with exception of the feed composition for MBR 2a. This included sodium nitrite and the initial nitrite concentration was then 650 mg NO_2^- -N/L, to regulate FNA concentration in the same range as that in MBR 1. Operation conditions are as shown in Table 7.1 and the sampling protocol was as described in Section 7.2.1.

7.3 Results and discussion

7.3.1 Effect of FNA on mitigation of membrane fouling

7.3.1.1 FNA regulated by pH

Table 7.2 summarizes the average FNA concentrations and effluent nitrogen compounds of the nitritation MBRs. There was no significant difference between the performance of MBR 1 and MBR 2. NAR of the two nitritation MBRs in phase 1 and phase 2 were 0.95 and 0.94, and 0.92 and 0.90, respectively. Overall, the FNA concentration in MBR 1 was one order of magnitude larger than that in MBR 2 (Table 7.2). The TMP profiles are as described in Figure 7.1. The TMP increase patterns of the two MBRs were similar at the early biofilm formation stage. However, differences in the TMP increase patterns became significant around one week after filtration re-initiated: TMP in MBR 2 exceeded 30 kPa over 19 days' filtration while TMP in MBR 1 was only 9.5 kPa (Figure 7.1). The differences became even more obvious when FNA in MBR 1 was increased to 4.6×10^{-2} mg HNO_2 -N/L in phase 2.

The biofilm samples collected from the two nitritation MBRs showed considerable difference. The biofilm mass in MBR 2 was 3.13 times that in MBR 1 (Table 7.2). To further analyse the differences in the biofilm, FESEM was conducted as shown in Figure 7.2. It was observed MBR 1 biofilm (Figure 7.2a) was not only

thinner than MBR 2 biofilm (Figure 7.2c), but also contained less EPS (Figure 7.2b) compared to MBR 2 biofilm (Figure 7.2d).

Figure 7.3 depicts the PN and PS concentrations in the SMP and effluent of MBR1 and 2. Results showed that the effluent PN and PS concentrations were comparable to those in the SMPs, but much less than those in LB and TB EPSs. Considering the similarities in concentrations, the membrane rejection of PN and PS in the SMPs in both reactors could be neglected (Zhang et al., 2016).

Table 7.2 Performances and average FNA concentrations in the nitrification MBRs

	MBR 1		MBR 2		MBR	MBR
	Phase 1	Phase 2	Phase 1	Phase 2	2a	2b
FNA ($\times 10^{-2}$ mg HNO ₂ -N/L)	2.7	4.6	0.63	0.85	3.2	0.78
Effluent NH ₄ ⁺ -N	0.25	32.64	5.76	23.75	58.42	46.68
(mg NO ₂ ⁻ -N	189.54	257.26	176.25	243.76	901.87	216.92
N/L) NO ₃ ⁻ -N	9.15	16.5	15.3	26.7	16.1	29.6
Biofilm thickness (μ m)	n.m.	6.64	n.m.	30.5	14.8	23.9
Biofilm mass (g VSS/m ²)	n.m.	3.64	n.m.	11.4	1.62	5.67

n.m. stands for not measured.

PS and PN concentrations were monitored to understand the effect of FNA on the bound EPS production in the suspended sludge and biofilm in both reactors (Figure 7.4). The MBR 1 sludge produced 3.4 mg PS/g VSS and 8.5 mg PN/g VSS in LB-EPS and 10.7 mg PS/g VSS and 33.3 mg PN/g VSS in TB-EPS. This was much less compared to that in MBR 2, which contained 5.7 mg PS/g VSS and 14.4 mg PN/g VSS in LB-EPS and 11.5 mg PS/g VSS and 45.1 mg PN/g VSS in TB-EPS. This was in accordance with the results in Chapter 6 which indicated high FNA results in reduction of bound EPS in the suspended sludge. The results in this chapter have added to the observation TB-EPS in the biofilm was also reduced by FNA stress (Figure 7.4). It was noted there was no difference between the bound EPS of the early biofilm in MBR 1 and 2. This explained the similar TMP development pattern in the early biofilm formation stage (Figure 7.1).

Figure 7.1 TMP profiles of MBR 1 and MBR 2; dash dot line represents the date when early biofilm was harvested while the full line represents the date when late biofilm was harvested.

Figure 7.2 FESEM pictures of late biofilm on the hollow fibre surface; a. cross-section of biofilm in MBR 1; b. biofilm surface in MBR 1; c. cross-section of biofilm in MBR 2; d. biofilm surface in MBR 2.

Figure 7.3 Concentrations of polysaccharide and protein in the effluent and SMP of MBR 1 and MBR 2.

Figure 7.4 Concentrations of polysaccharide and protein in the bound EPS of sludge and biofilm in MBR 1 and MBR 2.

7.3.1.2 FNA regulated by nitrite concentration

FNA is a function associated with nitrite concentration, pH, and temperature (Anthonisen et al., 1976). To exclude the possible effect of pH on microbial activity

and biofilm production, investigation on the effect of nitrite regulated FNA on the membrane fouling mitigation was conducted in MBR 2a and MBR 2b at pH 7.7. With the addition of nitrite in the feed, average FNA concentration in MBR 2a was 3.2×10^{-2} mg $\text{HNO}_2\text{-N/L}$ (Table 7.2). The ammonium removal rates in MBR 2a and 2b were comparable (Table 7.2), which suggested that AOB activities in MBR 2a were not inhibited by the higher FNA. Figure 7.5 shows the TMP development of MBR 2a and 2b. As expected, the TMP development patterns of MBR 2a and 2b in the first seven days were very similar. From day 8, the TMP in MBR 2b had increased faster and exceeded 30 kPa on day 21, while TMP in MBR 2a had remained at around 6.8 to 7.1. It was noted the hollow fibres in MBR 2b were covered with compact biofilm while only a few parts of the hollow fibres in MBR 2a had biofilm in them and that this tended to detach. FESEM pictures revealed the thinner biofilm and cleaner membrane surface in MBR 2a compared to that in MBR 2b (Figure 7.6). The biofilm mass of MBR 2a was 1.62 g VSS/m² while that of MBR 2b was 5.67 g VSS/m² (Table 7.2).

Figure 7.5 TMP profiles of MBR 2a and MBR 2b.

Figure 7.7 depicts the bound EPS PN and PS concentrations in the sludge and biofilm were lower, under the nitrite-regulated FNA stress in MBR 2a Compared to MBR 2b. This was in accordance with EPS reduction by pH-regulated FNA stress as discussed in previous part.

Overall, results of the FESEM and EPS contents showed that FNA up to 2.7×10^{-2} mg $\text{HNO}_2\text{-N/L}$ arising from either nitrite addition or pH manipulation led to considerable biofilm reduction on the hollow fibre surface. Chapter 6 had shown that cake layer formation was the main cause of membrane fouling. And in this chapter, it was shown FNA could mitigate such membrane fouling in the nitrification MBR.

Figure 7.6 FESEM pictures of late biofilm on the hollow fibre surface; a. cross-section of biofilm in MBR 2a; b. biofilm surface in MBR 2a; c. cross-section of biofilm in MBR 2b; d. biofilm surface in MBR 2b.

Figure 7.7 Concentrations of polysaccharide and protein in the bound EPS of sludge and biofilm in MBR 2a and MBR 2b

7.3.2 Mechanism of FNA on the reduction of EPS in the sludge and biofilm

7.3.2.1 EPS characterization by LC-OCD-OND

Although colometric PS and PN determinations have been widely used to characterize the EPS contents in the membrane fouling analysis, LC-OCD-OND was employed to further quantify the sub-fractions of the bound EPS based on their molecular weights. Based on their respective properties and molecular weights, the organic matters in the bound EPS were divided into hydrophobic DOC and hydrophilic DOC. These were further separated into biopolymers (>20 kDa, such as high molecular weight (HMW) PS, HMW PN and amino-sugars), humic substances (500-1000 Da), building blocks (300-500 Da), and low molecular weight (LMW) neutrals (such as alcohols, aldehydes, ketones and mono-oligosaccharides) (Lu et al., 2018).

Figure 7.8 Quantification of sub-fractions in the bound EPS of various samples by LC-OCD-OND; a. MBR 1 and MBR 2; b. MBR 2a and MBR 2b.

Overall, the five EPS sub-fractions in the sludge and biofilm were all reduced by FNA, when comparing the bound EPS in MBR 1 and 2a with those in the control

reactors (MBR 2 and 2b). It appears from Figure 7.8 that building blocks, LMW neutrals and hydrophobic DOC were the main sub-fractions in the bound EPS. It was observed that higher concentrations of hydrophobic DOC, LMW neutrals and building blocks in the EPSs are correlated to higher fouling rate (Figures 7.1, 7.5 and 7.8). This was in accordance with previous findings that the order of fouling potential of fractionated organic matters in a PVDF MF MBR was hydrophilic neutral > hydrophobic acids > transphilic acids > hydrophilic charged (Fan et al., 2001). It should be noted that the sub-fraction values of the bound EPS (Figure 7.8) had been normalized with the MLVSS or biofilm VSS concentrations. Considering the low VSS in the biofilm in MBR 2a (Table 7.2), the absolute values of hydrophobic DOC in the TB-EPS was then substantially lower than those in MBR 2b.

FNA also affected the succession of early biofilm to late biofilm. It was found the five sub-fractions of early biofilm TB-EPS in MBR 1 and 2 were comparable. This could have been due to the similar composition of the initial colonizers deposited on the membrane surface. However, under the same period, upon further exposure to FNA, the building blocks, LMW neutrals and hydrophobic DOC in the biofilm TB-EPS in MBR 1 were reduced by 68.6%, 34.3% and 39.9%, respectively, while those in MBR 2 increased by 26.8%, 32.4% and 4.3%, respectively.

7.3.2.2 EPS characterization by 3D-EEM

The bound EPS samples freshly extracted from suspended sludge and biofilm were characterized by 3D EEM spectra (Figure 7.9). Five main components, tyrosine-like proteins, tryptophan-like proteins, SMP like substances, fluvic acid like substances, and humic acid like substances, were identified from the EEM fluorescence spectra using the FRI technique (Chen et al., 2003). It was observed that the excitation intensities of the samples under FNA stress were weaker than those in the control MBRs. As is shown in Figure 7.10, the production of SMP like and humic acid like substances was largely reduced upon exposure to FNA, especially in the TB-EPS of the late biofilm, and this contributed to the slow TMP increase in MBR 1 and 2a.

However, it was noted there was more tyrosine-like proteins in the TB-EPS of sludge and biofilm samples under FNA stress compared to the control MBRs except

the TB-EPS in the late biofilms of MBR 1 and 2 (Figure 7.10a), while intensities of tryptophan like protein between FNA stressed MBRs and controls were comparable (Figure 7.10b). Protein has been reported to play a significant role in the defense mechanism upon toxic compounds (Sheng et al., 2005). More specifically, it was found that tyrosine like protein could react with oxidative radicals and incorporate nitro group ($-\text{NO}_2$) into protein tyrosines to form protein 3-nitrotyrosine, i.e. protein tyrosine nitration (Adgent et al., 2012; Radi, 2013). As FNA led to the formation of reactive nitrogen and oxygen species in the cytoplasm including nitric oxide (NO), nitrogen dioxide (NO_2), peroxyxynitrite (ONOO^-), hydroxide ion (OH^-) and hydrogen peroxide (H_2O_2) (Hartop et al., 2017), it was postulated that the tyrosine like protein detoxified the biocidal effect of FNA via protein tyrosine nitration, which explains the more tyrosine like protein in the TB-EPS of FNA stressed MBRs. As tyrosine nitration would modify the amino acid and lead to structural and functional changes (Radi, 2013), it might also have contributed to the less biofilm and less fouling in the MBRs under FNA stress. This would require further investigation.

7.3.2.3 EPS characterization by XPS

PS analysis was conducted on freeze-dried EPS samples over the energy range of 0-1100 eV to reveal the reduction of EPS by FNA from the atomic composition level. As shown in Table 7.3, the molar ratios of O to C and N to C fell in the range of 0.36-0.53 and 0.06-0.11, respectively. High-resolution scans of C 1s, O 1s and N 1s were deconvolved to identify the specific species to analyse the information on the chemical functional group in the EPS. Breakdown of the identified functional groups is summarised in Table 7.3.

The C 1s peak was decomposed into five peaks, with two main peaks - at 285.0 eV attributed to C-C and 285.9 eV to C-(O, N), respectively, and three minor peaks at 288.0 eV, 288.9 eV and 282.4 eV. The three minor peaks were contributed by C=O, COOR, and Carbide, respectively, with less than 10% of the total C (Table 7.3). Overall, compared to the TB-EPS in control reactors, there was more C-(O, N) but less C-C in the MBR 1 and 2a upon exposure to FNA (Table 7.3). The C-(O, N) would have existed primarily as amine, alcohols, or amide groups of proteins which are generally hydrophilic. The C-C would be with the more hydrophobic DOC

(Sinninghe Damsté et al., 2002). This is in accordance with the hydrophobic DOC results revealed by LC-OCD.

The O1s peak was a cluster of three peaks, i.e. peaks at 531.4 eV, 532.3 eV and 533.7 eV, with an extra one shake-up satellite at 536.2 eV corresponding to Na KLL. The peak at 531.4 eV corresponded with C=O, i.e. carbonyl, carboxylate, amide and ester groups, the 532.3 eV peak is associated with the aliphatic C-OH such as in alcohol, acetal or hemiacetal groups, and the 533.7 eV peak is associated with the aromatic C-OH. Overall, the TB-EPS extracted from MBR 1 and 2a under FNA stress contained more aliphatic C-OH while the control MBRs had more C=O. This was probably due to the oxidative effect of FNA.

Three peaks were resolved from the N 1s peak in each sample, i.e. peak at 399.9 eV which is associated with amines and amides ($N_{\text{non pr}}$), peak at 401.3 eV with protonated amines in the form of amino acids and amino sugars (N_{pr}) and peak at 403.5 with inorganic nitrite (Yin et al., 2015). The composition of $N_{\text{non pr}}$ and N_{pr} in the TB-EPS of the control MBRs was similar to that in the nitrification sludge previously reported (Yin et al., 2015). However, the N_{pr} was barely detected in the biofilm TB-EPS in MBR 1 and 2a. This is because that FNA could cause oxidative damage to the proteins, and down regulate the biosynthesis of various amino acids (Laloo et al., 2018).

Figure 7.9 EEM fluorescence spectra of the bound EPS samples; a. sludge LB-EPS of MBR 1; b. sludge LB-EPS of MBR 2; c. sludge TB-EPS of MBR 1; d. sludge TB-EPS of MBR 2; e. early biofilm TB-EPS of MBR 1; f. early biofilm TB-EPS of MBR 2; g. late biofilm TB-EPS of MBR 1; h. late biofilm TB-EPS of MBR 2; i. sludge LB-EPS of MBR 2a; j. sludge LB-EPS of MBR 2b; k. sludge TB-EPS of MBR 2a; l. sludge TB-EPS of MBR 2b; m. late biofilm TB-EPS of MBR 2a; n. late biofilm TB-EPS of MBR 2b.

Figure 7.10 FRI analysis of the EEM fluorescence spectra of the bound EPS samples; EB stands for the early biofilm and B stands for biofilm.

Table 7.3 Atomic ratio of O/C and N/C and functional groups revealed by XPS for the bound EPS in different samples.

	Molar ratio to the total C		C (peak energy, eV and corresponding chemical functions)					O (peak energy, eV and corresponding chemical functions)				N (peak energy, eV and corresponding chemical functions)		
	O/C	N/C	285.0 C-C	285.9 C- (O,N)	288.0 C=O	288.9 COOR	282.4 Carbide	532.3 C-OH (aliphatic)	536.2 Na KLL	533.7 C-OH (aromatic)	531.4 C=O	399.9 N _{non} pr	401.3 N _{pr}	403.5 nitrite
1-LB	0.43	0.10	0.48	0.36	0.07	0.05	0.04	0.52	0.27	0.03	0.19	0.88	0.00	0.12
2-LB	0.53	0.11	0.55	0.29	0.05	0.07	0.03	0.46	0.26	0.10	0.18	0.79	0.00	0.21
1-TB	0.45	0.11	0.28	0.53	0.08	0.08	0.03	0.38	0.36	0.04	0.22	0.94	0.03	0.03
2-TB	0.39	0.10	0.39	0.44	0.07	0.08	0.03	0.39	0.34	0.11	0.15	0.91	0.06	0.03
1-EB-TB	0.40	0.08	0.34	0.47	0.07	0.09	0.04	0.45	0.34	0.21	0.00	0.98	0.00	0.02
2-EB-TB	0.46	0.10	0.32	0.50	0.07	0.08	0.03	0.39	0.31	0.12	0.17	0.88	0.07	0.04
1-B-TB	0.45	0.06	0.36	0.42	0.04	0.10	0.08	0.39	0.33	0.14	0.14	1.00	0.00	0.00
2-B-TB	0.44	0.10	0.48	0.36	0.02	0.12	0.02	0.39	0.13	0.35	0.13	0.76	0.23	0.01
2a-TB	0.40	0.11	0.36	0.44	0.09	0.06	0.05	0.62	0.20	0.01	0.17	0.92	0.06	0.02
2b-TB	0.42	0.11	0.40	0.41	0.09	0.06	0.04	0.46	0.22	0.01	0.31	0.94	0.05	0.01
2a-B-TB	0.36	0.07	0.47	0.33	0.07	0.06	0.06	0.46	0.28	0.09	0.16	0.93	0.03	0.03
2b-B-TB	0.38	0.10	0.32	0.47	0.08	0.09	0.04	0.41	0.20	0.01	0.33	0.83	0.13	0.05

7.3.3 Practical implications for membrane fouling mitigation via FNA

A long run for the bioreactor while not being concerned about the fouling problem is essential. The adhesion of sludge suspension via EPS to the membrane surface and the subsequent formation of compact cake fouling layer lead to the development of membrane fouling (Zhang and Jiang, 2019). In this study, *in-situ* regulated FNA successfully mitigated the membrane fouling in the nitrification MBR. The EPS production in the suspended sludge was found to decrease due to the biocidal effect of FNA (Figures 7.4 and 7.7). Cake layer formation exhibited as biofilm mass and thickness was also suppressed as shown in Figures 7.2 and 7.6. These findings were helpful because fouling seriously disturbs the operation of a bioreactor for wastewater treatment. Inhibition on EPS excretion and biofilm disruption have been proposed as emerging biofouling control strategies (Xiong and Liu, 2010).

Following characterizations of EPS samples via LC-OCD-OND, EEM-FRI and XPS revealed the mechanisms of FNA-induced EPS reduction. The exposure of enriched *Nitrosomonas* biomass to high FNA stress not only decreased the amount of EPS in both suspended sludge and biofilm, but also induced the changes of EPS in compositions and main functionalities due to the oxidative effect. FNA reduced the low molecular weight neutrals and hydrophobic dissolved organic carbon which had high fouling potential. FNA stimulated the production of tyrosine-like proteins but reduced those of SMP like and humic acid like substances, which may further affect the adhesion between bacteria and membrane and contribute to the less biofilm and less fouling.

It should be noted that the applied FNA concentration did not break the cell membrane, which was different from FNA application of sludge pretreatment for anaerobic digestion (Wu et al., 2019; Zhang et al., 2020). In this study, the nitrification performance of MBRs under high FNA concentration was comparable or even better than that of control MBRs. This study also revealed that FNA regulated by pH and nitrite concentration led to similar membrane fouling mitigation. Therefore, from the practical point of view, further optimization of FNA via pH could serve as emerging strategy for both nitrification maintenance and biofouling control.

7.4 Concluding remarks

This chapter reported on the effectiveness of FNA regulated by pH and nitrite concentration on membrane fouling mitigation in nitrification MBRs. The biofilm occurrence on the membrane surface was markedly reduced by the *in-situ* generated FNA. It can be concluded that FNA significantly diminished the LMW neutrals and hydrophobic DOC in the bound EPSs of both the suspended sludge and the biofilm. The oxidative effect of FNA triggered slightly more tyrosine like protein in the TB-EPS but much less SMP and humic acid like substances. XPS analysis also revealed the different properties, from the atomic composition level, of the bound EPS in MBRs under high FNA stress and those in control MBRs. Consequently, this chapter showed that *in-situ* regulated FNA could be considered a feasible approach for mitigating membrane fouling in nitrification MBRs.

Chapter 8 Conclusions and Recommendations

8.1 Conclusions

Nitrification based nitrogen removal processes are promising technology with less requirement of aeration energy and external carbon as well as lower sludge production for high ammonium concentration wastewater treatment. Long-term maintenance of AOB activity adoration and NOB activity suppression, and membrane fouling are the two major problems affecting the nitrification MBR performance. The current study systematically investigated the feasible and sustainable strategies for stable nitrification maintenance in the long SRT MBR, from both biological and process operation perspectives. The biological behaviour of the enriched *Nitrosomonas* culture under high concentrations of FA and FNA with the aiming to maintain stable nitrification was discussed, i.e. nitrification performance, key functional bacteria dynamics, EPS production, and N₂O emission. Moreover, mechanism of membrane fouling in the nitrification MBR and the effectiveness of application of FNA to mitigate membrane fouling were explored, i.e. TMP development pattern, biofilm formation, EPS composition and dynamics and microbial community.

The strategy of combining low DO with high FA failed to maintain nitrification, or even partial nitrification. AOB was inhibited at FA concentration of 34.3 ± 6.85 mg NH₃-N/L while the threshold inhibiting concentration of FA was likely 23.6 ± 4.51 mg NH₃-N/L. Even though FA was reduced to 8.89 ± 0.95 mg NH₃-N/L in the following 37 days, after 70 days of operation under high FA, AOB cells became more sensitive to FNA. FNA concentration of 1.58×10^{-3} mg HNO₂-N/L caused a sharp decline in AOB cell numbers by 62.7%. The NOB had acclimated to the high FA conditions. This showed that long-term FA inhibition had adverse impact on process recoverability and subsequent failure threshold of the nitrification process.

The strategy that combined *in-situ* generated FNA shock with DO limitation successfully maintained stable nitrification in the long SRT MBR, from 200 mg NH₄⁺-N/L to 650 mg NH₄⁺-N/L, with ARR and NAR over 0.97 and 0.96, respectively.

Results showed that both activities of *Nitrospira* and *Nitrobacter* were suppressed and populations were reduced by the combined strategy of DO limitation and *in-situ* generated FNA shock. Moreover, no positive effect of the applied strategy on N₂O emission was observed. Overall, N₂O emission was $2.1 \pm 0.72\%$ of the ammonium removed. Slight reduction of N₂O was found after the *in-situ* generated FNA shocks due to the inhibition effect of FNA.

This study investigated the mechanism of membrane fouling in the nitrification MBR and the relationship between FNA and EPS production of an enriched *Nitrosomonas* culture. It was found that cake layer formation was the main cause of the membrane fouling and EPS played a critical role in the membrane fouling development process in the nitrification MBR. TB-EPS accounted for 84.3% of the bound EPS and more PN was found in the bound EPS. EPS PN and PS concentration in the suspended sludge were found to decrease by 23.4% and 19.0%, respectively when FNA concentration reached 1.47×10^{-2} mg HNO₂-N/L from day 180. Batch tests also verified the FNA stress reduced the production of LB and TB EPS but did not induce cell lysis to release PS and PN into SMP. Microbial community analysis showed that FNA also played a role in the microbial community composition, with *Nitrosomonas* the most abundant bacteria in both sludge and biofilm.

This study also systematically investigated the effectiveness of FNA on the membrane fouling mitigation in the nitrification MBR. It was found that FNA up to 2.7×10^{-2} mg HNO₂-N/L reduced both LB-EPS and TB-EPS in the suspended sludge and biofilm, and thus maintained the TMP in the low range compared to the control nitrification MBRs. Moreover, EPS sub-fraction analysis by LC-OCD-OND explicated that FNA caused the significant reduction of hydrophobic DOC and LMW neutrals, which played a critical role in the PVDF membrane fouling. The EEM-FRI technique illustrated the oxidative effect of FNA triggered slightly more tyrosine like protein in the TB-EPS but much less SMP and humic acid like substances. The XPS analysis also revealed the different compositions among the bound EPS of MBRs under the high FNA stress and those of control MBRs. As a result, biofilm formation, in terms of thickness and mass, was reduced by high FNA concentrations, which played a critical role in the membrane fouling mitigation in the nitrification MBRs.

The results in the current project give useful implications in the long-term maintenance of nitrification and membrane fouling control by *in-situ* generated FNA in the nitrification MBR. This project provides a detailed insight into the membrane fouling mechanism in the nitrification MBR, and sheds light on the employment of FNA into the fouling mitigation. Maintenance of nitrification in MBRs with *in-situ* generated FNA would reduce both of CAPEX and OPEX for the treatment of high concentration ammonium wastewater.

8.2 Recommendations and prospects

8.2.1 Optimization of nitrification performance and membrane mitigation by FNA in the nitrification MBR

In this project, FNA was found to be the feasible strategy for nitrification maintenance and the membrane fouling mitigation. From process safety point of view, spike FNA shock up to 0.61 mg $\text{HNO}_2\text{-N/L}$ within 12 hours successfully suppressed and decreased NOB population. From process stability point of view, continuous flow of FNA concentration in the range of 2.7 to 4.6×10^{-2} mg $\text{HNO}_2\text{-N/L}$ effectively mitigated the membrane fouling in the nitrification MBR.

However, despite the defence mechanisms of *Nitrosomonas* on exposure to high FNA, such as oxidative stress enzymes regulation, denitrification, and DNA and protein repair (Laloo et al., 2018), high FNA concentrations could have inhibitory effect on *Nitrosomonas*. It is reported that energy production capability of an 82%-enriched *Nitrosomonas* culture was inhibited by 50% at FNA concentrations of 0.40-0.63 mg $\text{HNO}_2\text{/L}$, while the growth process was completely inhibited at 0.40 mg $\text{HNO}_2\text{/L}$ (Vadivelu et al., 2006a; Vadivelu et al., 2007b). In Chapter 5, the enriched *Nitrosomonas* culture was not found to be inhibited at the spike FNA shock of 0.61 mg $\text{HNO}_2\text{-N/L}$. For one thing, this could be due to the shorter exposure duration. For another thing, the acclimatization via the increase of FNA concentration in the MBR also played a critical role in the tolerance of *Nitrosomonas* culture to FNA in this study. Due to the membrane separation, both MLVSS and particle size increased. Besides, the current study also reveals the coexistence of heterotrophic bacteria and *Nitrosomonas* provided the mutualistic symbiosis, to resist the biocidal stress of FNA.

Therefore, it is recommended to optimize the FNA concentrations for the nitrification performance and membrane fouling mitigation.

Biofouling is a common phenomenon in the MBR operations. Recently, biological fouling strategies have been proposed based on comprehensive understanding from microbial ecology perspective, including enzymatic disruption of EPS excretion, quorum quenching (Weerasekara et al., 2016) and biofilm disruption (Xiong and Liu, 2010). This study presented the effectiveness of FNA on the biofilm disruption to mitigate membrane fouling in the nitrification MBRs. Further research studies are also recommended to explore the further application of FNA on the biofouling control in other systems.

8.2.2 Further characterization of AOB and NOB responses under the FNA stress

This study shows the effectiveness of *in-situ* generated FNA shock on the selective inhibition of NOB but not to AOB. Although the duration of *in-situ* generated FNA shock was less than 12 hours, the FNA concentrations applied in this study exceeded both of AOB and NOB inhibitory concentrations. Therefore, the mechanism of *in-situ* generated FNA shock for long term maintenance of stable nitrification is pending to be addressed. Possible property to be taken into consideration is the morphology of AOB and NOB. i.e. in the form of suspension cells, flocs, granular or biofilm in the reactor. It has been reported that AOB tended to form large micro-colonies in the flocs while NOB formed small micro-colonies in the nitrification reactors (Coskuner et al., 2005; Degrange and Bardin, 1995; Picioroanu et al., 2016). The relatively larger average AOB colony sizes than those of NOB may lead to the higher tolerance of AOB to the FNA stress than NOB. Further investigation is required to elucidate the selective suppression of high FNA concentration to NOB but not AOB.

8.2.3 Further Application of FNA on the sludge fermentation and denitrification process

This study proves that nitrification could be maintained under long SRT MBR and provides a stable high concentration nitrite flow. One suitable approach to deal

with the nitrification effluent would be denitrification. However, one of the main problems in nitrogen removal at WWTPs is the unfavourable ratio of organic carbon to nitrogen in the reject water. As the use of commercially available carbon source, such as methanol ethanol, and acetic acid, would increase the operation expenditure of WWTPs, the research of readily available internal short chain carbon sources in WWTPs is of growing concern and practical interest (Frison et al., 2013). Tora et al. (2011) made a comparison about ethanol, acid-fermented primary sludge centrate, acid-fermented secondary sludge centrate, glycerol and landfill leachate and maximum specific nitrite removal rate of $0.25 \text{ g N g}^{-1} \text{ VSS d}^{-1}$ was achieved by using glycerol. Ji and Chen (2010) improved wastewater nitrification-denitrification process by using sludge fermentation liquid compared to acetic acid. Frison et al. (2013) investigate five available carbon sources and found that organic fraction of the municipal solid waste fermentation liquid was the best available external carbon source, achieving the specific denitrification rate of $0.65 \pm 0.07 \text{ kgNO}_2\text{-N/kgVSS day}$. Therefore, the sludge fermentation holds the promise to provide volatile fatty acids (VFAs) for the denitrification process for treating reject water.

Figure 8.1 Schematic flow of side-stream treatment of reject water by nitrification, sludge FNA pre-treatment and denitrification process

The scheme of sludge FNA pre-treatment is depicted as in Figure 8.1. With stable nitrification providing the flow of high nitrite, FNA could be utilized in the

sludge fermenter with WAS as feed under a prolonged SRT. With the biocidal effect of FNA, the organic matters in the WAS could be converted into soluble forms.

VFAs are produced in the acidogenic phase of fermentation, and then converted to methane in the presence of methanogenic bacteria. Therefore, during sludge fermentation process, the activity of methanogenic bacteria needs to be suppressed. Kluber and Conrad (1998) found nitrite inhibited methanogenesis process, but whether reversible or irreversible inhibition depended on the type of methanogenic bacteria as well as the nitrite concentrations. Adding nitrous acid would improve the biodegradability of secondary sludge (Pijuan et al., 2012), thus, the production of VFAs could be increased by dosing nitrite to the sludge fermentation process. In fact, sludge fermentation and denitrification could benefit each other. Introduction of nitrite to sludge fermentation process could enhanced sludge fermentation, as the nitrite added may form free nitrous acid, which could promote the decomposition and dissolution of sludge particles, and induce lipid peroxidation, and finally cause cell membrane damage. Moreover, denitrification process may also occur in the sludge FNA pre-treatment reactors, and consume VFAs, which would accelerate sludge decomposition and hydrolysis (Wu et al., 2014). Then the effluent is further pumped to the denitrification reactor for removal of nitrite to N_2 . The residual sludge in the FNA pre-treatment reactor could be pumped to anaerobic digester for further treatment (Figure 8.1).

Appendix A Nitrite and nitrate interference in colorimetric measurement of polysaccharide in biological wastewater treatment systems

A.1 Introduction

Bahaviour and characteristics of EPS and SMP are gaining more and more attention in biological wastewater treatment systems, due to their impacts on the granule formation, biofouling in membrane reactors, and treatment efficiency (Chen et al., 2016; Liang et al., 2007; Meng et al., 2017). The major components in EPS and SMP are PS, PN, humic substances, lipids and other small molecules (Le and Stuckey, 2016). Among these, PS are of great importance, which are regarded as the primary membrane foulant in MBRs (Meng et al., 2017).

Up to date, the colorimetric methods for the determination of PS is probably the most rapid, simple and cheap approach (Le and Stuckey, 2016). Phenol-sulfuric acid method devised by Dubois et al. (1956) is the most widely used and reliable for the PS measurement. By adding phenol and sulfuric acid (H_2SO_4) to the sample, the PS are dehydrated and formed into furfural derivatives, which then condense with phenols to form orange-yellow complexes for colorimetric quantification. However, due to the complex nature of biological wastewater treatment system, a large variety of solutes in the wastewater, such as sorbitol, ascorbic acid, etc, are reported to affect the color development of chromophore (Le and Stuckey, 2016). Compared to other colorimetric PS determination methods, 5% phenol-sulfuric acid method presented less interfering properties with other compounds (Le and Stuckey, 2016).

Drews et al. (2007) found that nitrite and nitrate would impair the photometric measurement and lead to elevated PS values. Thus, PS concentration was calibrated using Eq. A.1 (Drews et al., 2007).

$$C_{PS} = C_{PS,meas} - 0.099c_{NO_3-N} - 1.9c_{NO_2-N} \quad \text{Eq. A.1}$$

It should be noted that the nitrite and nitrate concentrations examined in their study were in the range of 0 - 60 mg N/L and 0 - 75 mg N/L, respectively. In practice, the treatment of high ammonium wastewater, i.e. landfill leachate, reject water generated from sludge anaerobic digestion, etc. would generate nitrite concentration more than 500 mg N/L via nitrification based processes. Nitrite has been reported to interfere the colorimetric determination of phosphorus (Greenberg et al., 1950), COD (Marouf-Khelifa et al., 2006) and hexavalent chromium (Cr(VI)) (He et al., 2015). Nitrite is relatively unstable, and could either be reduced by certain organic compounds, or be oxidized by KMnO_4 , or even serve as quencher of radical-based reaction due to the affinity for the hydrated electrons (Lopes et al., 2017).

Whilst the mechanism of nitrite interference with PS measurement is still not clear, the feasibility of calibration equation on the nitrite interference under high nitrite concentrations is also questioned. Several chemicals were reported to remove nitrite, i.e. sodium azide, o-Toluidine, and sulfamic acid. Among these, sulfamic acid had better performance in removing nitrite, compare to o-Toluidine (Pate et al., 1965), and had no toxic by-product, compared to sodium azide (Greenberg et al., 1950). Therefore, sulfamic acid had been used to remove nitrite for the accurate determination of nitrate, COD and Cr(VI).

However, either the efficiency of sulfamic acid in elimination of nitrite interference of PS determination, or the involvement of sulfamic acid in the PS-phenol-sulfuric acid system, had never been investigated elsewhere. Therefore, the main objective of this study was to elucidate the mechanism of nitrite interference and manifest the effectiveness of sulfamic acid in eliminating nitrite interference during PS determination. Nitrate interference in PS determination would also be discussed.

A.2 Experimental

A.2.1 Phenol-sulfuric acid method

PS concentration was determined by the phenol-sulfuric acid method (Dubois et al., 1956) with glucose as the standards. 1 mL of freshly prepared 5% phenol was added to 2 mL of a glucose standard or the sample, followed by 5 mL concentrated

H₂SO₄. The mixture was vortexed rapidly and allowed to stand at room temperature for 30 min. The absorbance was read at 490 nm by a double-beam Ultraviolet-visible spectrophotometer (UV-1800, Shimadzu). Triplicate measurements were conducted for each sample/standard. Full spectrum of absorbance was recorded with wavelength from 800 nm to 200 nm.

A.2.2 PS colorimetric analysis with interfering nitrite and nitrate

A series of known amount of nitrite (final concentration of 0-600 mg NO₂⁻-N/L) and nitrate (final concentration of 0-150 mg NO₃⁻-N/L) were deliberately added into known amounts of glucose standards. The concentrations of nitrite and nitrate were doublechecked according to the standard methods (APHA, 2017). Sulfamic acid was added to the sample to eliminate the nitrite interference (Greenberg et al., 1950; Marouf-Khelifa et al., 2006). A new standard curve was also prepared with sulfamic acid added. Triplicate measurements were conducted for each condition.

A.3 Results and discussion

A.3.1 Nitrite interference in PS determination and reaction mechanism

Figure A.1 shows the measured glucose concentration with different nitrite concentrations. Nitrite in the samples strongly interfered the PS determination. As the nitrite concentration increased, the reagent became dark purple and as little as 5 mg N/L in the test samples introduced considerable error in the determination.

Figure A.2 plots the full wavelength absorbance spectrum of different combination of 0-20 mg N/L nitrite and 0-20 mg/L glucose. It was found that with the presence of nitrite alone, there was a plateau peak from 500 nm to 550 nm, indicating nitrite interference. This also ruled out the possibility of reaction between nitrite and glucose. Nitrite has been used in the synthesis of nitroderivatives of aromatic compounds under strong acids (Uemura et al., 1978). Besides, upon UV irradiation, nitrite could react with phenol and form nitrophenols (Vione et al., 2001). Detailed reaction mechanism was elaborated by Patnaik and Khoury (2004): 2-nitrophenol was formed immediately while 4-nitrophenol appeared after induction periods, and this reaction could occur at acidic, neutral and alkaline conditions. As

the active species, nitrite attacks the phenol ring at the *ortho* position and undergoes a *para*-substitution with excess presence of nitrite (Patnaik and Khoury, 2004). Whereas in this study, 1 mL of 5% phenol solution (50 g/L) was added into the test samples for the PS determination, which consumed all the nitrite in the samples. The mechanism of nitrite interference in the PS determination was postulated that 2-nitrophenol was formed under nitrite and excess phenol conditions, and after PS was dehydrated and formed into furfural derivatives by 95% sulfuric acid, 2-nitrophenol together with excess phenol condensed with these furfural derivatives and formed the purple instead of orange-yellow complexes. Thus, as shown in Figure A.3, an elevated absorbance was found with the combination of 20 mg/L nitrite and 20 mg/L glucose.

A.3.2 Elimination of nitrite interference with sulfamic acid in PS determination

To accurately determine PS concentration with the presence of nitrite in the samples, it is necessary to explore an effective approach to eliminate nitrite interference. Drews et al. (2007) employed an empirical equation to calibrate the PS concentration impaired by nitrite interference. However, as shown in Figure A. 1 with the increase of nitrite concentrations, the interference was too severe to be corrected via subtracting factor of nitrite concentration. When nitrite concentration exceeded 100 mg N/L, none linear curve could be conducted between glucose concentration and measured absorbance.

To avoid the overstated reading of the measured PS concentration caused by the reaction between nitrite and phenol, it was better to remove nitrite before adding phenol to the test samples. In this study, the excess added sulfamic acid did not react with phenol or glucose (Figure A.2), and the improved method with sulfamic acid added generated the standard curve $y=0.0101x+0.0051$ ($R^2=0.9992$), which was almost the same as $y=0.0101x+0.0059$ ($R^2=0.9994$) in the sulfamic acid absent system (y stands for the absorbance at 490 nm and x the glucose concentration).

Figure A.1: Nitrite interference in the polysaccharide measurement using phenol-sulfuric acid method with nitrite concentration from 0 to 600 mg N/L.

Figure A.2: Full wavelength spectra of the mixed samples of nitrite and glucose. The first number stands for the nitrite concentration (mg N/L), while the second number the glucose concentration (mg/L). SA means the sample was added sulfamic acid

The efficiency of sulfamic acid in eliminating nitrite interference was also investigated. The reaction of nitrite and sulfamic acid was $HNO_2 + HSO_3NH_2 \rightarrow N_2 + H_2SO_4 + H_2O$. In this study, 10 mg sulfamic acid was added per 1 mg NO_2^- -N to ensure total nitrite elimination, and a prolonged incubation period was adopted to release nitrogen gas sufficiently. As shown in Figure A.3, with the nitrite concentrations up to 600 mg N/L, the measured PS concentration using this improved method recovered 95.7-103.8% of the added PS value from 0 to 50 mg/L.

Figure A.3: Elimination of Nitrite interference by sulfamic acid for polysaccharide measurement using phenol-sulfuric acid method with nitrite concentration from 0 to 600 mg N/L.

A.3.3 Nitrate interference and correction

Nitrate in the samples also interfered the determination of PS using phenol-sulfuric acid method (Figure A.4a). The nitrate interference became more severe with the increase of nitrate concentrations from 0 to 150 mg N/L. However, different from nitrite interference, nitrate interference seemed to elevate the whole baseline to the absorbance (Figure A.5) and did not have the absorbance peak at 490 nm without the presence of glucose.

Figure A.4: Nitrate interference in the polysaccharide measurement using phenol-sulfuric acid method with nitrate concentration from 0 to 150 mg N/L; a. nitrate interference; b. corrected PS concentration using empirical equation.

Figure A.5: Full wavelength spectra of the mixed samples of nitrate and glucose.
a. different nitrate concentrations without glucose; **b.** different nitrate concentrations with 20 mg/L glucose. The first number stands for the nitrate concentration (mg N/L), while the second number the glucose concentration (mg/L).

Like nitrite, nitrate could also be removed via different approaches. Lopes et al. (2017) evaluated different approaches to eliminate nitrate interference with photochemical vapor generation, such as reduction with formaldehyde, photochemical reduction, precipitation with nitron, and reduction with hydrazine sulfate/copper. However, none of these approaches fit for the PS measurement, either because of the low efficiency, or due to the long incubation duration, which could lead to the PS degradation. Thus, an empirical equation was applied to correct the nitrate interference in the PS determination based on the different combinations of nitrate and glucose concentrations, as shown in Eq. A.2. This was in accordance with (Drews et al., 2007). The corrected concentration of PS recovered 98.5-103.7% of the added glucose concentrations from 0 to 50 mg/L (Figure A.4b).

$$c_{PS} = r c_{PS, meas} - 0.067 c_{NO_3-N} \quad \text{Eq. A.2}$$

where r is the dilution ratio by the addition of sulfamic acid for the nitrite elimination, $c_{PS, meas}$ is the measured concentration of the PS concentration, and C_{NO_3-N} is the nitrate concentration of the sample.

A.4 Concluding remarks

Nitrite could severely interfere the PS determination using phenol-sulfuric acid spectrophotometric method, leading to overstated readings at 490 nm, while nitrate interference elevated overall readings from 800 nm to 350 nm. The nitrite interference mechanism was investigated that nitrite as the active species would react with phenol and thereby formed the purple complexes. Addition of sulfamic acid was an efficient and effective approach to eliminate the nitrite interference. Based on the data obtained from this study, an empirical equation was established for eliminating nitrite and nitrate interference in determine PS concentration using phenol-sulfuric acid method.

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