PILOT-SCALE INTEGRATED FIXED FILM ACTIVATED SLUDGE SYSTEMS FOR WASTEWATER BIOLOGICAL NUTRIENT REMOVAL

by

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ABSTRACT

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Under the supervision of Dr. Yang Liu

In this study, the performance of two identical pilot-scale biological nutrient removal - activated

sludge (BNR-AS) reactors were assessed as their influent flow rate was increased from 30L/min

through 45L/min and up to 60L/min. The bacteria community structure for nitrifiers and

denitrifiers as well as the protein to polysaccharide ratio (PN/PS) of the extracellular polymeric

substances (EPS) in both reactors were monitored during each flow rate. The experimental results

showed that both reactors maintained excellent chemical oxygen demand (COD) removal,

however, the removal of ammonia and phosphorous deteriorated at some point during 40L/min

influent flow rate and beyond. Furthermore, bacteria community analysis showed that Nitrobacter

was the more dominant of the two nitrite-oxidizing bacteria (NOB) communities that were

investigated. The EPS analysis demonstrated that the PN/PS of sludge EPS significantly decreased

as the solids retention time (SRT) decreased from 9.3 days to 2.2 days. Furthermore, PN/PS content

had the tendency to temporarily increase in response to an increase in flow rate before decreasing

back to a steady value. This study also compared the performance of a BNR-AS reactor side-by-

side with a biological nutrient removal – integrated fixed-film activated sludge (BNR-IFAS)

reactor. The experimental results showed that the proliferation of red worms identified as

Aeolosoma hemprichi in the BNR-IFAS reactor led to the significant deterioration in the BNR-IFAS reactor performance especially in ammonia removal, although COD and phosphorous removal seemed to be unaffected by the red worms. Of the different strategies applied to eliminate the red worms, the most effective was shutting off the dissolved oxygen in the aerobic zone for 48 hours in addition to cutting off influent feed supply, stopping nitrified liquor recycling and maintaining low mixed liquor suspended solids (MLSS) concentration.

PREFACE

This MSc work was carried out under the supervision or Dr. Yang Liu. The research project was funded by EPCOR and Abdul Mohammed from EPCOR supported the project. All the experiments included in this thesis were conducted by myself with some assistance.

More specifically for chapter 3: Dr. Shengnan Xu performed part of the data analysis and co-wrote the proceeding paper from chapter 3 with me. Dr. Yang Liu was involved in the planning, experimental design, discussion and paper writing. Abdul Mohammed was involved in planning, design and discussions. Yao Tang was involved in performing the EPS and qPCR experiments.

For chapter 4: Dr. Shengnan Xu performed the qPCR experiments*, part of the data analysis, and co-wrote the proceeding paper from chapter 4 with me. Dr. Yang Liu was involved in the planning, experimental design, discussion and paper writing. Abdul Mohammed was involved in planning, design and discussions and paper editing.

Chapter 3 of this thesis has been accepted for publication in the Journal of Environmental Engineering with the title "Effect of Flow Rate Increase on the Performance of a Pilot-Scale Biological Nutrient Removal Reactor" by Kingsley Nze, Shengnan Xu, Yao Tang, Abdul Mohammed and Yang Liu. Chapter 4 is currently being authored and edited with more data for submission to a journal.

*The qPCR experiments performed by Dr. Xu for chapter 4 is not included in this thesis, however, it will be incorporated into the proceeding paper.

DEDICATION

I would like to dedicate this research to the loving memory of my sister

Victoria Chinwe Nze, who left us a little too early while I was in the middle of this study. Your

passion for excellence will always remain an inspiration.

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LIST OF ABBREVIATIONS

AOB: Ammonia oxidizing bacteria

AS: Activated sludge

BNR: Biological nutrient removal, composed of biological nitrogen removal and enhanced biological phosphorus removal (EBPR) (Metcalf and Eddy 2003)

BNR-AS: Biological nutrient removal using a conventional activated sludge system

BNR-IFAS: Biological nutrient removal using integrated fixed film activated sludge

CAS: Conventional active sludge

DENs: De-nitrifying organisms

EBPR: Enhanced biological phosphorous removal

F/M: Food-to-microorganism ratio

HRT: Hydraulic retention time

IFAS: Integrated fixed-film active sludge

MBBR: Moving bed bioreactor

ML: mixing liquor

MLE: modified Ludzak Ettinger

MLR: Mixed Liquor Recirculation

MLSS: Mixed liquor suspended solids concentration

MLVSS: Mixed liquid volatile suspended solids

NLR: Nitrified liquor recycle

NOB: Nitrite oxidizing bacteria

PAOs: Polyphosphate accumulating organisms

qPCR: Quantitative polymerase chain reaction

RAS: Recycling activated sludge

SRT: Solids retention time, it is also called mean cell retention time (MCRT)

SVI: Sludge volume index

VFA: Volatile fatty acid

CHAPTER 1. INTRODUCTION

1.1 Importance of Municipal Wastewater Treatment

The discharge of improperly treated wastewater is the primary cause of quality deterioration in ecosystems such as rivers, lakes, and oceans (Chan et al. 2009). The impact load of discharged wastewaters on these ecosystems in terms of chemical oxygen demand (COD), biochemical oxygen demand (BOD) and total suspended solids (TSS) is estimated to be in the tens of thousands mg/L (Chan et al. 2009). Municipal wastewater is particularly rich in phosphorous (P) and nitrogen (N); these two elements are primarily linked to eutrophication in rivers and lakes (Oldham and Rabinowitz 2001). The discharge of nitrogen and phosphorous fosters the growth of algae and aquatic plants, thereby depleting the oxygen content of these receiving water bodies and rendering them inhabitable to fish and undesirable for domestic or recreational use (Oldham and Rabinowitz 2001). In order to protect water resources, the regulations controlling the discharge limits of N and P into receiving water bodies are becoming stricter; for instance in the province of Alberta, Canada, in 2015 the main municipal wastewater treatment company, EPCOR, was licenced to operate with a discharge limit of 5.0mg/L for NH_3-N during the summer months, 10mg/L for NH_3-N during the winter months and 1.0 mg/L total Phosphorous all year round (Thomas et al. 2015). Given the increasingly stringent regulations, most secondary treatment plants in the world have recently been converted to biological nutrient removal (BNR) facilities; "BNR" referring to the removal of nitrogen and phosphorous using a biological mechanism (Metcalf & Eddy et al. 2014)

1.2 Overview of biological nutrient removal - activated sludge system (BNR-AS)

Although It is possible to remove phosphorous and nitrogen from municipal wastewater using physical and chemical processes, the use of biological processes for nutrient removal is preferred because they generate less waste sludge which can easily be converted and used for agricultural land applications (Oldham and Rabinowitz 2001, Metcalf & Eddy et al. 2014); furthermore, biological processes are less expensive compared to chemical processes (Barnard 1974, Vaiopoulou et al. 2007, Kim et al. 2009) which makes them attractive to treatment companies. The activated sludge (AS) process is a biological process which employs a large mass of microorganisms to convert organic matter and nutrients in wastewater to gasses and cell tissue (Metcalf & Eddy et al. 2014). These microorganisms are usually suspended in aerobic process treatment tanks using aeration or mixing (Metcalf & Eddy et al. 2014).

Historically, the use of activated sludge processes dates as far back as the early 1900s (Metcalf & Eddy et al. 2014); However, the development of biological nutrient removal (BNR) through the activated sludge process (BNR-AS) to a level of controllable and predictable removal efficiency gained significant advancement in the 1970's in South Africa (Barnard 1974, Oldham and Rabinowitz 2001). To target nitrogen removal, a key modification to the known processes at the time was the introduction of a pre-anoxic zone upstream of the aerobic zone(Ludzack and Ettinger 1962). The unaerated but mixed pre-anoxic zone received nitrified liquor which was recycled from the aerobic zone and mixed with the BOD - rich influent to enhance denitrification. In the subsequent years, different process configurations targeting nitrogen removal were developed; including the modified Ludzak Ettinger (MLE) and the Bardenpho process amongst others. To achieve enhanced biological phosphorous removal (EBPR), Barnard showed the necessity of

having an anaerobic contact zone between the aeration zone and the influent wastewater (Barnard 1974). This led to the development of bioreactor configurations such as the 5-stage bardenpho process with the capability to distinctly handle biological phosphorous and nitrogen removal (Barnard 1974, 1976, Oldham and Rabinowitz 2001). Typically, in the BNR-AS system, sludge is either transferred from the anoxic or aerobic zone through a membrane as in the case of a membrane bioreactor (MBR); alternatively and more commonly, sludge is transferred from either the anoxic or aerobic zone to a secondary clarifier where floc particles of 50 - 200 mm in size form and settle out by gravity, and are either wasted or recycled to the anoxic zone as return activated sludge (RAS) (Metcalf & Eddy et al. 2014).

Nitrification, being the key mechanism of nitrogen (N) removal in a BNR process, engages the two-step oxidation of ammonium (NH₄-N); first to nitrite (NO₂-N) and then from nitrite to nitrate (NO₃-N) by aerobic chemoautotrophs termed ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) respectively (Metcalf & Eddy et al. 2014). Nitrate and nitrite are then reduced to nitrogen through biological denitrification which is carried out by a group of bacteria collectively termed denitrifiers (DEN) (Metcalf & Eddy et al. 2014, Lu et al. 2014). The two-step oxidation process that brings about the conversion of ammonia to nitrite (nitritation) and the conversion of nitrite to nitrate nitrification is depicted in equations 1 and 2 respectively;

$$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 4H^+ + 2H_2O \qquad \text{(Equation 1)}$$

$$2NO_2^- + O_2 \rightarrow 2NO_3^- \qquad \text{(Equation 2)}$$
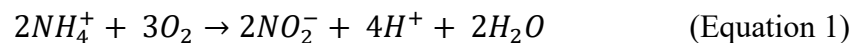
and the complete oxidation reaction that brings about nitrification is depicted in the equation 3

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O \qquad \text{(Equation 3)}$$
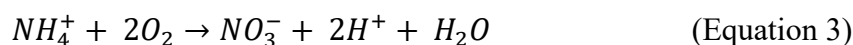
Enhanced biological phosphorous removal (EBPR) employs phosphorous accumulating organisms (PAOs) to take up and store readily biodegradable COD (rbCOD) under anaerobic conditions as poly-β-hydroxyalkanoate (PHAs). PHA can then be used as an energy source to incorporate soluble orthophosphate (O-PO_4) into the PAO bacteria cell either under aerobic conditions, or under anoxic conditions for denitrifying PAOs (Metcalf & Eddy et al. 2014, Xu et al. 2014).

1.3 Overview of BNR with Integrated Fixed-Film Activated Sludge (BNR-IFAS)

Another variant of the BNR process is the Integrated Fixed-film Activated Sludge (IFAS), which is characterized as a hybrid process (Metcalf & Eddy et al. 2014). The BNR-IFAS builds upon the advantages of the BNR-AS process by incorporating media material into the suspended biomass growth in a BNR-AS reactor; this media provides additional surface for biomass growth.

The IFAS media material can either be suspended in the activated sludge and be moved around due to air sparging as in the case of the mobile media used for Moving Bed Bioreactors (MBBR) (Fig. 1. A & B), or the media material could be Fixed in the aeration tank (fixed-media) (Fig. 1. C & D). A key feature of the BNR-IFAS over the BNR-AS is that the provision of attached growth surface results in a total equivalent mixed liquor suspended solids (MLSS) concentration that may be 1.5 to 2.0 times the AS MLSS concentration alone (Metcalf & Eddy et al. 2014). This in turn provides a longer solids retention time (SRT) for the slow growing nitrifiers (Kim et al. 2011b). Earlier studies showed that an IFAS system retrofitted into an activated sludge was successfully used to enhance nitrification and denitrification at a shorter hydraulic retention time (HRT) of 6

hours and at a lower temperature, while saving millions of dollars from the projected construction cost of building additional reactor tanks which could have occurred if there was no IFAS retrofitting (Randall and Sen 1996).

From 1996 until now, significant advancements have been made on the biofilm attachment media for IFAS. However, these improvements have not been without setbacks. One of the earlier challenges was the proliferation of red worms observed with the use of rope-type "Ringlace" IFAS media (Jones et al. 1998) (Fig. 1. D). Other occasions of uncontrollable worm blooms with IFAS have been recorded (Onnis-Hayden et al. 2007). These red worms have been reported to negatively impact consistent nitrification performance (Sriwiriyarat and Randall 2005b, Ye et al. 2009). This in turn has encouraged the use of media material made with polyethylene, Poly Vinyl Chloride (PVC) and more especially structured sheet media (SSM) (Fig.1.C.) (Li et al. 2015).
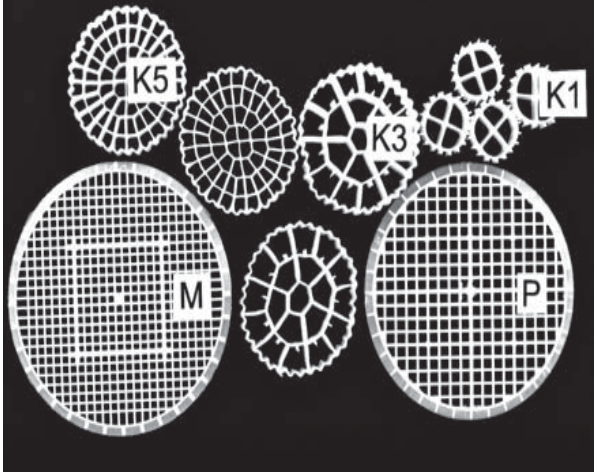
A. 1-5mm in diameter range (AnoxKaldness™ mobile biofilm carriers provided courtesy of Veolia, Inc.) (Metcalf & Eddy et al. 2014)	B. AnoxKaldnes mobile plastic media carriers (courtesy of Veolia) http://www.veoliawatertechnologies.co.uk/ waterandwastewater/municipal/technologie s-solutions/MBBR/
C. AccuFAS structured sheet Fixed-film Distribution and Vertical Flow media (Courtesy of Brentwood Industries) http://www.brentwoodindustries.com/w ater-wastewater-products/accufas/	D. Ringlace media installation in Annapolis (Hubbell et al. 2006)

Figure 1 Different types of IFAS media material

1.4 Overall study goal, objectives, and thesis structure

The overall goal of this study was to comparatively assess the inclusive performance of the BNR-AS and the BNR-IFAS systems under different flow rates, and to gain a better understanding of the microbial community structure that influences nitrification performance of both systems.

This study was divided into two major parts, the first part (detailed in Chapter 3) dealt exclusively with BNR-AS. The specific objectives of the first part are listed below:

➢ To assess N, P and COD removal efficiency in a BNR-AS system and to determine the microbial community changes under different influent flow rates (30L/min, 45L/min and 60L/min).

➢ To monitor the effect of flow rate change on the protein-to-polysaccharide ratio (PN/PS) of the sludge extracellular polymeric substances (EPS).

The second part of this study (detailed in chapter 4) compared BNR-AS and BNR-IFAS side-by-side. However, during the reactor operations, red worms identified as *Aeolosoma hemprichi* bloomed in the BNR-IFAS reactor; this bloom led to the significant deterioration in the BNR-IFAS reactor performance. The objectives achieved in this part of the study are listed below:

➢ To comparatively assess the N, P and COD removal efficiency between BNR-AS and BNR-IFAS under different influent flow rates (30L/min and 45L/min)

➢ To identify effective mitigation strategies to stop the proliferation of red worms without resorting to chlorinating the entire train

CHAPTER 2. LITERATURE REVIEW

2.1 Comparative studies on BNR – IFAS system and BNR-AS system

Comparative studies between BNR-IFAS and BNR-AS show different pros and cons to both systems. A study conducted by Stricker et al. (2009), in Ontario, Canada, compared a floating media IFAS system to a AS system at full-scale. Both reactors were operated in parallel and received equivalent influent loading. It was noted that the IFAS system had 50% more biomass when the attached phase was considered. The findings from this study confirmed that during the winter months, the IFAS system nitrified more consistently and had a higher capacity than the AS system which was at critical solids retention time (SRT). However, in the summer months, this IFAS nitrification was similar to AS. McQuarrie et al. (2004) also confirmed a better overall ammonia removal and a lower effluent concentration of total suspended solids (TSS) in the BNR-IFAS process compared to the BNR-AS. Operationally, the IFAS system requires more air flow to maintain the required dissolved oxygen (D.O.) concentration of >3mg/L (due to the higher MLSS content) compared to the AS system (Stricker et al. 2009, Rosso et al. 2011); this directly increases the operational cost of running an IFAS system. Performance-wise, it has been reported that the BNR-IFAS system is more stable than the BNR-AS in response to changes in HRT, SRT and temperature (Stricker et al. 2009, Onnis-Hayden et al. 2011). There are however, mixed results on sludge settleability between IFAS and AS; Settleability in IFAS is either better (McQuarrie et al. 2004, Li et al. 2015), the same (Sriwiriyarat et al. 2008) or worse (Stricker et al. 2009, Kim et al. 2010) than in AS. Taken together, although there are several advantages and disadvantages to both systems, the BNR-IFAS consistently produces a better effluent quality compared to the BNR-

AS. However, very few studies have comparatively investigated the changes in bacterial community structure in both systems in response to different stressors.

2.2 Nitrification studies on BNR-AS and BNR-IFAS

It is generally agreed upon that nitrification rates are higher in the BNR-IFAS compared to the BNR-AS (Azimi et al. 2007, Kim et al. 2011a, Onnis-Hayden et al. 2011). The only conflicting report to this stance was (Kim et al. 2011b), however, the drawback to this study was that a higher concentration of D.O. was provided to the activated sludge compared to the IFAS. Nitrification rates will continue to increase up to D.O. concentrations of 3-4mg/L (Metcalf & Eddy et al. 2014). Nitrification rates directly correlate with the presence and activity of AOBs and NOBs in a system. Theoretically, the BNR-IFAS should provide better growth conditions for nitrifiers by providing attachment surfaces and increasing MLSS, consequently the research results explored thus far are just as expected. Alkalinity and pH are also important considerations during for nitrification. Autotrophic nitrifiers need an optimum pH range of 7-8 for metabolism and growth (Zhang et al. 2012). A high influent COD concentration has the potential to adversely affect nitrification rates, as per Kim et al. (2011a); this could be because of competition between heterotrophic bacteria, which utilizes organics, and the autotrophic nitrifiers. Other factors that affect nitrification rates include; influent ammonia concentration, carbon-to-nitrogen ratio (C/N), as well as water temperature (Randall and Sen 1996, Onnis-Hayden et al. 2011).

2.3 Biological Phosphorous removal studies in BNR-IFAS vs BNR- AS

Enhanced biological phosphorous removal (EBPR) can be incorporated into the IFAS system by providing the right conditions such as an anaerobic/anoxic contact zone, alkalinity balance, supply of readily biodegradable COD (rbCOD) or volatile fatty acids (VFA), and short aerobic SRT (Metcalf and Eddy 2014). The SRT is important because at excessively long SRTs, endogenous decay begins to occur and the amount of biomass produced and wasted becomes less, thereby reducing the entire phosphorous removal efficiency. An SRT that is slightly higher than what is needed for nitrification produces the best results for EBPR. Metcalf and Eddy (2014) recommend $SRT \geq 2.5$ days for a BNR-AS system that is operating with a water temperature of 20^oC. Adequate D.O. in the upstream of the aerobic tank has been shown to be critical to phosphorous uptake. Rapid phosphorous uptake kinetics has been observed in the first 20 percent of the aerobic reactor volume relative to the subsequent volume of the aerobic tank; without sufficient oxygen in the upstream of the aerobic tank, EBPR suffers regardless of higher D.O. in the remainder of the aerobic tank volume (Narayanan et al. 2006). Sriwiriyarat and Randall (2005a) conducted a pilot plant comparison of EBPR performance between an AS system, an IFAS system with fixed media in the aerobic zone, and an IFAS system with fixed media in the aerobic and anoxic zones. It was observed that the EBPR efficiency was similar between the AS system and the IFAS system with fixed media in the aerobic zone. EBPR efficiency was slightly lesser in the IFAS system with media in both the aerobic zone and the anoxic zone. It was suggested that perhaps this slight decrease in efficiency was caused by the aerobic conditions within the biofilm in the anoxic zone which resulted in phosphorous release (Sriwiriyarat and Randall 2005a).

2.4 Operational parameters of interest that influence plant performance (Influent flow rate, SRT, HRT, temperature, sludge settleability)

In both BNR-AS and BNR-IFAS, the aeration rate goes hand in hand with the D.O. in the suspended phase. The aeration rate and mixing rate in reactors are important, especially for the IFAS media reactors, because they affect the thickness and the density of biofilm growing on the media (Lodhi et al. 2010). As mentioned earlier, the D.O. plays a very critical role in the operation of BNR systems. It is well known that molecular oxygen is the terminal electron acceptor during nitrification. Studies have confirmed that even IFAS systems (which typically have a better nitrification efficiency than the AS) will experience lesser nitrification if the D.O. supplied is not enough (Kim et al. 2011b). Heterotrophic bacteria can tolerate lesser D.O. concentrations than nitrifiers; in fact, substrate oxidization will continue in heterotrophic bacteria until D.O. is less than 0.2mg/L. On the other hand, NOB oxidation of NO_2 becomes inhibited very quickly at low D.O. concentrations. A minimum of 0.7mg/L D.O. is needed to initiate nitrification, however for most AS systems, the recommended DO concentration for the Aerobic tank is 1.5- 2mg/L. A DO concentration of >4.0mg/L may not necessarily improve the AS system performance, instead it will lead to high aeration cost and may potentially encourage the growth of foaming organisms (Metcalf & Eddy et al. 2014). Sludge biomass density is said to correlate positively with the presence of PAOs and EBPR activity (Schuler and Jang 2007). Good sludge density in turn brings about good sludge settleability in the secondary clarifier. However, the presence of filamentous growth decreases the settleability of sludge. Operationally, combating filamentous growth in activated sludge typically involves adjusting D.O. levels, mixing and SRT.

2.5 Bacterial community distribution

Biofilm growth pattern in IFAS systems typically depend on the kind of media being used i.e. whether it is suspended media or fixed media. A comparison of the distribution of biofilm on the fixed IFAS media to the distribution on mobile IFAS media showed that there is thicker biomass on the fixed IFAS media (Lodhi et al. 2010). This may be related to the fact that due to mixing currents and motion of the mobile IFAS media, biomass constantly sloughs from the media into the mixed liquor. The thickness and density of biomass on media is expected to influence the distribution of the bacterial community on both media. A comprehensive examination of the bacterial community in both IFAS and AS using pyrosequencing indicated that the attached phase had distinct bacteria communities that were either fewer or not present in the suspended phase. 37% of the operational taxonomic units (OTUs) present in the attached phase was shared with the suspended phase while only about 17.8% of OTUs in the suspended phase was shared with the attached phase (Kwon et al. 2010). Using qPCR for analysis, the total bacteria genomic copies and the amount of AOB detected in the mixed liquor (ML) of an IFAS plant was similar to what was detected in the ML of an AS plant (van den Akker et al. 2010). However, the gene copies of AOB and NOB are higher in the attached biofilm compared to the mixed liquor (Kim et al. 2011, Van den Akker et al. 2010). Per (Onnis-Hayden et al. 2011), PAOs and enhanced biological phosphorous removal (EBPR) activity were more readily found in the mixed liquor than in the biofilm, while nitrifiers and nitrification activity were higher in the biofilm. Thus, the N-removing and the P-removing bacteria (which typically had conflicting SRTs) could be decoupled to allow for an overall higher efficiency in N and P removal. For IFAS systems that had media in their aerobic zones, the PAOs preferred the mixed liquor because they need to alternate between aerobic

and anoxic/anaerobic zones to efficiently incorporate phosphorous into their biomass (Metcalf and Eddy, 2014).

2.6 Overview of Extracellular Polymeric Substances (EPS)

Another important aspect of bacteria behavior is the secretion of extracellular polymeric substances (EPS). These secretions are a complex of high molecular weight polymers combined with hydrolysis products from cells and macromolecules, and some waste water organic matter; EPS comprises of carbohydrates, proteins, and humic substances, amongst others (Sheng et al. 2010).

EPS is inextricably linked to the functions of microbial aggregates. Sheng et al. (2010) elucidated functions such as mass transfer, surface charge, flocculation, and settleability, among others. For instance, the sludge volume index (SVI, a measure of settleability) of a floc is said to increase as the EPS content increases (Liao et al. 2001); more specifically the protein content of EPS has a positive relationship with SVI (Sheng et al. 2010).

The production of EPS is influenced by different factors in a system, such as the nutrient conditions, growth phase, and external conditions. Janga et al. (2007) found that EPS concentration increased as the food to microorganism ratio (F/M) increased in the MBR process. The SRT of a system is agreed to influence EPS production, however, the findings from the literature are inconsistent as to whether this influence is positive, negative or even neutral (Liao et al. 2001, Sesay et al. 2006, Li and Yang 2007). Liao et al. (2001) monitored the EPS as the SRT increased

in a sequencing batch reactor SBR, and Sesay et al. (2006) monitored replicate "semi-continuous" reactors set at specified SRTs. Both studies concluded a positive correlation between SRT and protein to carbohydrate ratio, however, Li and Yang (2007) concluded that there was no correlation between SRT and tightly bound EPS (TB-EPS). The study described in this report investigated the effect of a decrease in SRT and a corresponding decrease in HRT on the protein to carbohydrate ratio in a BNR process.

2.7 Overview of red worms in wastewater treatment plants

Wastewater treatment plants (WWTPs) are typically rich in organics as well as bacteria, thus they provide a very favorable environment for metazoan organisms such as *Annelida* and *Rotifera*. (Ratsak and Verkuijlen 2006, Elissen et al. 2008). It is unclear how *Annelida* ends up in WWTPs, however, shear condition was identified as an important factor controlling the proliferation of red worms (Menniti and Morgenroth 2010). Contrarily, Wang et al. (2011) has reported that increased aeration will decrease floc size thereby supplying more food for the worms. There are several classes of Annelida in WWTPs, the most common two are *Aphanoneura* (which includes *Aeolosomatidae*) and *Oligochaeta* (which consist of *Tubificidae* [including the *Naidinae*]) (Elissen et al. 2008, Navaratna et al. 2014). Although they can attach to surfaces as well, *Aeolosomatidae* and *Naidinae* mostly occur as free-swimmers in the activated sludge (Elissen et al. 2008), while *Tubificidae* and other WWTP *Oligochaeta* are mainly "sessile" – needing attachment surfaces such as media material to grow on (Elissen et al. 2008). For clarification purposes, most literatures have classified *Aeolosoma hemprichi* as *Oligocheata* (Liang et al. 2006a, Ratsak and Verkuijlen 2006).

A study of 4 WWTPs in the Netherlands targeted the most abundant free-swimming Oligochetes in that region (*Nais spp., Aeolosoma hemprichi, Pristina aequiseta, Aeolosoma variegatum, Chaetogaster diastrophus, and Aeolosoma tenebrarum.)* (Elissen et al. 2008). It was reported that the worms were present throughout the year (even in winter) with an average yearly population peak ranging from 2-3 months for each worm. The doubling time during the peak periods were 2-6 days. During the peak period for a particular worm specie, the worms grew faster than normal and also multiplied faster relative to other worms due to stable and excellent spatial temporal and environmental conditions. (Elissen et al. 2008).

2.7.1 Effects of red worms on plant performance

Most of the literature reporting on aquatic worms in wastewater treatment plants has focused on the cultivation and use of aquatic worms for sludge reduction in activated sludge systems (Wei and Liu 2005, Liang et al. 2006a, 2006b, Song and Chen 2009b). Impacts of worm predation on wastewater bioreactor performance showed that sludge settleability and SVI correlates can be significantly affected by red worms, especially at high densities (Wei et al. 2003, Liang et al. 2006a, Wang et al. 2011). Menniti and Morgenroth (2010) reported that *A. hemprichi* proliferated in an MBR under lower shear conditions after 37 days of operation. Within 5 – 7 days of proliferation, *A. hemprichi* altered the floc composition by essentially eliminating the filamentous bacteria population. Filamentous bacteria are typically implicated in sludge bulking (Graham and Smith 2004). Furthermore the presence of *A. hemprichi* encouraged an increase in smaller protozoa and metazoa (Menniti and Morgenroth 2010).

2.7.2 Effects of red worms on nutrient removal

The reported literature on the effect of red worms' proliferation on nutrient removal varies considerably from one author to the next. There is no generally accepted conclusion as to whether red worms are detrimental to nutrient removal or not. This section will explore different studies and their stance on the subject matter, discuss a possible link between red worms and nitrifying bacteria, and thirdly, examine three key studies where poor nutrient removal (especially nitrification) has been reported in relation to the proliferation of red worms.

2.7.2.1 Studies showing the effect of red worms on COD and phosphorous removal

Some studies reported that the presence of aquatic worms (*Aeolosoma hemprichi* and *Tubificidae*) had no effect on COD removal; however, the total phosphorous removal efficiency was decreased (Wang et al. 2011) or stable (Liang et al. 2006a). Wei et al. (2003) reported that nitrification was unaffected during worm blooms and the COD and PO_4^3-P removals were by the reactor types and dominant worm species (i.e., *Nais or Aeolosoma*).

2.7.2.2 Exploring the link between red worms and nitrifying bacteria

To the best of my knowledge, no study has unequivocally stated and demonstrated that red worms such as *Aeolosoma hemprichi* particularly feeds on nitrifying bacteria; however, selective feeding of aquatic worms on bacteria has been well reviewed by Ratsak and Verkuijlen (2006). The

selective feeding strategy creates mutualism and co-existence which in turn ensures the survival of a worm species. A food selectivity test was conducted for *Aeolosoma hemprichi,* using monoxenic culture, and the report indicated a strong preference towards gram-negative bacteria (Inamori et al. 1990, Ratsak and Verkuijlen 2006). Nitrifying bacteria (Nitrosomonas, Nitrobacter etc.) are mostly gram-negative bacteria. Although no monoxenic experiments have been conducted on *A hemprichi* and nitrifying bacteria specifically, we infer that *A hemprichi* feeds on nitrifying bacteria as the detrimental effects of red worm predation on nitrification in IFAS systems has been observed in lots of recent studies including this current study.

2.7.2.3 Three key studies that report poor nitrification related to red worms' predation

Most publications reported the detrimental effects of red worms on nitrification in the IFAS system (Jones et al. 1998, Sriwiriyarat and Randall 2005b, Hubbell et al. 2006, Jackson et al. 2007, Sen et al. 2007). A select few will be highlighted in this review.

In a full-scale system at the Waterdown Sewage Treatment Plant in Ontario Canada, the nitrification performance of an IFAS system (using the "Ringlace and Biomatrix looped chord" media) was reduced due to red worm blooms (Jones et al. 1998). In the same study, they have made several attempts to eliminate the worms, including chlorinating the RAS, turning off the aeration, and stopping the feed for a period. Turning off aeration for 48 hours was most effective strategy for red worm reduction (Jones et al. 1998).

In the Evaluation of Two different configurations of IFAS bioreactors set up alongside a control CAS, Sriwiriyarat and Randall (2005b) observed a lesser nitrification in one of the IFAS bioreactors, this lesser nitrification was attributed to "a large population" of red worms on the media installed in the IFAS bioreactor Sriwiriyarat and Randall (2005b).

Thirdly, in another full-scale demonstration project at the Mamaroneck WWTP, a MLE configuration IFAS system (using "flexible strand media") was operated in parallel with another MLE configuration CAS as control (Psaltakis et al. 2003). Compared to the CAS control, the nitrification in the IFAS system was not consistent. Red worm infestation was attributed to this poor nitrification (Psaltakis et al. 2003).(Hubbell et al. 2006) also reports several instances of red worms blooms that occurred after a dramatic change in influent loading and the chlorination and anoxic measures taken to contain the worms.

2.7.3 Physical factors that influence oligochaete growth in reactors

The reports on the effect of physical factors on aquatic worms have been shown to vary. Kuniyasu et al. (1997) reported that the optimum pH in which *A hemprichi* thrives is 30^0C while it's optimum growth pH ranges from 6-8. Most conventional water treatment systems operate at a pH of 7-8. Elissen et al. (2008) monitored oligochaetes in 4 wastewater treatment plants and could not establish any relationship between the temperature and the presence of the worms. Liang et al. (2006a) demonstrated that F/M had significant impact on *A hemprichi* proliferation. A F/M less than 0.7 mg-COD/ (mg-VSS day) was necessary to allow *A hemprichi* to proliferate to densities higher than 20 ind/mL. In a comparative study, Wei et al. (2003) demonstrated nine tested operational parameters (i.e., TSS, HRT, SRT, F/M, recycle ratio, temperature, pH, and DO) all

affected worm. Depending on the reactor and the worms considered, different parameters influenced worm growth while the rest did not. For instance, the growth of *Aeolosoma* in the MBR was affected by HRT, recycle ratio, temperature, TSS, and pH while the remaining four factors had no effect.

2.7.4 Controlling red worm blooms

There is currently no consensus in the literature regarding how to control the bloom of aquatic worms in WWTPs, in fact they are still deemed as uncontrollable (Wei et al. 2003, Elissen et al. 2008, Hendrickx et al. 2009). According to (Wei et al. 2003), the potential control measures vary depending on the species of worms that are in the system, as well as the kind of bioreactor being used. SRT has been identified as an effective red worm control factor(Liang et al. 2006a, Song and Chen 2009a). Other studies reported that SRT did not affect worm growth in a CAS reactor (Wei et al. 2003). Other attempts to control the worm blooms included cutting off the influent, chlorinating the RAS and cutting off oxygen in order to render the aerobic tank anaerobic (Jones et al. 1998, Hubbell et al. 2006). It was concluded that shutting off the D.O. was the most effective control mechanism (Jones et al. 1998)

CHAPTER 3. EFFECT OF FLOW RATE INCREASE ON TWIN PILOT-SCALE BNR-AS REACTORS

This chapter marks the first part of the current study, the influent flow rate of a twin pilot-scale BNR reactor (Fig. 2) was increased by 50% and then further increased by 100% (doubled). The performance and the bacteria community structure was monitored for the twin reactors over a period of 265 days. Ammonia, nitrate, orthophosphate phosphorus, and dissolved chemical oxygen demand (COD) were monitored in the influent and effluent of both bioreactors. Biomass concentration and sludge settleability in terms of SVI were also measured. qPCR was used as a molecular tool to investigate the relative abundance of target AOB, NOB, DEN and Total bacteria genes.

Figure 2 Image of Twin pilot-scale plant at EPCOR's Goldbar Wastewater Treatment Plant Edmonton, Alberta, Canada.

3.1 Materials and methods

3.1.1 Reactor Setup

The EPCOR's Gold Bar Wastewater Treatment Plant houses twin pilot-scale reactors (see Fig. 2.). The process configuration of both reactors is an adapted version of the Westbank EBPR process with an anoxic, anaerobic, and a pre-anoxic zone included before the aerobic zone (see Fig. 3). Both reactor trains have identical dimensions (length × width × height = 7m × 0.98m × 3.07m), with an effective volume of $19m^3$ each. The aerobic zone holds approximately $13.5m^3$ while the remaining volume is contained in the anoxic, anaerobic, and a pre-anoxic zone. The pre-anoxic zone holds almost double the volume of the anoxic and anaerobic zones each. The operational temperature for both reactors ranged from 23 °C during the spring/summer months (days 1 to 175) to 14.5 °C during the fall months (days 175 to 265).
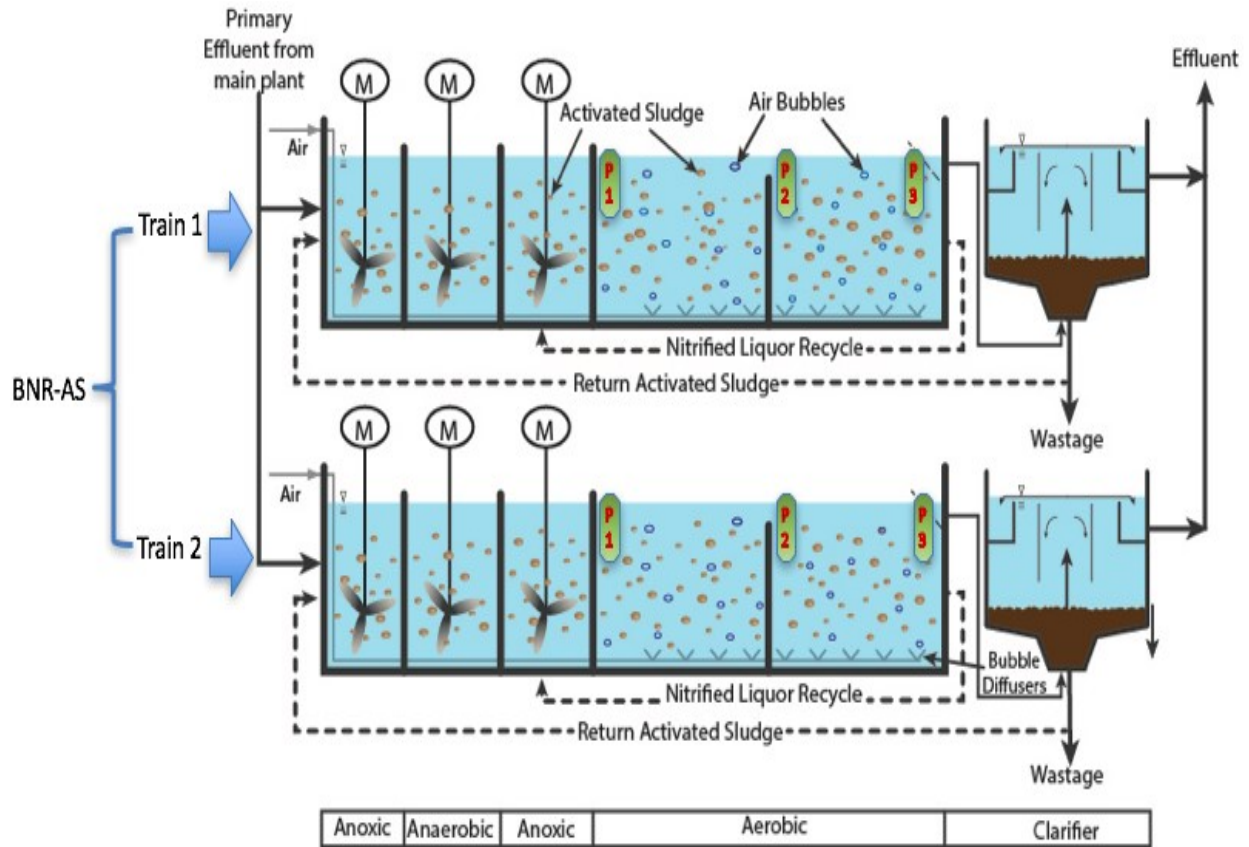
Figure 3 A schematic diagram of the BNR activated sludge process (BNR-AS) showing Train (reactor) 1 above and Train (reactor) 2 below.

The operational conditions and parameters for both trains were the exact same. Both reactors were operated as BNR activated sludge systems at three different influent flow rates; 30L/min, 45L/min and 60L/min which corresponded to Phase I, Phase II and Phase III respectively (see Table 1). During Phase 1 the HRT was 10.5 hours and the average SRT was calculated to be 9.33 ± 1.4 days for each train. Phase 2 HRT was calculated to be 7.0 hours and the average SRT was 5.76 ± 0.16 days. Phase 3 HRT was calculated to be 5.3 hours and the average SRT was calculated to be 2.2 ± 0.1 days. DO was maintained in the aerobic zone of both reactors at 2ppm using three "online" DO probes P1, P2 and P3 (Fig 3) to monitor DO levels. Data from the "online" DO probes was

automatically fed back into the delta V automation control system which controlled a pneumatic actuator that opened and closed air supply lines to the aerobic diffusers. The return activated sludge (RAS) was maintained for both reactors at approximately 80% of the influent flow rate, this was determined based on a simple mass balance calculation around the secondary clarifier boundary considering the mixed liquor volatile suspended solids (MLVSS) of the aerobic zone, mixed liquor, and the MLVSS of the waste activated sludge (WAS), and the loading rate of the system (Metcalf and Eddy 2006). The nitrified liquor recycle was maintained at 2.5 x the influent flow rate. The primary effluent (PE) from the Goldbar wastewater treatment plant was used as influent for both reactors.

Table 1 Parameter settings for CAS Trains (reactors) 1 and 2

Parameter settings for Train 1 and 2	Phase 1	Phase 2	Phase 3
Influent Flow rate (L/min)	30	45	60
RAS Flow rate (L/min)	24.5	40	35
Nitrified liquor recycle (NLR) from Aerobic to 2nd anoxic zone (L/min)	78	95 - 100	95 -100*
Average WAS Flow rate$^{\$}$	2mins of wasting per 90mins cycle	2mins per 90mins cycle	2mins per 90mins cycle.
D.O. probe values(ppm)	Set point at 2.0, 2.0 and 1.5	Set point at 2.0, 2.0 and 1.5	Set point at 2.0, 2.0 and 1.5

* At the time of the experiments the available pump was at maximum capacity and could be operated to exceed this flow rate. $ The WAS flow rate was constantly adjusted to maintain a target mixed liquor concentration of 2000L/mi

3.1.2 Reactor performance

Influent, effluent and occasionally in-train process water was sampled and filtered with a 0.45 μm pore size syringe filter. Afterwards, dissolved COD, ammonia, nitrate, and orthophosphate phosphorus were measured using commercially available test kits from Hach company USA; TNT 821 (method 8000), TNT 830 (method 10205), TNT835 (method 10020), and TNT 844 (method 10209) respectively. Furthermore, the sludge volume index (SVI) was determined as the volume in millilitres occupied by 1 gram of solids from the aerobic zone mixed liquor after 30 minutes of settling in a 1L graduated cylinder (Bridgewater and Rice 2012). The mixed liquor suspended solids (MLSS), and MLVSS were also measured per standard methods (Bridgewater and Rice 2012).

3.1.3 Microbial community analysis

3.1.3.1 qPCR analysis

qPCR was performed to examine the changes in bacteria population in each reactor as the influent flow rate increased. Specifically, AOB (represented by amoA gene), NOB (represented by the 16S rDNA of Nitrospira spp. and Nitrobacter spp.), DEN (represented by nirK gene), and total bacteria populations were targeted. Prior to the qPCR procedure, DNA was isolated from composite samples using MO BIO PowerSoil® DNA Isolation Kits (MoBio Laboratories Inc. Carlsbad, California). qPCR assays were performed using a CFX96TM Real-Time Detection System (Bio-RAD, California, USA) per Kim et al. (2011). Primers for qPCR are listed in Table 2.

Table 2 Primers used in *q*-PCR (Huang et al. 2015)

Target	Primer	Sequence (5'-3')	Reference
Bacterial 16S rDNA	*341f*	5'-CCTACGGGAGGCAGCAG-3'	(Muyzer et al. 1993)
	907r	5'-CCGTCAATTCCTTTRAGTTT-3'	(Muyzer et al. 1993)
amoA gene	*amoA-1F*	5'-GGGGTTTCTACTGGTGGT-3'	(McTavish et al. 1993)
	amoA-2F	5'-CCCCTCKGSAAAGCCTTCTTC-3'	(McTavish et al. 1993)
Nitrospira spp. 16S rDNA	*NSR 1113f*	5'-CCTGCTTTCAGTTGCTACCG-3'	(Dionisi et al. 2002)
	NSR 1264r	5'-GTTTGCAGCGCTTTGTACCG-3'	(Dionisi et al. 2002)
Nitrobacter spp. 16S rDNA	*Nitro 1198f*	5'-ACCCCTAGCAAATCTCAAAAAACCG-3'	(Graham et al. 2007)
	Nitro 1423r	5'-CTTCACCCCAGTCGCTGACC-3'	(Graham et al. 2007)
nirK gene	*nirK 876*	5'-ATYGGCGGVCAYGGCGA-3'	(Henry et al. 2004)
	nirK 1040	5'-GCCTCGATCAGRTTRTGGTT-3'	(Henry et al. 2004)

3.1.3.2 EPS analysis

Composite samples representing all the zones (anoxic, anaerobic, pre-anoxic and aerobic) in each reactor were designated for EPS extraction. A variation of the formaldehyde–NaOH method (Liu and Fang 2002) was used for EPS extraction. 10 mL of sludge from the composite samples were added together with 0.06 mL of 36.5% formaldehyde to 15 mL polyethylene centrifuge tubes.

The tubes were inverted several times to ensure adequate mixture of their contents, and cooled at 4 °C for 1 hour. After 1 hour, 4 ml of 1 N NaOH was added to each tube, with inversion and storage for 4 hours at 4 °C. Afterward, the tubes were centrifuged at 6000g for 20 minutes and the supernatant was filtered through a 0.22 μm pore filter syringe and the filtrate was dialysed using a membrane with a molecular weight cut-off (MWCO) of 3.5KD. Phenol-sulfuric acid method (DUBOIS et al. 1956) was used in determining the carbohydrate content while the protein content of the EPS was measured using the Bradford method (Bradford 1976).

3.1.4 Statistical analysis

One-way ANOVA analysis was conducted to determine if there was a statistically significant difference in the comparison of the performance of both reactors, with p values greater than 0.05 indicating no statistical significance.

3.2 Results and Discussion

3.2.1 Effect of flow rate change on biomass concentration and settling

As shown in Fig. 4, the average MLSS concentrations were 2138 ± 913 mg/L and 1864 ± 850 mg/L in reactor 1 and reactor 2 respectively (p=0.44 for the comparison between the two reactors) in Phase I (influent flow rate was 30 L/min and lasted for 174 d). In Phase II, the influent flow rate was 45 L/min and lasted for 57 days; the MLSS concentration was 1722 ± 386 mg/L and 1662 ± 538 mg/L in reactor 1 and reactor 2 respectively (p=0.96 for the comparison between the two reactors). In Phase III, influent flow rate was 60 L/min and lasted for 36 days; the MLSS concentration was 934 ± 144 mg/L and 952 ± 438 mg/L in reactor 1 and reactor 2 respectively (p=0.98 for the comparison between the two reactors). From Phase I to Phase II, the sudden increase in influent flow rate from 30 L/min to 45 L/min caused a slight decrease of 11-20% in biomass concentration for both reactors (p=0.11 for reactor 1 and p=0.46 for reactor 2, for the comparison between Phase I and Phase II). However, when influent flow rate increased further from 45L/min to 60L/min as from Phase II to Phase III, both reactors experienced a significant decrease in biomass concentration by 43-45% (p=0.004 for reactor 1 and p=0.0001 for reactor 2, for the comparison between Phase II and Phase III)
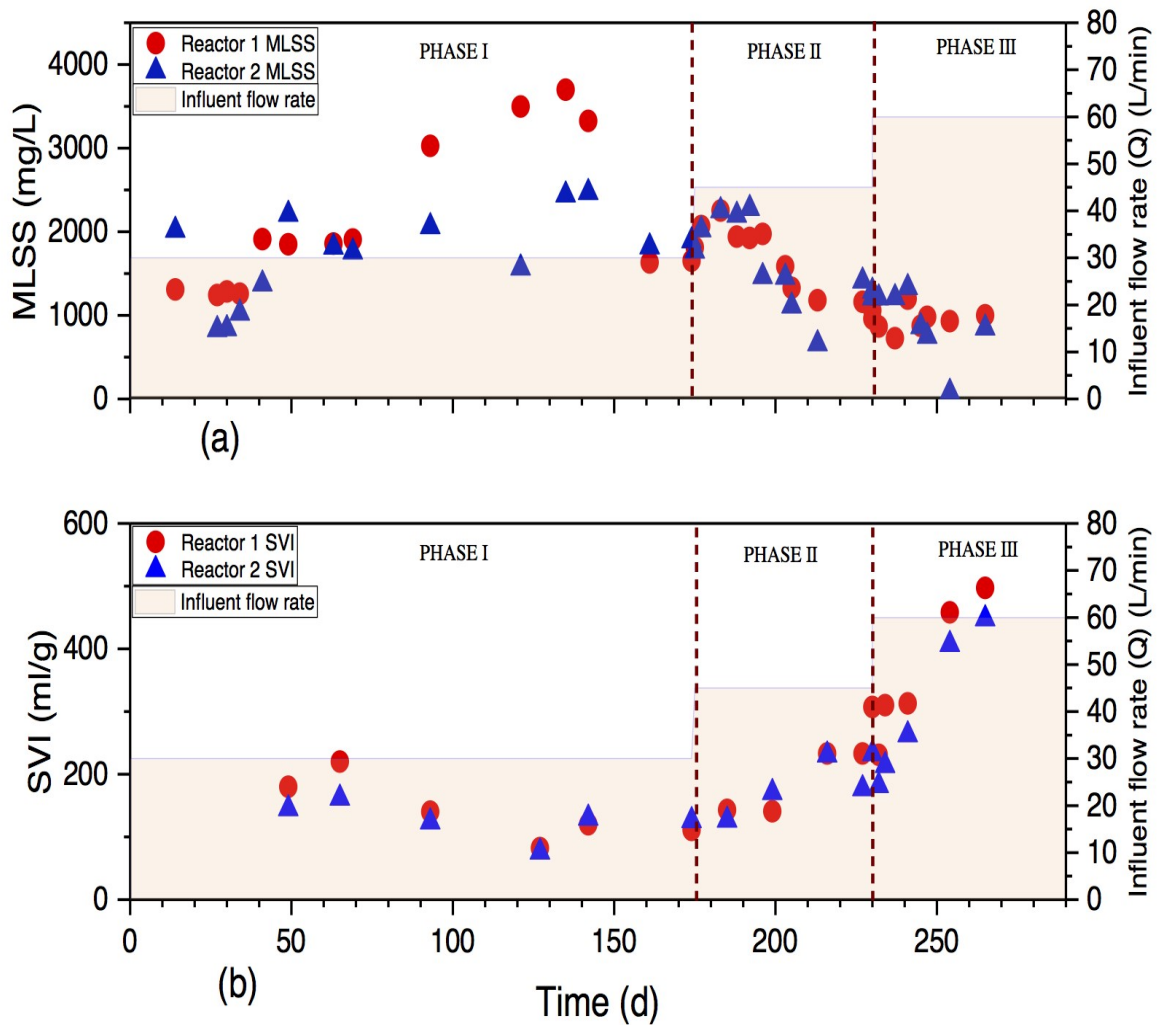
Figure 4 MLSS concentrations (a) and corresponding SVI values (b) of both reactors 1(●) and

reactor 2 (▲)

The increase in the influent flow rate caused the proportional decrease in HRT, and the decreased

HRT led to the washout of biomass arising from the increased hydraulic pressure (Pan et al. 2004).

This phenomenon was similar to the previous research that biomass concentration in the bioreactor

depends on the HRT (Xu et al. 2014). The rate of MLSS decrease when influent flow rate increased

from 30 L/min to 45 L/min was relatively lower than that from 45 L/min to 60 L/min. The sludge started to suffer with slight sludge bulking from the late Phase II (SVI=211 ± 70 in reactor 1 and SVI = 187 ± 45 in reactor 2), and worse sludge bulking in Phase III (SVI = 270 ± 56 in reactor 1 and SVI = 198 ± 23 in reactor 2), as high SVI (>150) indicates a poor sludge settlement. The increased influent flow rate lead to adverse effects on activated sludge. The average MLVSS/MLSS ratios in Phase I, Phase II and Phase III were relatively consistent, which were 0.84 ± 0.05 and 0.82 ± 0.07, 0.84 ± 0.03 and 0.83 ± 0.04, and 0.82 ± 0.05 and 0.84 ± 0.05 for reactor 1 and reactor 2 respectively. This indicates the influent flow rate change did not cause significant change in biomass mineralization and biological activity (Ouyang and Liu 2009).

3.2.2 Effect of flow rate change on COD removal

Given the influent COD concentrations of 141 ± 26, 157 ± 15 and 178 ± 19 mg/L in Phase I, II, and III respectively, the corresponding influent organic loading rate was increased significantly from Phase I to Phase III (6.1 ± 1.1, 10.1 ± 1.1, and 14.9 ± 1.1 kg/d), the effluent COD concentrations were 32 ± 5 mg/L and 32 ± 6 mg/L for reactor 1 and reactor 2 in Phase I; 35 ± 6 mg/L and 36 ± 4 mg/L for reactor 1 and reactor 2 in Phase II; and 38 ± 4 mg/L and 46 ± 21 mg/L for reactor 1 and reactor 2 in Phase III, respectively (Fig. 5), with corresponding COD removal efficiencies of 77 ± 5% and 77 ± 5% in Phase I, 78 ± 4% and 77 ± 3% in Phase II, and 78 ± 4% and 75 ± 10% in Phase III respectively. The statistical analysis showed throughout the whole operation period, reactor 1 and reactor 2 have a similar COD removal performance (p=0.95, 0.63 and 0.33 in Phase I, II and III respectively). There was no significant difference in COD removal

efficiency between the operation at influent flow rate of 30 L/min and 45 L/min (p = 0.08 in reactor 1) and influent flow rate of 45 L/min and 60 L/min (p = 0.09 in reactor 1). It seems that the COD removal efficiency was stable although the influent flow rate was increased, which was also observed in our previous studies (Sheng et al. 2016).
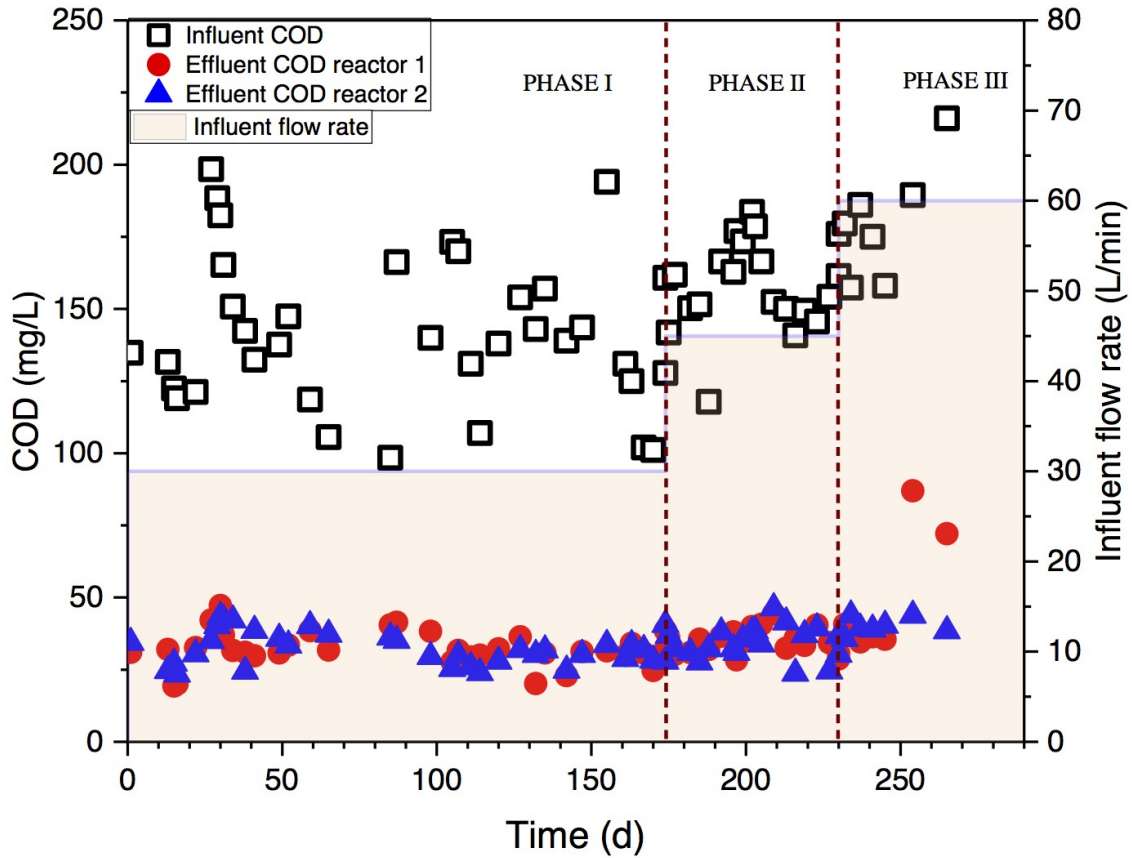
Figure 5 Influent (□) and effluent COD concentrations for Reactor 1(●) and Reactor 2 (▲)

3.2.3 Effect of flow rate change on ammonia removal

At the influent NH_4^+-N concentration of 38.5 ± 8.4, 38.7 ± 6.0, and 40.0 ± 3.3 mg/L (See Fig. 6), and corresponding influent NH_4^+-N loading rate of 1.7 ± 0.4, 2.5 ± 0.4, and 3.5 ± 0.3 kg/d in Phase I, II, and III, the average effluent NH_4^+-N concentrations in Phase I, Phase II, and Phase III were 1.6 ± 2.8 and 0.7 ± 1.3 mg/L, 4.6 ± 6.1 and 7.1 ± 8.4 mg/L, and 30.1 ± 6.2 and 28.9 ± 5.8 mg/L for reactor 1 and reactor 2, respectively (Fig. 6). The effluent NH_4^+-N concentration was similar between the two reactors throughout the three phases (p=0.07 in Phase I, p=0.28 in Phase II, and p=0.65 in Phase III). However, the effluent NH_4^+-N concentration was significantly lower in Phase I than that in Phase II (p=0.048 and <0.001 for reactors 1 and 2 respectively), and further lower in Phase II than Phase III (p <0.001 for both reactors). The average NH_4^+-N removal efficiencies in reactor 1 and reactor 2 were $96 \pm 7\%$ and $98 \pm 3\%$, $88 \pm 16\%$ and $82 \pm 21\%$, and $25 \pm 12\%$ and $28 \pm 11\%$ in Phase I, II, and III respectively. The increased hydraulic pressure due to the increased flow rate could cause washout of bacteria especially slow-growing bacteria such as AOB and NOB. The decrease in NH_4^+-N removal efficiency from Phase II to Phase III was 66-71%, which was much higher than that from Phase I to Phase II (8-17%). This could be also attributed to the serious sludge bulking situation in Phase III in both reactors, which could cause a significant sludge loss from aerobic zone including the AOB population. The effluent NH_4^+-N concentrations in both reactors did not meet the discharge limit (3 mg/L) after influent flow rate increased to 45 and 60 L/min.

Since most of the NH_4^+-N was oxidized to NO_3^-N via nitrification, the NO_3^-N production in the effluent was also monitored. The effluent NO_3^--N concentrations in Phase I, II and III were 5.0 ± 2.4 and 6.7 ± 2.4 mg/L, 3.9 ± 2.5 and 3.0 ± 2.6 mg/L, and 0.3 ± 0.1 and 0.3 ± 0.1 mg/L of reactor 1 and reactor 2, respectively (See Fig. 6). Except for Phase I, where there was a significant difference in effluent NO_3^--N concentration between reactor 1 and reactor 2 (p=0.005), the performance of both reactors was similar during the subsequent operational periods (p=0.25 in Phase II and p=0.98 in Phase III). Moreover, the effluent NO_3^--N concentrations in reactor 1 did not experience a significant change from Phase I to Phase II (p=0.2) whereas significantly reduced from Phase II to Phase III (p<0.001). Meanwhile, the effluent NO_3^--N concentrations in reactor 2 reduced significantly from Phase I to Phase II (p < 0.001) and from Phase II to Phase III (p = 0.07). The decrease in effluent NO_3^--N concentration was consistent to the increase in effluent NH_4^+-N due to the adverse effect caused by increased influent flow rate.
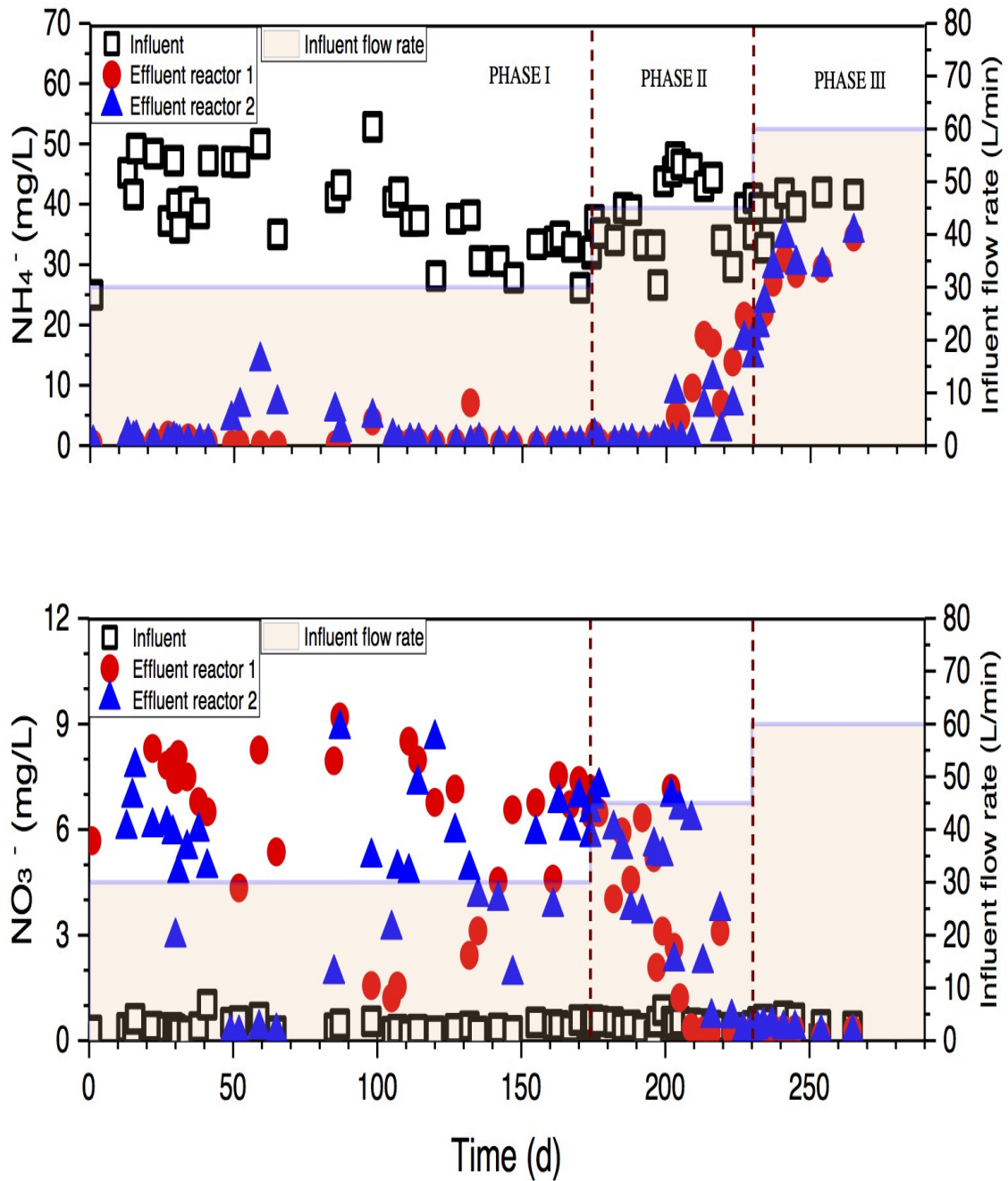
Figure 6 Influent ($\square$) and effluent concentrations of NH_3-N and NO_3-N for Reactor 1($\bullet$) and

Reactor 2 ($\blacktriangle$)

3.2.4 Influence of flow rate change on nitrifying bacteria community structure

The change in NH_4^+-N removal efficiency with influent flow rate was also indicated by the change in nitrifying bacterial population. The qPCR analysis specifically targeting AOB and NOB indicated that the AOB genera primarily consisted of *Nitrosomonas* spp. (targeted by amoA gene) and the NOB genera contained both *Nitrospira* and *Nitrobacter* in the activated sludge process. The figures 7 and 8 represent the relative abundance of each bacterial species. We found that among NOB population, *Nitrobacter* was the dominant NOB (15.1% in Phase I, 20.5% in Phase II and 8.8% in Phase III of reactor 1; 19.9% in Phase I, 17.2% in Phase II and 3.7% in Phase III of reactor 2) in both reactors throughout the whole operation period.
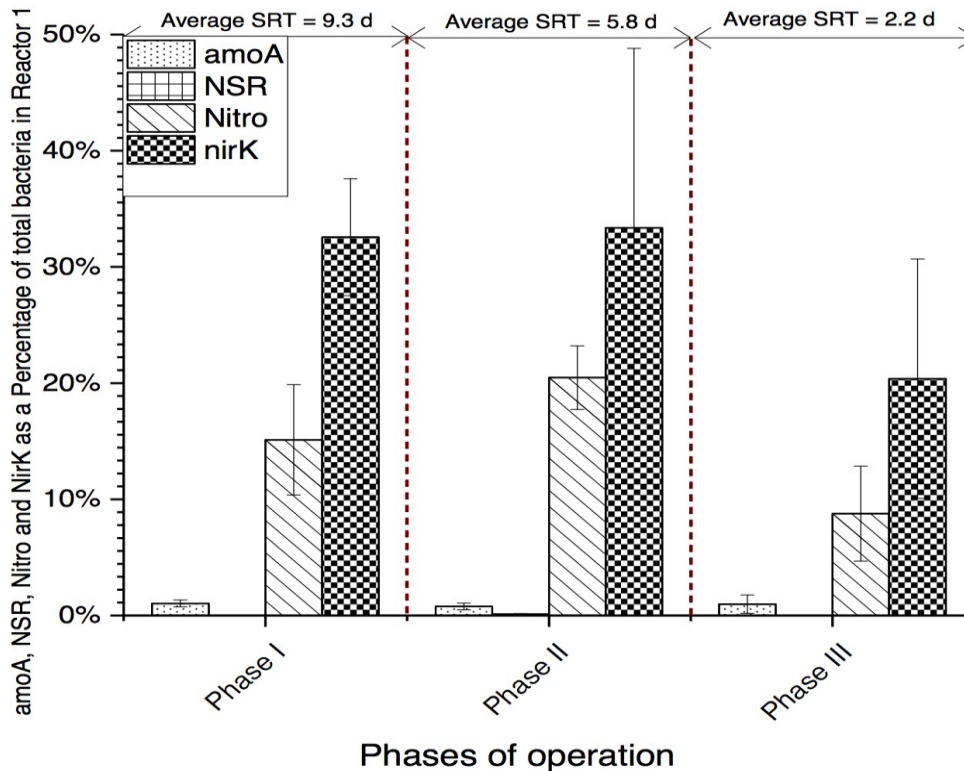
Figure 7 Relative abundance of amoA, NSR, Nitro and NirK in reactor 1 during the three operational phases
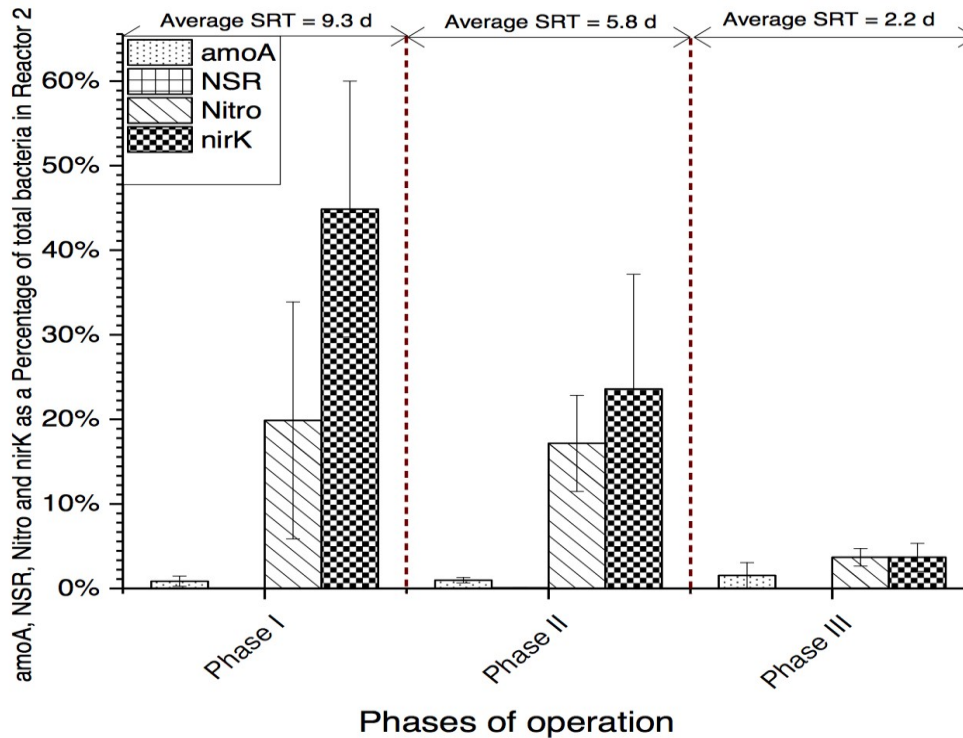
Figure 8 Relative abundance of amoA, NSR, Nitro and NirK in reactor 2 during the three

operational phases

The average of gene copies found in both reactor 1 and reactor 2 combined is shown in Figure 9. The lower copy numbers of genes found in Phase 2 sample 3 (S3) and Phase 2 S4 are representative of periods of severe sludge bulking and subsequent sludge washout. Both *Nitrosomonas* (AOB) and *Nitrobacter* (NOB) at higher influent flow rate appeared to exhibit lower population abundance than those at lower influent flow rate, which could be explained by the above-mentioned washout theory at high hydraulic pressure. Relative to AOBs and NOBs detected, denitrifiers were dominant. Through the detection of denitrifiers (targeting *nir*k gene), we found the population of denitrifiers (heterotrophs) could also be washed out significantly, which could

lead to a worse process performance. With an increased flow rate, increased influent nutrient loading, and a decreased biomass concentration, a higher food to microorganism ratio (F/M) was reached. The SRT decreased from 9.3 days to 2.2 days when the influent increased, and microorganisms that grow relatively faster were selectively retained in the bioreactor. Thus, the sludge age became younger and the SVI became higher. On the other hand, higher sludge concentration at lower influent flow rate in activated sludge operation could provide better retention of slowly growing bacteria (Holakoo et al. 2007) and those bacteria especially nitrifiers could form healthy sludge flocs and clusters by close contact (Ni et al. 2008).
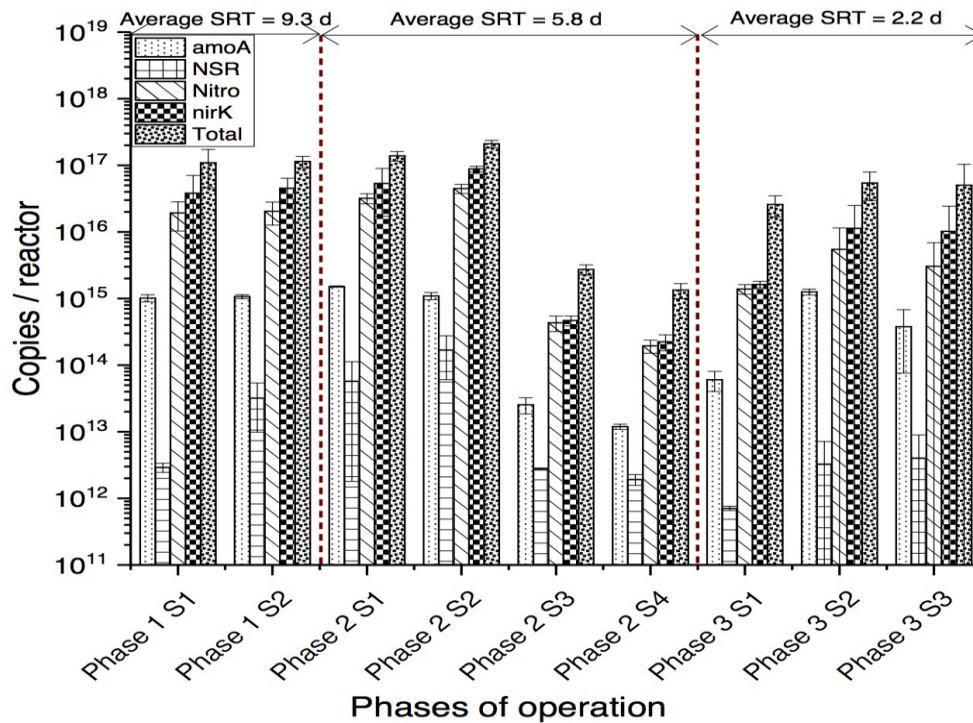
Figure 9 Gene copies per bioreactor of the AOB(amoA), Nitrospira(NSR), Nitrobacter (Nitro) denitrifying bacteria (nirK) and the total bacteria in flocs sampled during Phase 1, Phase 2 and Phase 3.

3.2.5 Effect of flow rate change on Phosphorous removal

The effluent PO_4^{3-}-P and PO_4^{3-}-P removal results showed that there was significant difference in P removal efficiency at different HRTs. At the influent PO_4^{3-}-P concentration of 5.0 ± 1.2, 4.3 ± 0.6, and 4.9 ± 1.1 mg/L in Phase I, II, and III respectively, with corresponding PO_4^{3-}-P loading rate of 0.2 ± 0.1, 0.3 ± 0.0, and 0.4 ± 0.1 kg/d, the effluent PO_4^{3-}-P concentrations in reactor 1 and reactor 2 were 2.0 ± 1.6 and 2.1 ± 1.3 mg/L, 1.0 ± 0.8 and 1.1 ± 0.9 mg/L, and 2.4 ± 1.1 and 1.9 ± 0.7 mg/L in Phase I, II, and III, respectively (see Figure 10). Both reactors performed a similar PO_4^{3-}-P removal efficiency throughout the operation periods (p=0.85 in Phase I, p=0.41 in Phase II, and p=0.34 in Phase III). The average PO_4^{3-}-P removal efficiencies in reactor 1 and reactor 2 were $63 \pm 26\%$ and $60 \pm 21\%$, $76 \pm 18\%$ and $74 \pm 20\%$, and $51 \pm 15\%$ and $60 \pm 14\%$ in Phase I, II, and III respectively. Although the influent PO_4^{3-}-P loading rate was increased from Phase I to Phase II and to Phase III, the average PO_4^{3-}-P removal efficiencies were not correspondingly increased or decreased. PO_4^{3-}-P removal efficiencies largely depended on the PAO growth, therefore in this research the reduced HRT could not provide sufficient VFAs for PAO growth, which was supposed to result in lower PO_4^{3-}-P removal efficiencies. However, PO_4^{3-}-P removal could also occur through adsorption and precipitation with sludge (HU et al. 2014), so the sludge loss in Phase II and III could actually improve PO_4^{3-}-P removal efficiencies.
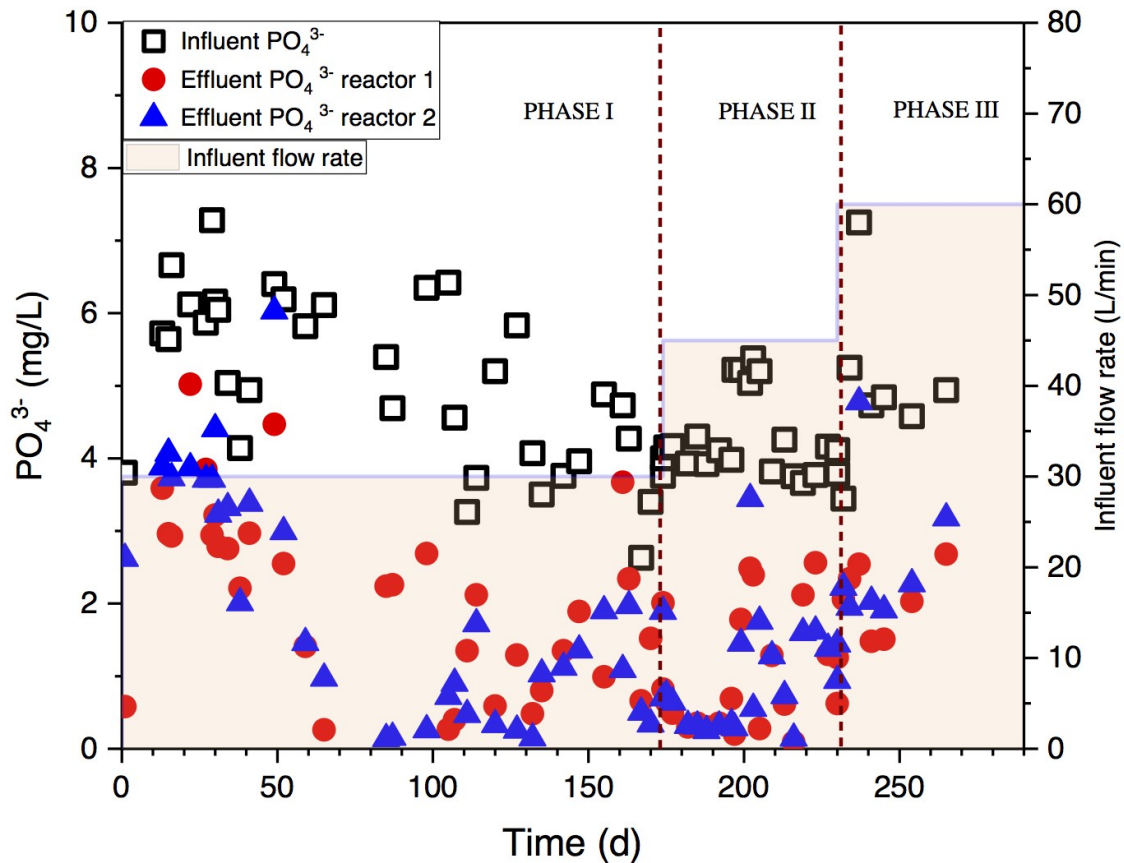
Figure 10 Influent (▢) and effluent PO$_4$-P concentrations for reactor 1(●) and reactor 2 (▲)

3.2.6 Effect of flow rate change on EPS

Throughout all three phases of this study, protein and polysaccharide ratio (PN/PS) was measured in both reactor reactors, and there was no significant difference between the two reactors in EPS generation (p=0.6). The average value of PN/PS during Phase I was 1.2 ± 0.02 for both reactor 1 and reactor 2 (p=0.38); during Phase II, the average PN/PS value was 1.7 ± 0.27 and 1.27 ± 0.25 for reactor 1 and reactor 2 respectively (p=0.45); during phase 3 the average PN/PS value was 0.92

± 0.06 and 0.46 ± 0.32 (P=0.03) for reactor 1 and reactor 2 respectively. The significant difference (P<0.05) observed between reactor 1 and 2 during Phase III of their operation could be partially attributed to the relatively short duration of sampling at this influent flow rate (60L/min). It was thought that if enough time was allowed for steady-state in both reactors during Phase III, the measurements from both reactors would have been more similar; however, this was not the focus during this phase of the study. In comparing the average PN/PS between phase 1 and phase II for both reactors, there was no significant difference between both phases; (P=0.89 and P=0.81 for reactor 1 and reactor 2 respectively). However, it was observed that a few days after the operating conditions were changed from phase I to phase II (i.e. influent flow rate increased by 50%) the PN/PS increased significantly from 1.2 to 1.6 (Figure 11). This 33% increase was short-lived because about one week after the phase change, the PN/PS returned to approximately the same as it was before the phase change. Again, there was a repeat of this sudden temporary increase in PN/PS in response to a flow rate increase after an unintended surge increased the influent flow rate from 45L/min to 55L/min (Figure 6). Shortly after this unintended surge, the PN/PS ratio increased from approximately 1.2 to approximately 1.6 before reducing back to previous levels. This "temporary" responsive change of EPS content to SRT was similarly observed by (Li and Yang 2007). Comparing phase II to phase III on both reactors the PN/PS reduces significantly in reactor 2 (P=0.0003) but not significantly in reactor 1(P=0.1). Again, this could be attributed to the relatively short time for measurements as well as the excessive overflow of sludge from reactor 2 before steady-state. After comparing the PN/PS ratio in Phase 1 (SRT = 9.3 days) to Phase III (SRT = 2.2 days) in both reactors; and the PN/PS ratio in Phase III was shown to be significantly smaller than in Phase I (P=0.004 and P=0.03 for reactor 1 and reactor 2 respectively). This result is consistent with findings from (Liao et al. 2001 and Sesay et al. 2006) where low SRT

corresponded with low PN/PS ratio. The results of the present study are further understood through experiments conducted by (Higgins and Novak 1997) where the adding a protein-hydrolyzing enzyme to an activated sludge sample demonstrated that higher PN content in EPS was critical to sludge flocculation. The lower PN content during Phase III corresponded with poor sludge settleability and compressibility as evidenced by SVI (Figure 4).
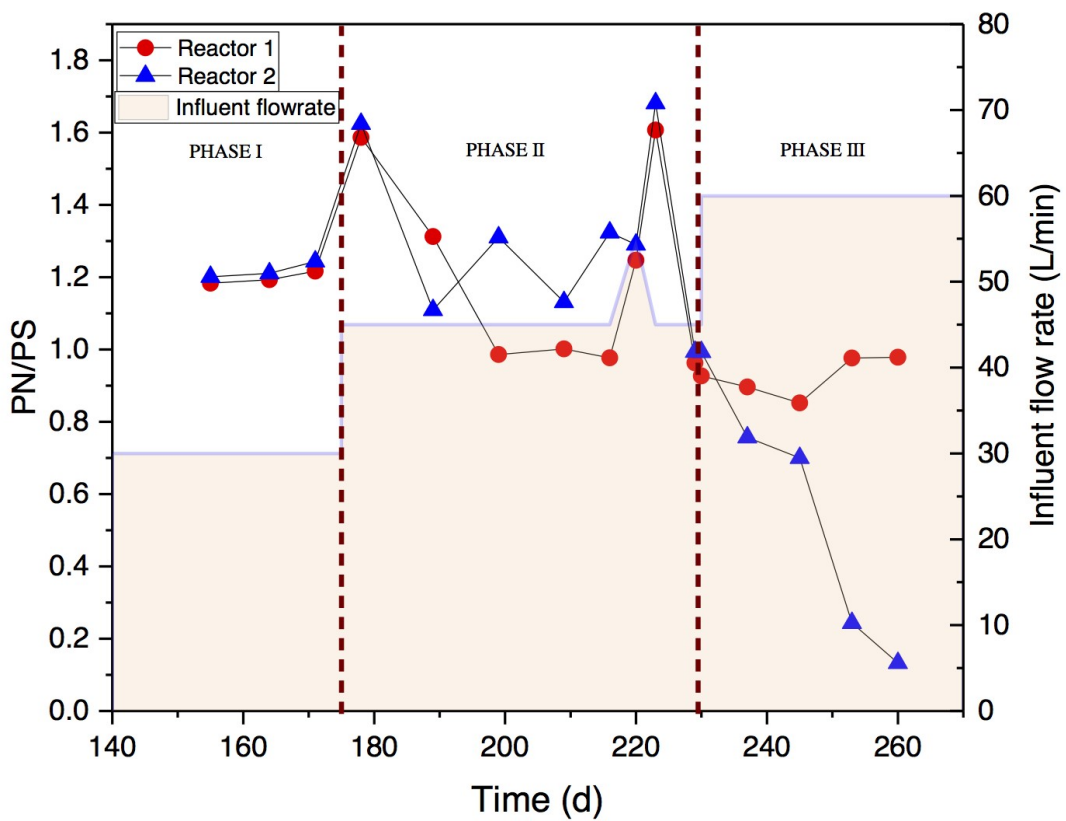
Figure 11 PN/PS for reactor 1(●) and reactor 2 (▲) as observed from Phase I through Phase III

3.3 Conclusions

This study investigated the effect of increasing flow rate by 50% and 100% on the performance of two identical BNR – activated sludge reactors operated in parallel and receiving influent from the same source. Moreover, the study also evaluated the impact of this increased flow rate and its ramifications (shorter SRT) on the bacteria community structure of nitrifiers and denitrifiers as well as the effects on the PN/PS content of extracellular polymeric substances. Performance-wise, both reactors maintained excellent COD removal rates for the three different flow rates monitored (30L/min or Phase 1, 45L/min or Phase 2 and 60L/min or Phase 3). NH_4-N and PO_4-P removal were severely impacted sometime during Phase II of the reactor operation, mainly due to sludge bulking and washout. Q-PCR results from the bacteria community analysis showed that of the two NOBs targeted in both reactors, Nitrobacter was much more abundant than Nitrospira. It was also observed that a significant portion of the bacteria community was susceptible to washout during sludge bulking event. The EPS results showed that the PN/PS temporarily increased in response to an increase in influent flow rate, however a significant decrease in SRT (from 9.3 days to 2.2 days) also brought about a decrease in PN/PS.

CHAPTER 4: RED WORM PROLIFERATION AND ELIMINATION STRATEGIES IN PILOT-SCALE BNR-IFAS REACTOR.

This chapter marks the second part of the current study and was conducted From January to September 2016 beginning with the retrofit of an existing BNR-AS train with a fixed-film structured sheet media material to have a BNR-IFAS train. The initial goal was to compare BNR-IFAS to BNR-AS systems in terms of nutrient removal efficiency and bacteria community structure at different flow rates; specifically, by measuring the ammonia, nitrate, phosphorous and COD concentration in the influent and effluent as flow rate was being increased from 30 L/min through 60 L/min, and by performing a q-PCR at all stages of the flow rate increase. The BNR-AS reactor (Train 1) was retrofitted with the IFAS media in January 2016, by February 20th 2016 the retrofitting was complete and the reactor was commissioned for operation and seeded with mixed liquor from the already operating BNR-AS reactor (Train 2). For reference purposes February 22nd is considered and represented as day "1" in all the graphs and table in section 4.3. Four months after operations started approximately around June 17th, 2016, (or day 118) Red worms were visibly observed in the BNR-IFAS train. The specific trigger for the occurrence of red worms in this study is unclear, however, the installation of the IFAS media material provided additional surface area for biofilm attachment and growth, thus increasing the sludge age in the train and requiring more dissolved oxygen supply for effective treatment; this may have created favourable conditions to encourage red worm proliferation.

It is also thought that red worms proliferated due to the frequent influent flow rate change (Hubbell et al. 2006). The influent flow rates in both reactors increased from 35 L/min in phase I to 45 L/min in Phase II, and then reduced a little to 40 L/min in Phase III. More so, both reactors experienced
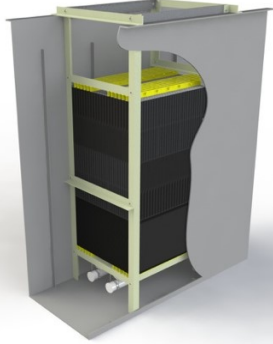
very high or shock influent flow rate (over 45L/min) over several hours due to over pressuring of the lines as evidenced by the spikes in early Phase III. Interestingly, red worms were only observed in IFAS reactor. The incidence of red worms necessitated that the focus of this study be re-directed to the identification of strategies for combating red worms. Another reason for adopting this new focus was because of the deterioration in ammonia removal efficiency of the BNR-IFAS train observed during the presence of red worms. Thus, the specific objectives of this study were:

- To comparatively assess the N, P and COD removal efficiency between BNR-AS and BNR-IFAS under different influent flow rates (30L/min and 45L/min).
- To identify effective mitigation strategies to stop the proliferation of red worms without resorting to chlorinating the entire train.

The mitigation strategies explored were grouped into strategies 1, 2 and 3. Strategy 1 was to pause the internal mixed liquor recirculation to avoid cycling red worms through the oxygen-rich aerobic zone. Strategy 2 combined strategy 1 together with increased sludge wasting so that the MLSS concentration remained slightly below 1000mg/L. This was done to hopefully waste away the red worms while drastically reducing the sludge age (a red worm favourable condition) as well as their food source i.e. bacteria in sludge. Strategy 3 combined strategies 1 and 2 together with temporarily stopping the influent feed and stopping the oxygen supply in the aerobic zone for over 48 hours. More details about the mitigation strategies are provided in section 4.3.5. This chapter will begin with an overview of the IFAS media installation for the retrofit, an overview of the red worms found in the IFAS train, and follow with sections on materials & methods, results & discussion. A literature review on red worms has been presented in chapter 2 (section 2.7).

4.1 IFAS media installation for retrofitting existing CAS train 1

Figure 12 shows a picture chart (Figure 12(b) to Figure 12(f)) of how the two fixed film media towers were retrofitted into the aerobic zone of the BNR-AS Train 1 with a fill ratio of 40 to 50%. The media material in the towers were made of thermoformed corrugated polyvinyl chloride (pvc) sheets which allowed for even distribution of liquid and air for continuous mixing and scouring of biofilm. Each media tower was layered with 0.717 m^3 (total surface area = 141 m^2) of distribution media (DM) module at the bottom closest to the circular membrane fine bubble diffusers (Figure 12(h)), and 1.434 m^3 of vertical-flow (VF) media module (total surface area = 450 m^2) on the top (Figure 12(i)). Both DM and VF modules were specifically designed to enhance nitrification and BOD removal, however the DM was designed to enhance distribution of air, while the VF allowed air bubbles to be directed upwards for circulation of mixed liquor by airlift pumping thus avoiding clogging (Brentwood presentation material).

(a) Media installation Concept (courtesy of Brentwood industries)	(b) Drained CAS train. Diffusers at the bottom are removed and circular membrane diffuser were installed	(c) Media supporting frames are installed
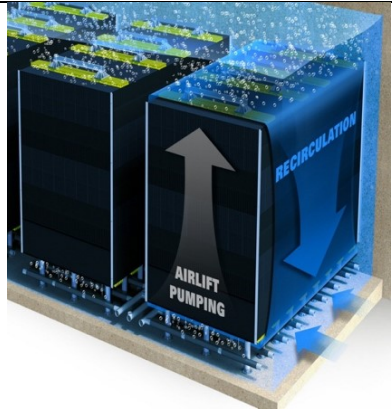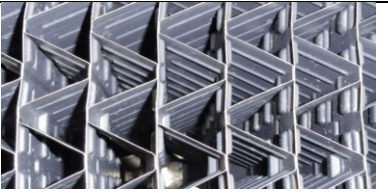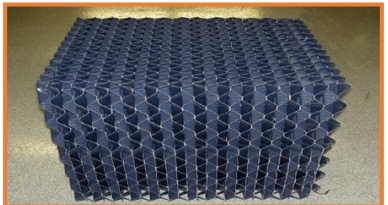
(d) Circular membrane fine bubble diffusers are installed	(e) First media tower is installed	(f) Retrofit is complete with media coupons for testing embedded
(g) Expected flow pattern of airlift pumping and recirculation (image curtesy of Brentwood industries)	(h) Vertical flow media VF-1900 on top (Top view) (i) Distribution media DM (also called cross-flow media CF-1900DM) (bottom)	(j) Sample media coupon strips embedded in the media modules for measuring biofilm thickness and other analysis.

Figure 12 Overview of IFAS media tower retrofit in CAS train 1

4.2 Materials and Methods

4.2.1 Reactor setup

The reactor set-up for this study was like the set-up described in chapter 3 (reactor setup) in the first part of the study, except that one of the CAS reactors (Train 1) was retrofitted with IFAS media thereby converting it to BNR-IFAS while Train 2 was operated in CAS mode. Furthermore, the configuration of both reactors was an adapted version of the A^2/O process in the anaerobic-anaerobic-anoxic-aerobic operation sequence. The effective volumes of the anaerobic, anoxic, aerobic chambers and settler were 2.2 m^3, 3.3 m^3, 13.2 m^3 and 5.7 m^3 respectively. The D.O. probes and air supply were linked and automated by a proportional-integral-derivative controller system known as DeltaV. Three D.O. probes were arranged on the aerobic zones for both the BNR-IFAS and the BNR-AS trains such that one probe, (P1), was closer to the anoxic zone upstream, another probe, (P3), was closer to the clarifier (downstream) and a third probe, (P2), was in the middle of the aerobic zone as shown in Figure 13. The minimum air flow rate for providing sufficient share to discourage red worm attachment onto the media surface was 11.0 m^3 per hour. Figure 13 shows a schematic of the twin pilot-scale reactors with the BNR-IFAS or Train 1 (top) retrofitted with structured sheet media towers provided by Brentwood industries. The bottom train or Train 2 in Figure 13 shows the BNR-AS which was operated alongside the BNR-IFAS as a control. Table 3 shows a selection of operational parameters that were used during different flow rate regimes.
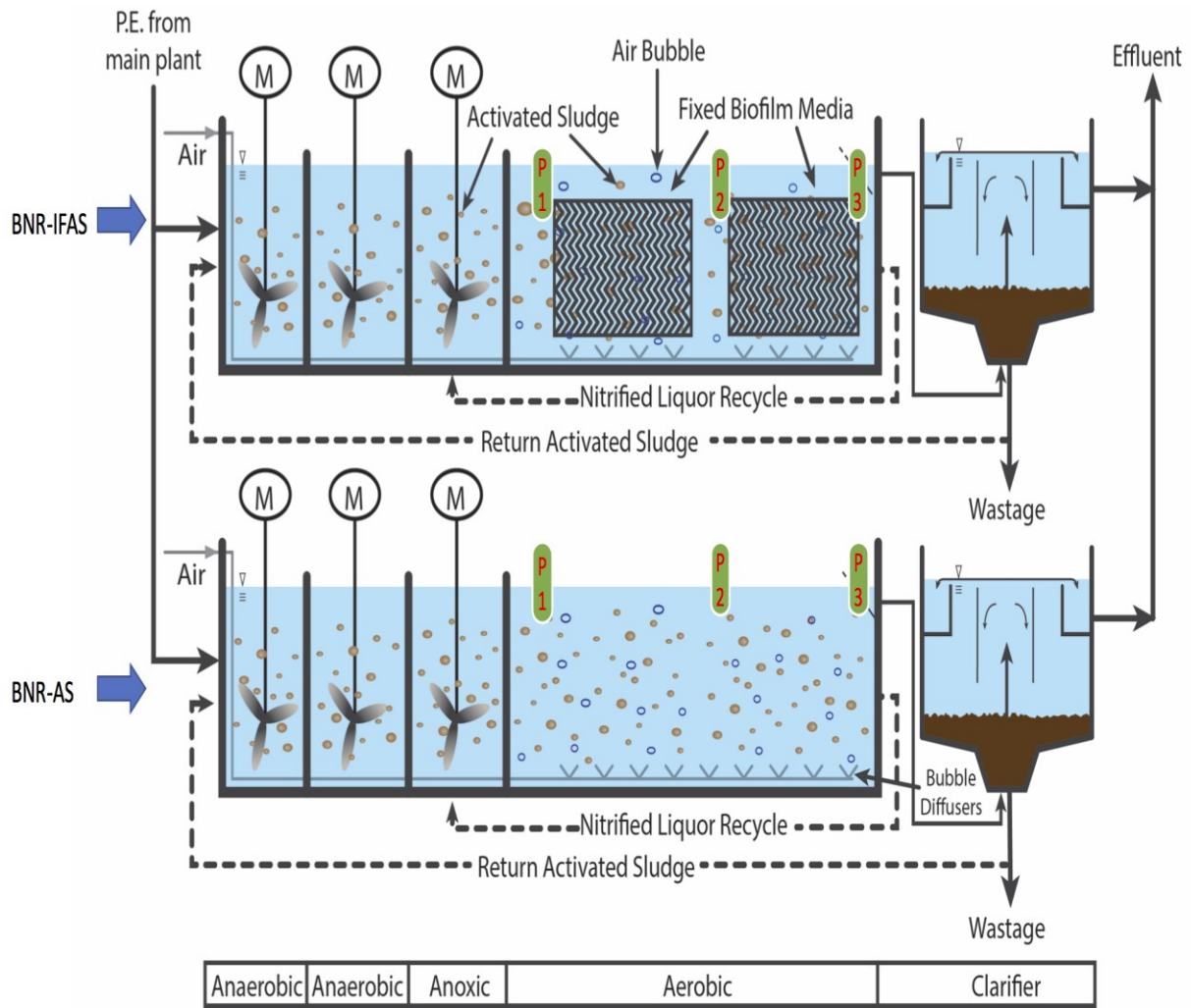
Figure 13 A schematic diagram of the BNR process showing IFAS fixed biofilm media retrofitted into train 1 above and CAS train 2 below.

Table 3: Parameter settings for IFAS Train 1 and CAS Train 2

Parameter settings for Train 1 and 2	Settings used for different flow rates		
Operation Phases	Phase I (Day 1-60)	Phase II (Day 61-Day 72)	Phase III (Day 73-Day 228)
Influent Flow rate (L/min)	30	45	40
RAS Flow rate (L/min)	24.5	40	35
Mixed Liquor Recirculation (MLR) from Aerobic to anoxic chamber (L/min)	78	95 - 100	95 -100*
Average Waste Activated Sludge (WAS) Flow rate$	2mins of wasting per 90mins cycle	2mins per 90mins cycle	2mins per 90mins cycle.
D.O. probe set point for BNR-IFAS train 1. Probes P1, P2 & P3 respectively (ppm)#	2.0, 2.0 and 1.5	3.0, 3.0 and 2.5	3.0, 3.0 and 2.5
D.O. probe set point for BNR-AS train 2. Probes P1, P2 & P3 respectively (ppm)#	2.0, 2.0 and 1.5	2.0, 2.0 and 1.5	2.0, 2.0 and 1.5
Minimum air flow rate (m^3 per hour)	11	11	11

* At the time of the experiments the available pump was at maximum capacity and could be operated to exceed this flow rate. $ The WAS flow rate was constantly adjusted to maintain a target mixed liquor concentration of 2000L/min

Although the set points were fixed, sometimes, the actual perceived values of D.O. was +/- 0.5 ppm from the set point

4.2.2 Reactor performance chemical analysis

Wastewater Influent and effluent were sampled and filtered with a 0.45 μm pore size syringe filter. Afterwards, dissolved COD, ammonia nitrogen, nitrate nitrogen, and orthophosphate phosphorus were measured using commercially available test kits from Hach company USA; TNT 821 (method 8000), TNT 830 (method 10205), TNT835 (method 10020), and TNT 844 (method 10209) respectively. Furthermore, the sludge volume index (SVI) was determined as the volume in milliliters occupied by 1 gram of solids from the aerobic zone mixed liquor after 30 minutes of settling in a 1L graduated cylinder (Bridgewater and Rice 2012). The mixed liquor suspended solids (MLSS), and the mixed liquor volatile suspended solids MLVSS were also measured per standard methods (Bridgewater and Rice 2012).

4.2.3 Biofilm thickness measurement using Confocal Laser Scanning Microscopy (CLSM)

The methodology for measuring biofilm thickness was replicated in entirety from (Huang et al. 2015) supplementary materials. The proper mixture of the SYTO9 and propidium iodide stains added to the prepared biofilm samples rendered bacteria with intact cell membranes fluorescent green while bacteria with damaged cell membranes will be stained fluorescent red. SYTO9 stain has an excitation/emission maxima of 480/500 nm while propidium iodide's is at 490/635 nm. Media coupons which were inserted at the start-up of the IFAS system (Figure 12(j)) were retrieved and used for this analysis. Coupon samples were directly stained by using LIVE/DEAD ®

BacLightTM Bacterial Viability Kits (which contain SYTO9 and propidium iodide stains). The distribution of live and dead bacteria was determined using fluorescence.

To begin this analysis, biofilm coupons retrieved from the IFAS train were cut using a sterile surgical scalpel to generate mini coupon samples (approximately 5 x10 mm). The mini coupon samples were washed three times in phosphate buffered saline (PBS) solution to remove loose cells. Approximately 250µL of the appropriate dilution dye mixture (3 µL of the dye mixture for each mL) was applied to the mini coupon biofilm samples. The samples were incubated in the dark at room temperature for 1 hour. Afterwards, 0.85% NaCl was used to rinse the mini coupon samples thrice to remove any residual dye mixture solution. Immediately after the staining process, biofilm samples were analyzed using a confocal laser scanning microscope (CLSM) (Huang et al. 2015).

Biofilm samples were observed at a 10x objective under condition optimized green and red fluorescence. Three view positions were randomly chosen to evaluate each sample. The thickness of biofilm was determined by first scanning on the surface of media and marking this stage position as origin. The stage was moved until the top surface of a cell cluster came into focus. The thickness was measured as the difference between the stage positions (Huang et al. 2015).

4.2.4 Red worm observation and quantification

The identification and quantification of *Aeolosoma hemprichi* occurred after red worms had proliferated in the IFAS reactor for over a month. The identification of the red worms present as

Aeolosoma hemprichi was done using the Carl Zeiss Axio imager upright microscope (Carl Zeiss, Oberkochen, Germany). A drop of mixed liquor from the aerobic zone of the IFAS train was placed on a microscope slide and covered with a cover slip and immediately viewed under 10× magnification. For quantification, mixed liquor was obtained from the BNR-IFAS train aerobic zone with proper mixing, and transferred into a petri dish for counting. Most of the worms were visible to the naked eye. Each sample was analyzed for at least 15 times.

4.2.5 Statistical analysis

Anova was conducted using Microsoft excel to determine if there was a statistically significant difference in the comparison of the performance of both trains, with p values less than 0.05 indicating statistical significance.

4.3 Results and Discussion
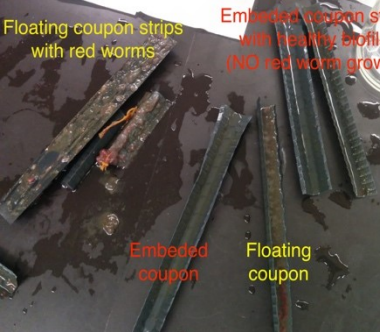
4.3.1 Red worm Identification and Quantification

The dominant specie of red worm identified in the BNR-IFAS reactor (train 1) was *A. hemprichi*; it was recognizable by its characteristic red pigmentation as seen in Figure 14 (a), (b) and (c). Further confirmation was seen under the microscope by observation of its ciliated prostomium, lipoidic inclusions, transparency of worm body (See figure 14 (g), (h) and (i)) and general

comparison with known representative images from other authors (Herlant-Meewis 1950, Menniti and Morgenroth 2010). They range in size from 500 to 1500 micrometers (Wei and Liu 2005, Menniti and Morgenroth 2010). They mainly reproduce asexually and their population doubling time is 2-6 days (Elissen et al. 2008). From physical observation in the BNR-IFAS train, *A. hemprichi* exhibited the characteristics of an obligate aerobe, tending to concentrate in areas where the D.O. concentration was likely to be highest. In the IFAS train, the worms *A. hemprichi* were found to be both sessile and free- swimming.

The structured PVC IFAS media has been reported to be less favorable to red worm growth compared to other types of IFAS media due to the physical characteristic of the PVC media (Metcalf & Eddy et al. 2014). Therefore, it was importantly noted that the dedicated aeration coupled with airlift pumping (Figure 14 (g)) in the media towers provided sufficient scouring and mixing at a minimum air flow rate of $11m^3$/hr such that biofilm growth was maintained inside the media tower and at the same time red worms were not able to attach to the media modules. However, outside of the media, the high DO and low F/M ratio conditions might have promoted worm growth. Ye et al. (2009). Observing the embedded media coupons were used for biofilm thickness analysis and later qPCR analysis, it was noted that the firmly embedded coupons developed healthy biofilm and barely had red worms attached to their surface. These firmly embedded coupons were representative of the internal condition of the media modules. However, some of the media coupons were not firmly embedded in the module, and came loose due to the force of the airlift pump, and drifted for several days in the process stream. These loose coupons which were left floating and drifting in the mixed liquor stream provided ready attachment surfaces for red worm growth. Essentially, the sessile worms were not found attached to the structured sheet fixed film media modules, instead, they were mostly found attached to other surfaces that were

near the top of the waterline in the reactor such as the D.O. probes (Figure 14 (a)), cotton strings used to hold media coupons in place for future analysis, floating media coupons, the side walls of the trains and clarifier (Figure 14 (c)) etc. The sessile worms were also more likely to agglomerate in clusters (Figure 14 (a)), while the free-swimming worms could be found all over the mixed liquor.

(a) Clusters of red worms on D.O. probe	(b) Biofilm media coupon strips (embedded) with no red worms) and (floating)with red worms)	(c) Red worms observed on the edge of the clarifier for BNR-IFAS
(d) Red worm quantification in petri dish	(e) Red worm quantification in petri dish (closer)	(f) Red worm quantification in petri dish (closer) showing worms by mm ruler

(g) Red worm identification under a microscope	(h) Bright field view of *aeolosoma hemprichi* identification under a microscope showing transparent body	i) Bright field view of *aeolosoma hemprichi* identification under a microscope showing ciliated prostomium
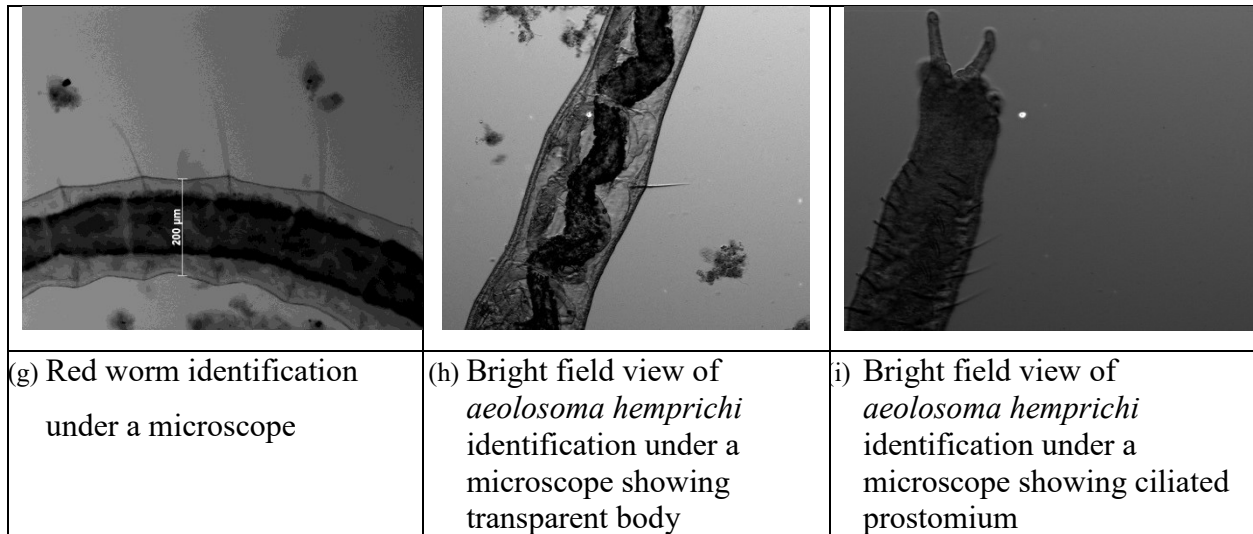
Figure 14 Images of Red worm *Aeolosoma hemprichi* showing physical observation on the IFAS train (a, b, c) quantification from nixed liquor in petri dish (d, e, f) and identification under the microscope (g, h, i)

4.3.2 Effect of red worms on nutrient removal

4.3.2.1. Effect of red worms on COD removal

With average influent CODs of 170.5 ± 27.9 mg/L, and the effluent CODs in BNR-IFAS and BNR-AS reactors were 35.1 ± 13.6 mg/L and 33.7 ± 5.8 mg/L, respectively. There was no significant difference in CODs removal efficiencies between the two reactors (p=0.49), which were $78.7 \pm 10.5\%$ in IFAS and $79.7 \pm 5.4\%$ in AS, respectively. On Day 118 of the testing period, Train 1 was suffering from a red worm bloom, which however did not affect the COD removal efficiencies (p=0.28, $80.2 \pm 5.7\%$ vs $78.8 \pm 4.7\%$ before and after red worm occurrence). Given that aerobic heterotrophic bacteria are typically involved in the aerobic oxidation and removal of

organics from treatment streams, it was inferred that the presence of *Aeolosoma hemprichi* did not functionally affect aerobic heterotrophs in any significant way. This finding agrees with previous reports that the red worms had limited impacts on COD removal (Liang et al. 2006a, Wang et al. 2011). Figure 15 shows the influent and effluent COD concentrations for the BNR-IFAS train (Top); it can be compared with Figure 15 (Bottom) which shows the same measurements for the BNR-AS train. The points indicated by letter "A" on the BNR-IFAS train shows the moment when red worms were visibly observed in the BNR-IFAS train as pictured in Figure 15 (a) and (c). Points B, C and D represent the times when different intervention strategies were applied to eliminate red worms in the BNR-IFAS train. At point B, intervention strategy I was applied (i.e. Mixed liquor recirculation (MLR) was eliminated) at point C, intervention II was applied (i.e. a combination of no MLR and increased wasting 4 mins per 60 mins), at point D, intervention III was applied which is a combination of intervention I & II and no D.O.). Points A, B, C and D are identified in the COD graph and identified in subsequent nutrient removal graph
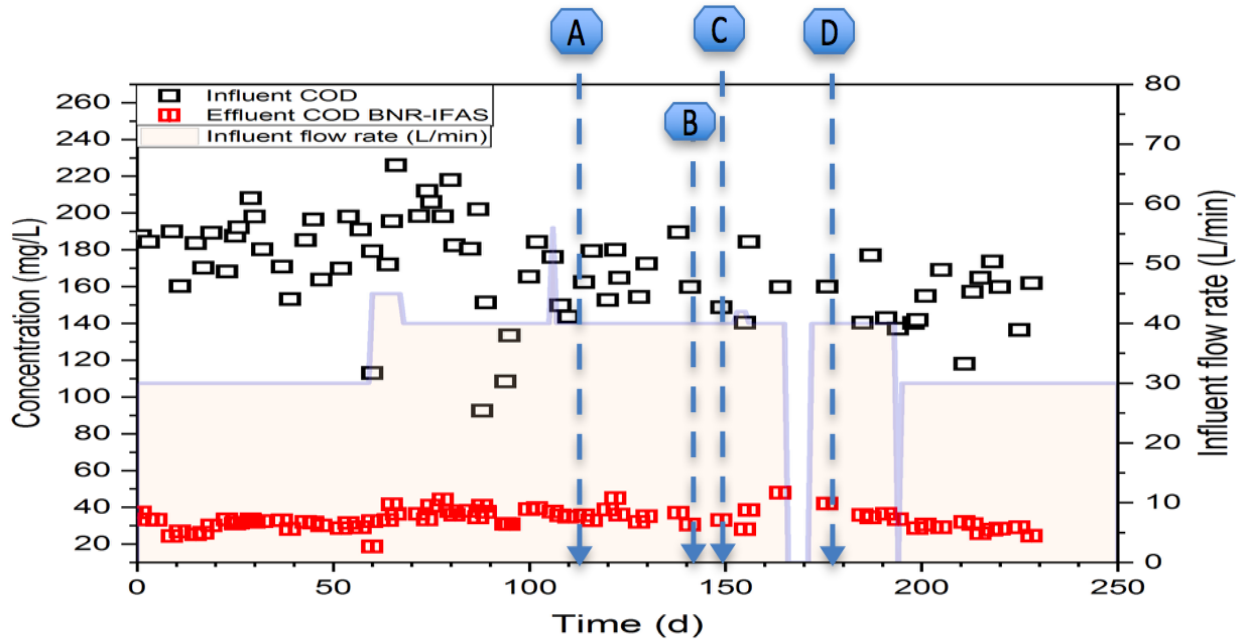
(a)
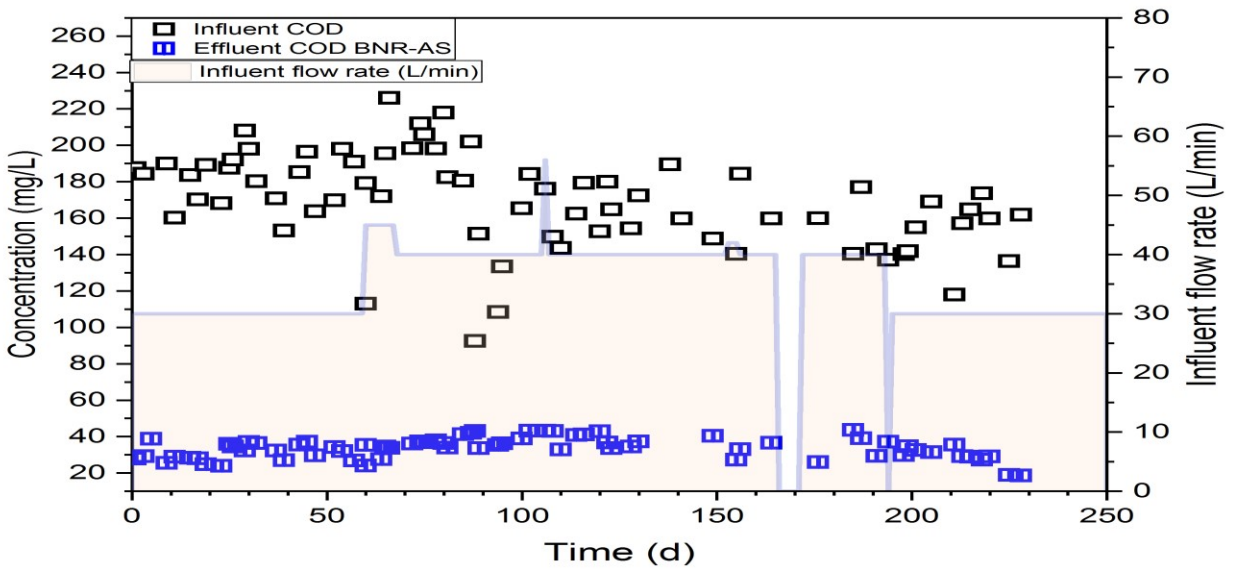
(b)

Figure 15 Reactor performance on COD removal showing influent and effluent COD in the

BNR-IFAS train (a) and the BNR-AS train (b). Arrow A in BNR-IFAS indicates when red

worms were first observed. Arrows B, C and D indicate when mitigation strategies 1, 2 and 3

were applied respectively.

4.3.2.2. Effect of red worms on nitrification

At an average influent ammonia concentration of 43.5 $\pm$ 6.9 mg/L, the ammonia removal efficiencies were 91.5 $\pm$ 10% and 96 $\pm$ 3% in BNR-IFAS and BNR-AS reactors respectively in Phase I (p=0.1), 50.0 $\pm$ 21% and 50.7 $\pm$ 25% in BNR-IFAS and BNR-AS reactors respectively in Phase II (p=0.87), and 79.1 $\pm$ 20% and 88 $\pm$ 15% in BNR-IFAS and BNR-AS reactors (p=0.23) respectively in Phase III. Prior to the observation of red worms in the BNR-IFAS train (approximately between day 73 until day 117 in Fig. 16), the performance of both reactors in terms of nitrification was similar. However, with the red worm bloom in IFAS during day 118 to day 143, there was a significant difference between BNR-IFAS and BNR-AS in ammonia removal (P=0.0004) since the ammonia removal efficiencies in IFAS decreased significantly (p<0.0001) from 79.1 $\pm$ 20% to 24.5 $\pm$ 14% because of red worm proliferation. Our results show that BNR-IFAS did not demonstrate a better ammonia removal performance than BNR-AS at different influent flow rates but it more easily favored the growth of red worms when suffering the significant flow rate change. After performing the mitigation strategies to eliminate the red worm bloom in IFAS, the ammonia recover efficiencies were increased significantly (p=0.009) from 24.5 $\pm$ 14% to 58.9 $\pm$ 30% during day 184 to the end of testing period (day 228) but still much lower than that of AS reactor (91.2 $\pm$ 16%, p<0.001).

(a)

(b)

Figure 16. Reactor performance on ammonia removal showing influent and effluent ammonia in the BNR-IFAS reactor (a) and the BNR-AS reactor (b). Arrow A in BNR-IFAS indicates when red worms were first observed. Arrows B, C and D indicate when mitigation strategies 1, 2 and 3 were applied respectively.

Since most ammonia was oxidized to nitrate via nitrification, nitrate production in the effluent was measured as well. The effluent nitrate concentrations in Phase I, Phase II and the period prior to the red worm bloom in Phase III were 4.9 ± 2.4 and 6.2 ± 3.2 mg/L (p=0.17), 0.2 ± 0.04 and 0.2 ± 0.05 mg/L (p=0.63) and 2.0 ± 2.2 and 2.8 ± 3.1 mg/L (p=0.38), respectively. Like the ammonia removal performance, the effluent nitrate concentrations before the occurrence of red worms in BNR-IFAS has no significant difference between both reactors. However, with the red worm bloom (during day 118-143 Fig. 17), the nitrate concentrations in BNR-IFAS were 0.3 ± 0.2 mg/L, which was significantly lower than that before the occurrence of red worm in BNR-IFAS (p=0.04), and significantly lower than that in BNR-AS process (4.3 ± 5.9, p<0.001). During day 184-228, the effluent nitrate concentrations (3.4 ± 2.5 mg/L) in BNR-IFAS became significantly (p=0.005) higher than that in Phase III before performing the mitigation strategies. In this period, the effluent nitrate concentration in BNR-IFAS and BNR-AS were 3.4 ± 2.5 mg/L and 8.6 ± 4.2 mg/L respectively and significantly different (p=0.0004). The results are consistent with the ammonia removal performance. Therefore, it is inferred that after the red worm bloom elimination strategies worked, it will take more than 45 days for a completely recovery in ammonia removal.
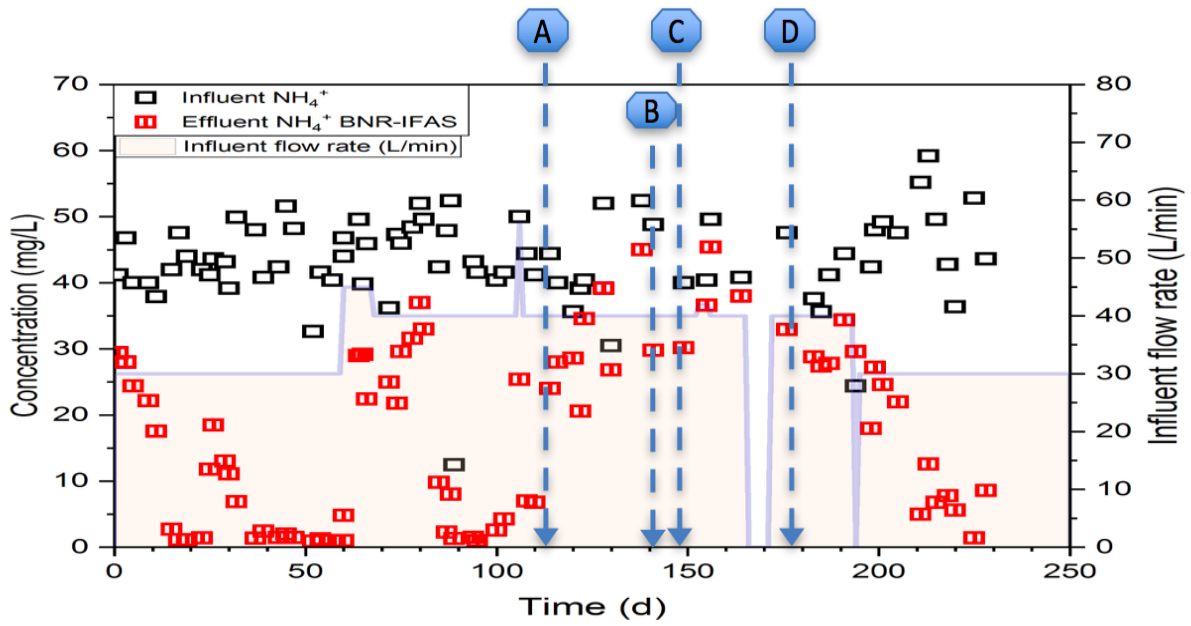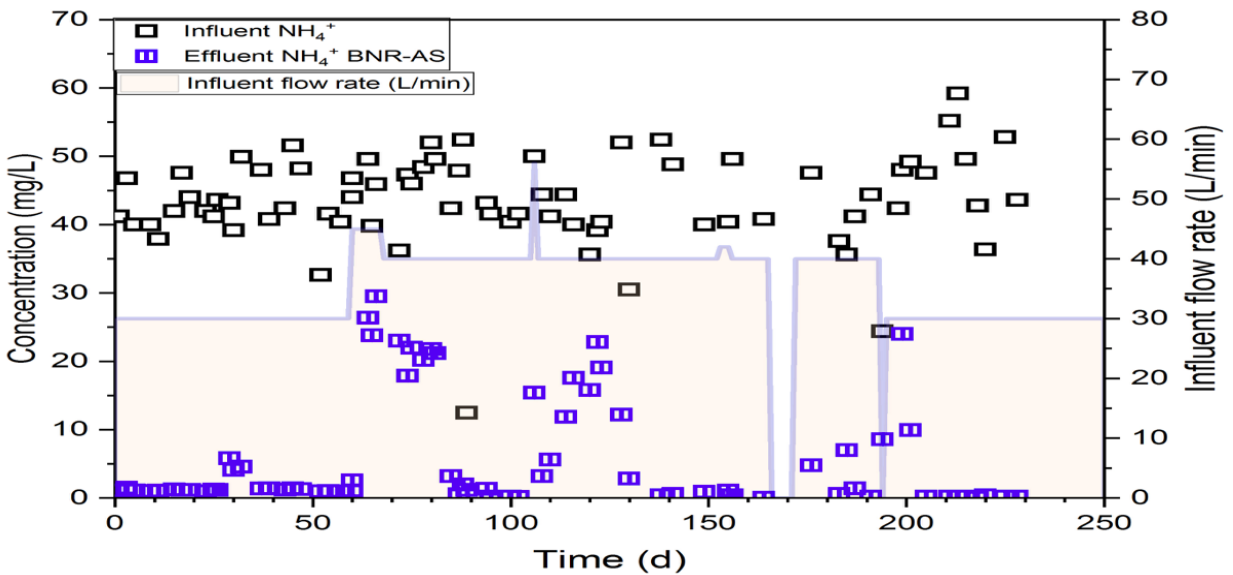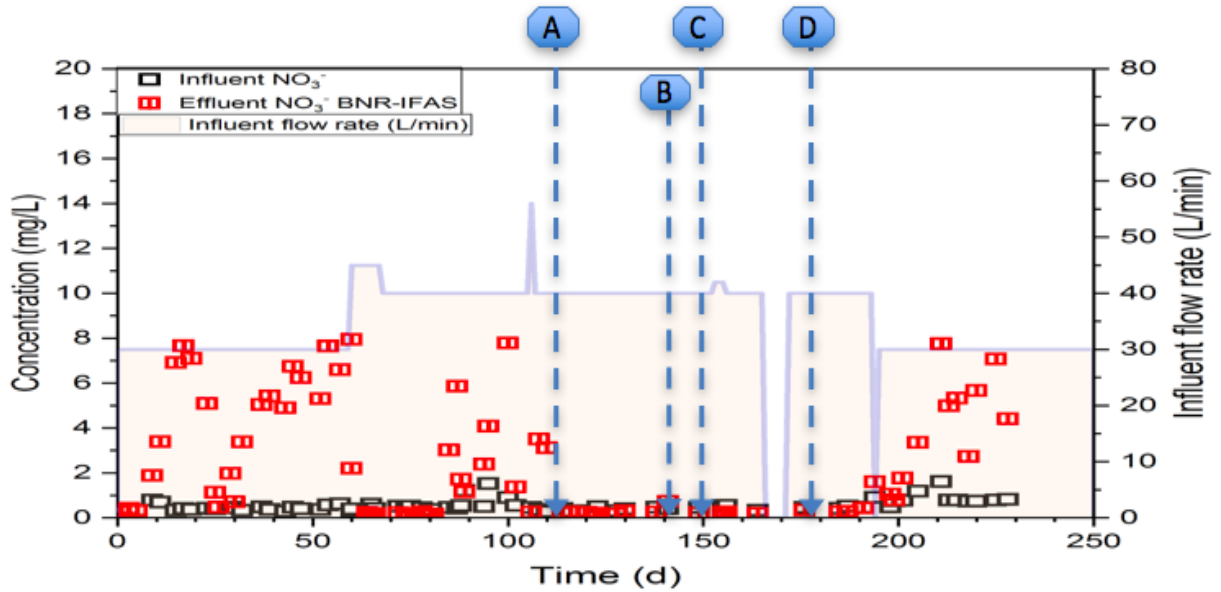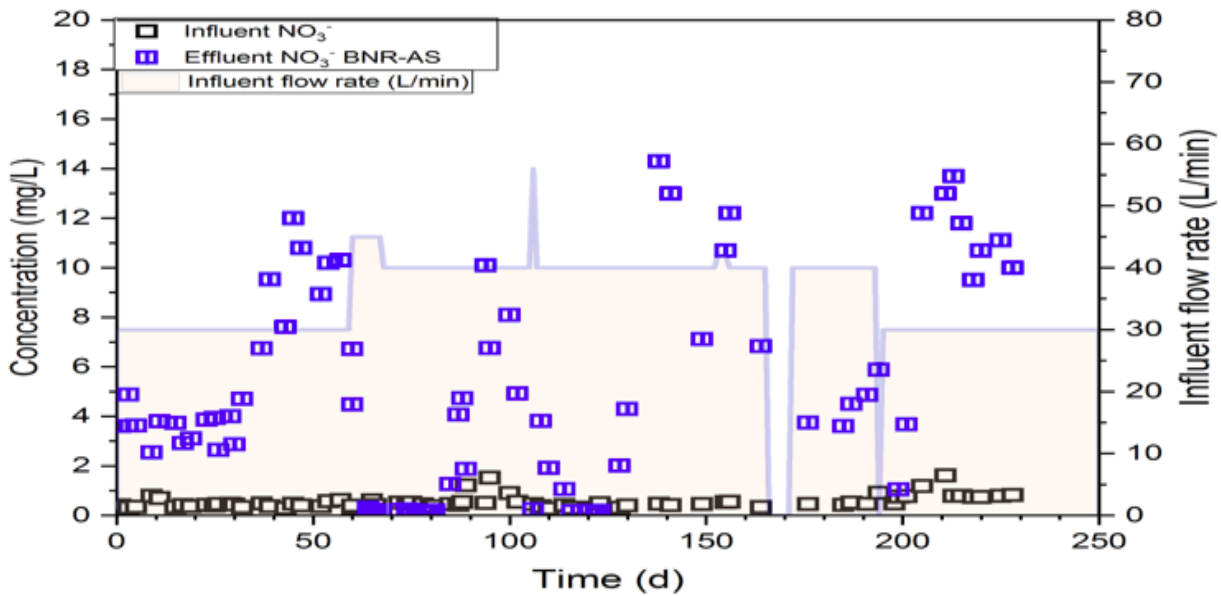
Figure 17 Reactor performance showing influent and effluent nitrate in the BNR-IFAS train (a) and the BNR-AS train (b). Arrow A in BNR-IFAS indicates when red worms were first observed. Arrows B, C and D indicate when mitigation strategies 1, 2 and 3 were applied respectively.

4.3.2.3. Effect of red worms on Phosphorous removal

At an average influent PO_4-P of 5.4 ± 1.3 mg/L, before the occurrence of red worms, the average effluent PO_4-P were 2.2 ± 3.6 mg/L and 1.6 ± 2.5 mg/L respectively in BNR-IFAS and BNR-AS reactors without significant difference (Fig. 18). The PO_4-P removal efficiencies in Phase I were 81 ± 22 % and 90 ± 9.6 % respectively in BNR-IFAS and BNR-AS (p=0.07). In Phase II, the PO_4-P removal were 33% and 40 ± 27 % respectively (p=0.79), and in Phase III, the PO_4-P removal were 57 ± 33% and 59 ± 31 % respectively (p=0.89) before the red worm occurrence. In BNR-IFAS reactor, after the red worm occurrence, the PO_4-P removal efficiency was slightly increased from 57 ± 33% to 68 ± 22% (p=0.4), which indicates that heterotrophic bacteria such as PAO won't be significantly affected by red worms. Then after applying the worm mitigation strategies, the PO_4-P removal efficiencies were increased to 79 ± 18 % which was probably due to the sludge wasting rate increase. Meanwhile, during this period, the PO_4-P removal efficiency in BNR-IFAS reactor was also significantly different from that in BNR-AS reactor (p=0.037).
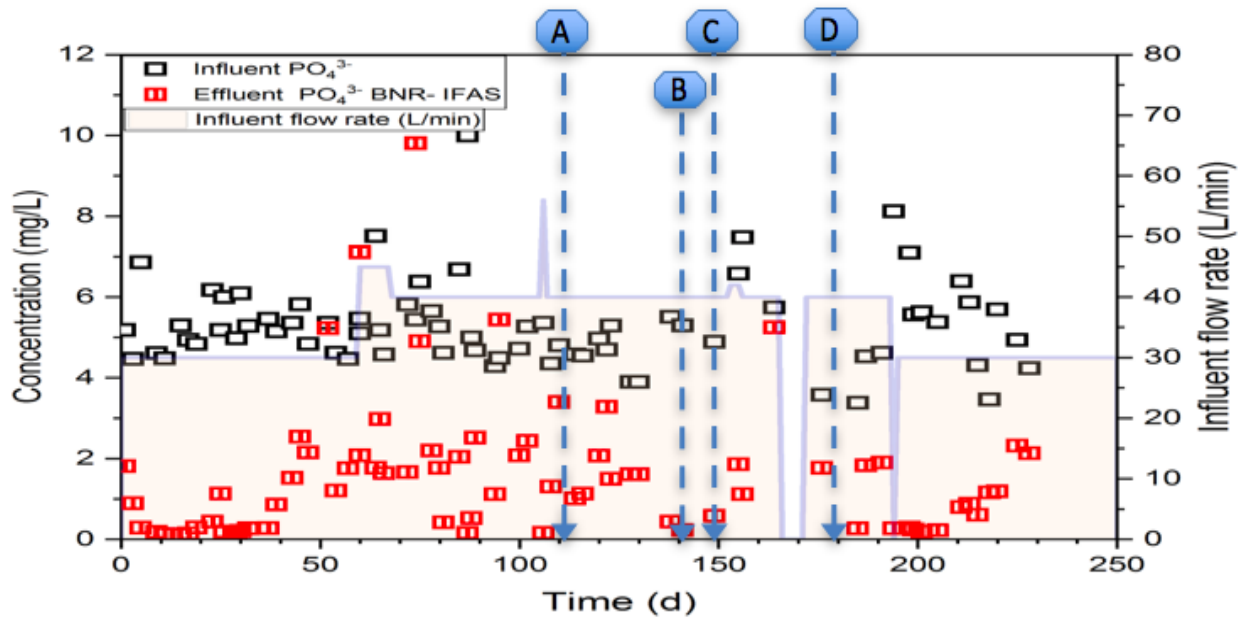
(a)
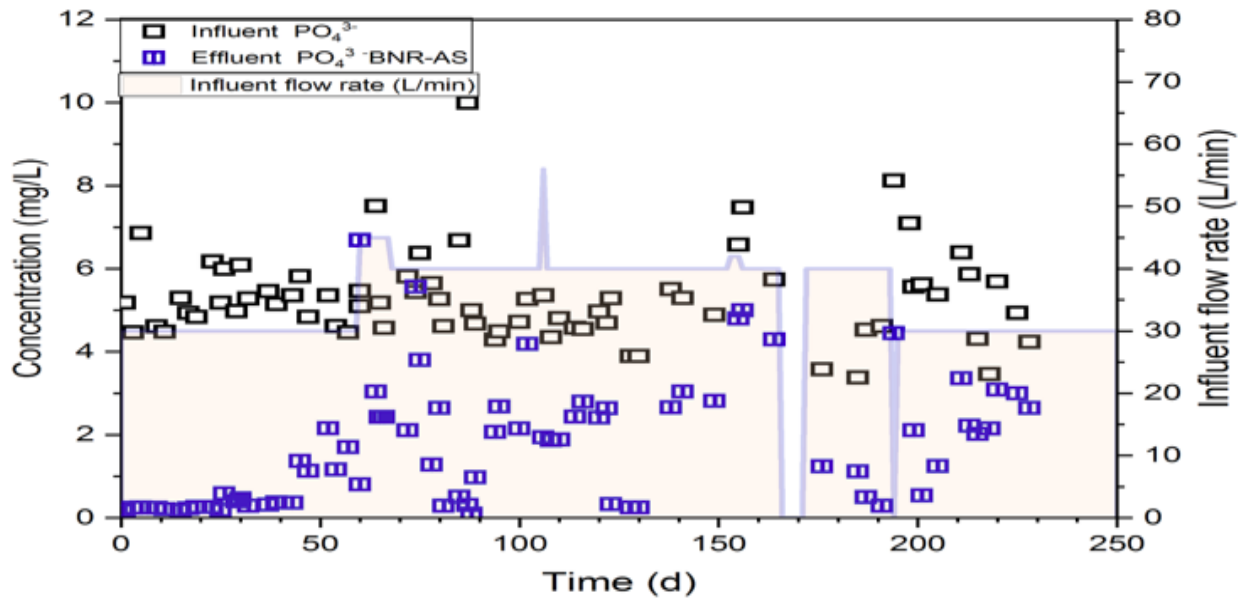
(b)

Figure 18 Reactor performance on PO₄-P removal in the BNR-IFAS reactor (a) and the BNR-AS reactor (b). Arrow A in BNR-IFAS indicates when red worms were first observed. Arrows B, C and D indicate when mitigation strategies 1, 2 and 3 were applied respectively.

4.3.3 Food to microorganism ratio (F/M) Comparison

The measurement of F/M showed a significant difference ($P < 0.05$) between BNR-IFAS and the BNR-AS (Figure 15). Generally, the F/M ratio of the BNR-IFAS system was less than that of the BNR-AS system which is expected because the biofilm growth on the media material in the BNR-IFAS system was indicative of a higher bacteria and microorganism concentration than in the BNR-AS system which had no fixed media, even though both systems were receiving the same amount of food in terms of organic loading concentration. During the days of increased flow rate as observed on (Day 60 and day 101 on figure 19), the flow rate increased from 30 L/min to 45 L/min and from 45 L/min to 56 L/min on both days respectively. The BNR-AS F/M increased well beyond 0.7 mg-COD/ (mg-VSS day), however the F/M for BNR-IFAS barely reached 0.7 mg-COD/ (mg-VSS day). The fact that *Aeolosoma hemprichi* proliferated in the BNR-IFAS train is supported by previous studies by (Liang et al. 2006b) where F/M of less than 0.7 mg-COD/(mg-VSS day) was determined to be ideal for red worm multiplication.
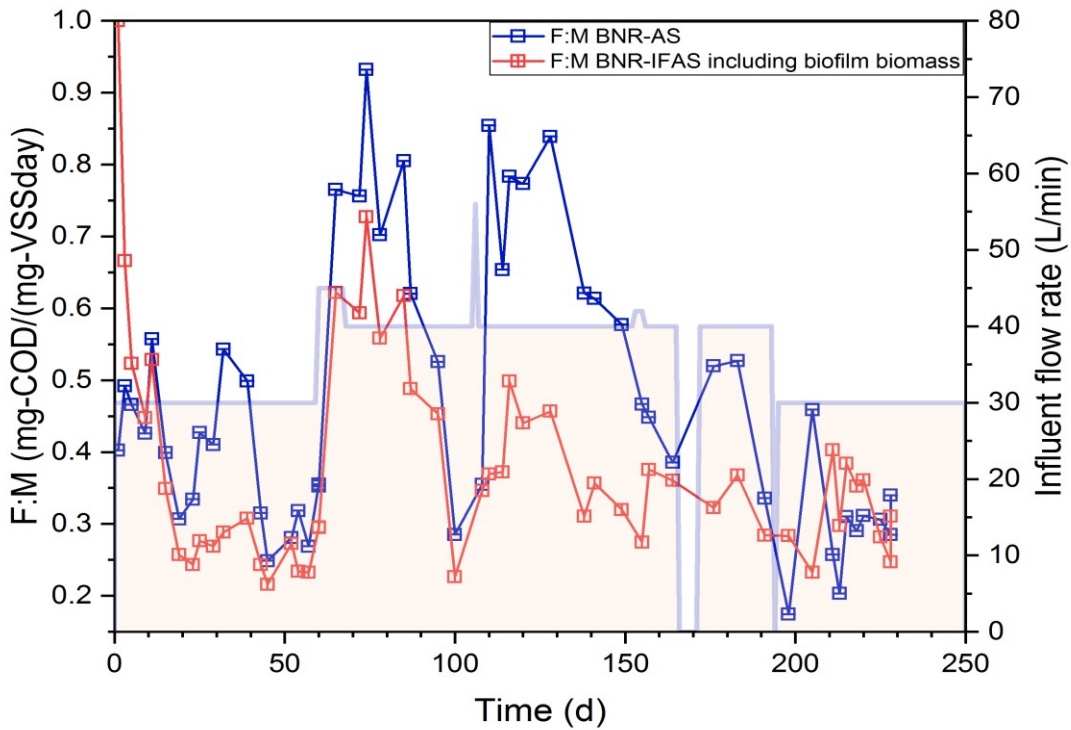
Figure 19 Food to microorganism ratio for BNR-IFAS and BNR-AS

4.3.4 Biofilm thickness

After retrofitting the BNR-IFAS train with fixed media, and prior to seeding the train with mixed liquor, representative samples of media coupons provided by Brentwood industries which were made of the exact same material and mould as the installed VF and DM media modules were embedded in the modules so that they could be harvested later for analysis. These coupons were a practical alternative used in lieu of lifting the entire media tower from the process stream and cutting off a piece of media material for analysis. The BNR-IFAS train was allowed approximately

3 weeks from the start of seeding and operation before the biofilm measurement. Biofilm thickness was measured periodically to ascertain that sufficient biofilm had grown on the media to support nutrient removal. Maas et al. (2008) Identified that biofilm solids would require over 50 days to reach a quasi-steady-state. Thickness measurements were performed by harvesting the coupons and staining them for CLSM analysis as described in section 4.2.3. Most of the coupons were embedded in the media tower that was at the end of the aerobic zone (closest to the clarifier) where the D.O. was 2ppm. A few coupons were embedded upstream of the aerobic zone closer to the pre-anoxic zone where the D.O. was set to approximately 3.5ppm. The measured thickness varied depending on the dissolved oxygen (DO) concentration in the aerobic section where the media coupon was harvested from. Coupons nearest to the anoxic zone where the DO concentration was higher at 3.5ppm had more biofilm than coupons nearer to the clarifier where the D.O. was 2ppm. (Fig 20).
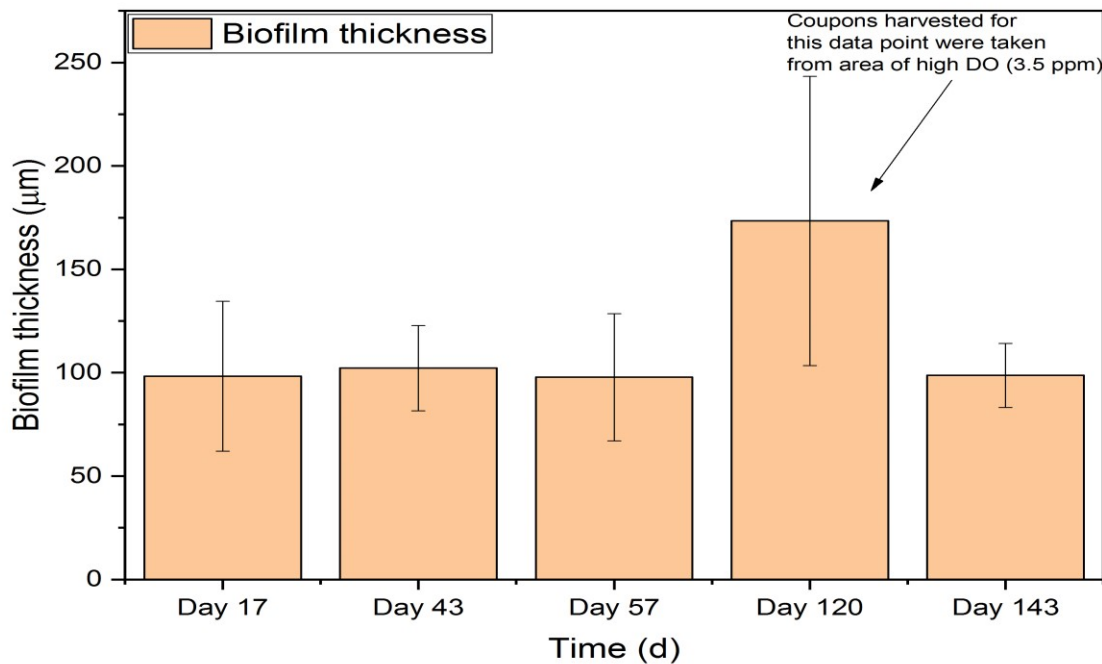
Figure 20. Biofilm thickness measured using confocal laser scanning microscope

In summary, there was no significant change in biofilm thickness observed prior to or after the red worm bloom. The only difference in biofilm thickness was dependent on oxygen supply.

4.3.5 Recap of strategies to mitigate red worms

4.3.5.1 Pausing mixed liquor recirculation (Arrow "B" in nutrient removal graphs)

The internal Mixed liquor recirculation (MLR) line in the BNR-IFAS process train recycles mixed liquor from the end/outlet of the aerobic zone back into the anaerobic zone. To eliminate the persistence of red worm in the IFAS bioreactor, it was decided to turn off the MLR to limit the recirculation of red worms containing sludge through the oxygen rich aerobic zone. This intervention was carried out initially by itself for a few days (days 141 to 144) before the combination with the reduction of the MLSS in the reactor through increased wasting (discussed in 4.4.2). However, there was no effect on red worm density ammonia removal.

4.3.5.2 Increasing Sludge wasting in combination with paused mixed liquor recirculation (Arrow "C" in nutrient removal graphs)

It has been concluded by some researchers that high MLSS concentration is indeed beneficial to *Aeolosomatidae* and consequently there is a positive relationship between high SRT and high worm density (Elissen, Hellen J.H. 2008). Thus, it was postulated that by increasing the wasting rate of the sludge, the worms would have been eliminated from the reactor. The challenge however

was to maintain certain MLSS concentration to support the reactor performance while maximizing the sludge wasting rate for worm elimination. Our strategy was to increase the wasting rate from 2min/90min to 4 min/60min from day 144 until day 172 and turn off the MLR line to eliminate the persistence of red worms in the entire IFAS process at the same time. During the 28 days, the 3-time increase in sludge wasting rate lead to a significant decrease (p=0.04) in MLSS concentration from 1808 ± 395 mg/L to 1278 ± 694 mg/L (Table 4). Meanwhile average red worm concentration decreased significantly (p=0.002) from 1.64 ind./mL to 0.18 ± 0.23 ind./ml with the decreased MLSS concentrations. However, on day 176, the red worm density has a slight recovery as soon as the previous operation was resumed. From day 173 when the previous sludge wasting rate (2min/90min) was resumed, the MLSS started to recover to 1703 ± 369 mg/L, and the red worm density was maintained as low as 0.045 ± 0.09 ind./mL (Table 4).

Table 4: Average red worm density in BNR-IFAS reactor with corresponding reactor MLSS

concentration

Day	Average red worm density (ind./mL)	MLSS (mg/L)
141	1.64	2250
149	0.633	2470
150	0.13	1350
151	0.128	950
155	0.137	1590
157	0.03	660
164	0.01	650
173	0.045	1703
176	0.18	1910
183	0	2220
191	0	2270
198	0	1680

4.3.5.3 Rendering entire reactor anaerobic + paused nitrified liquor recycle + maintaining

MLSS < 1000mg/L through excessive sludge wasting (Arrow "D" in nutrient removal graphs)

Since red worms are strictly aerobes, it was decided that the entire BNR-IFAS reactor be rendered anaerobic for a while as another mitigation option. On day 181 to day 183, the influent flow and DO supply was shut off in the bioreactor. This anaerobic condition existed for 48 hours straight, after when the influent flow and the aerobic zone DO was restored. This treatment completely wiped off all the red worms (Table 4).

About 1 month after the entire train was rendered anaerobic for 48 hours (i.e. day 215). The BNR-IFAS reactor was observed to recover in terms of ammonia removal such that NH3-N concentration in the effluent was less than 10 mg/L. The reason for this recovery time could be that this is how long it took for nitrifiers to be healthy enough to carry out nitrification in the absence of red worms. Mass et al. (2008) Identified that a start-up IFAS process reached high nitrification rate within weeks

4.4 Conclusion

Red worms identified as *Aeolosoma hemprichi* were found to have proliferated in a BNR-IFAS train shortly after a shock increase (50%) in the influent flow rate. Subsequent fluctuations in influent flow rate sustained the perpetuation of the red worms. The proliferation of these worms seemed to directly impact nitrification although COD removal and PO$_4$-P removal were relatively unaffected. A combination of measures was taken to eliminate the red worms. The only combination which eliminated the worms involved the stopping of aeration for 48 hours, in addition to stopping the influent feed, maintaining a low MLSS and stopping the mixed Liquor recirculation.

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