Ammonia removal: biofilm technologies for rural and urban municipal wastewater treatment

Xin Tian

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Ottawa-Carleton Institute for Environmental Engineering
Department of Civil Engineering
Faculty of Engineering
University of Ottawa

Abstract

The new Canadian federal wastewater regulations, which restricts the release of ammonia from treated wastewaters, has resulted in upgrade initiatives at many water resource recovery facilities across the country to reduce the discharge of ammonia into our natural waters. The objective of this dissertation is therefore to investigate and optimize the performance of two attached growth technologies for rural and peri-urban/urban municipal ammonia removal. In particular, the first specific objective of this dissertation is to investigate the performance and microbial response of the BioCord technology as an upgrade system for the post-carbon removal nitrification of rural wastewaters. The second specific objective is to study the start-up of an attached growth anammox technology to enhance current knowledge pertaining to anammox biofilm attachment, growth and maturation.

The results pertaining to the first specific objective of this research, a study of the design and optimization of the BioCord technology, demonstrates a recommended design rate for the post-carbon removal, nitrifying BioCord system of a surface area loading rate (SALR) of 1.6 NH_4^+-N/m^2·d and up to 1.8 NH_4^+-N/m^2·d with steady ammonia-nitrogen removal efficiencies greater than 90% and steady and low solids production rate up to 0.26 g TSS/d. A loss of system stability and biofilm sloughing, identified as fluctuating ammonia removal rates and solids production rates, were observed at elevated SALRs of 2.1 and 2.4 g -N/m^2·d. The microbial results indicate that the meso-scale structure of the biofilm and the micro-animal population are directly affected by operational conditions. Enhanced air scouring configuration is shown to be a potential optimization strategy to prevent the clogging of biofilm pores and improve the system stability in terms of solids production rate in the BioCord technology.

The results pertaining to the second specific objective of this research, the study of inoculation and carrier modification strategies for the rapid start-up of attached growth

anammox technology, demonstrates significantly higher kinetics, faster biofilm growth and greater anammox bacteria enrichment on the silica-functionalized carriers and pre-seeded denitrifying carriers in a system inoculated with detached anammox biofilm mass during the early stages of attachment and growth of start-up. The study suggests that the use of the silica-functionalized and pre-seeded denitrifying carriers along with detached anammox biofilm inoculation has the potential to accelerate the anammox biofilm attachment, growth and maturation.

Preface

This dissertation is an original work performed by Xin Tian under the supervision of Dr. Robert Delatolla. The dissertation contains four manuscripts that are published or "submitted" for publication in peer reviewed journals. Versions of these manuscripts are published in chapters 3 to 6 of the dissertation. References for each manuscript along with author contributions to each of the manuscripts are presented below.

Chapter 3:

A version of the following manuscript has been published in this chapter: Tian, X., Ahmed, W., & Delatolla, R. (2019). *Nitrifying BioCord reactor: Performance optimization and effects of substratum and air scouring.* Environmental Technology, 40(4), 480-488.

Xin Tian performed all experiments, collected and analyzed the data, prepared and revised the manuscript.

Warsama Ahmed contributed to data analysis and manuscript revision.

Robert Delatolla (supervisor) designed and planned the study, developed the research question, directed the study, and revised the manuscript.

Chapter 4:

A version of the following manuscript has been published in this chapter: Tian, X., & Delatolla, R. (2019). *Meso and micro-scale effects of loading and air scouring on nitrifying BioCord biofilm.* Environmental Science: Water Research & Technology, 5(6), 1183-1190.

Xin Tian contributed to the experimental design, performed all lab work and data analysis, wrote and revised the manuscript.

Robert Delatolla (supervisor) conceived and designed the study, provided direction and feedback on the experiments, and revised the manuscript.

Chapter 5:

A version of the following manuscript has been submitted to the journal of Bioresource Technology in 2020. Tian, X., Schopf, A., Amaral-Stewart, B., Christensson, M., Morgan-Sagastume, F., Vincent, S. & Delatolla, R. *Anammox attachment and biofilm development on surface-modified carriers with planktonic- and biofilm-based inoculation.*

Xin Tian performed all microbial experiments and data analysis, contributed to research guidance and data interpretation, and outlined, wrote and revised the manuscript.

Alex Schopf and Bianca Amaral-Stewart operated and maintained the reactors, contributed to data collection, biofilm/biomass images acquisition and research discussion.

Magnus Christensson, Fernando Morgan-Sagastume and Simon Vincent were the industrial collaborators, they contributed to the research question and the experimental design of the study and contributed to research discussion and the manuscript revision.

Robert Delatolla (supervisor) contributed to the conception and design of the study, directed the study, provided data interpretation, and contributed to the revision of the manuscript.

Chapter 6:

A version of the following manuscript has been submitted to journal titled Chemosphere in 2020. Tian, X., Schopf, A., Amaral-Stewart, B., Christensson, M., Morgan-Sagastume, F., Vincent, S., St-Pierre, J., Mercier, E., Zhang, X. & Delatolla, R. *Carrier surface modification for enhanced attachment and growth of anammox biofilm.*

Xin Tian performed all microbial experiments and data analysis, contributed to research discussion and data interpretation, prepared and revised the manuscript.

Alex Schopf and Bianca Amaral-Stewart operated and maintained the reactors, performed constituent analysis, contributed to biofilm/biomass images acquisition and research discussion.

Magnus Christensson, Fernando Morgan-Sagastume and Simon Vincent were the industrial collaborators, they contributed to the research question and the experimental design of the study and contributed to research discussion and the manuscript revision.

Jean-Philippe St-Pierre and Élisabeth Mercier produced and validated the dextran-modified and silica-modified carriers.

Xiaojing Zhang contributed to research discussion and manuscript edits.

Robert Delatolla (supervisor) contributed to the conception and design of the study, directed the study, provided data interpretation, and contributed to the revision of the manuscript.

I am aware of the University of Ottawa Academic Regulations, I certify that I have obtained written permission from each of the co-authors to include the above materials in my thesis and the above material describes work completed during my full-time registration as a graduate student at the University of Ottawa.

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Finally, heartfelt thanks to my loving family. To my parents for always being my warm harbor and giving me unconditional support on my growing path. To my husband for always being at my side at every moment when I feel lost. Their love, understanding and encouragement accompanied me to today, and will accompany me to continue moving forward.

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List of Acronyms

ANAMMOX	Anaerobic Ammonium Oxidation
ANOVA	Analysis of Variance
AOB	Ammonia Oxidizing Bacteria
$CBOD_5$	Five Day Carbonaceous Biochemical Oxygen Demand
CLSM	Confocal Laser Scanning Microscope
COD	Chemical Oxygen Demand
CSTR	Continuous Stirred Tank Reactors
DDPCR	Digital Droplet Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
EPS	Extracellular Polymeric Substances
FA	Free Ammonia
FNA	Free Nitrous Acid
HRT	Hydraulic Retention Time
IFAS	Integrated Fixed Film Activated Sludge
LC50	Median lethal concentration
MBBR	Moving Bed Biofilm Reactor
MTBL	Mass Transfer Boundary Layer
NOB	Nitrite Oxidizing Bacteria
SALR	Surface Area Loading Rate
SARR	Surface Area Removal Rate
SBR	Sequencing Batch Reactor
SCOD	Soluble Chemical Oxygen Demand

SRT	Solids Retention Time
TAN	Total Ammonia Nitrogen
TSS	Total Suspended Solids
VPSEM	Variable Pressure Scanning Electron Microscope
WSER	Wastewater Systems Effluent Regulations
WRRF	Water Resource Recovery Facility

1 Chapter 1 – Introduction

1.1 Background

As population and commercial activity grow in communities municipal sewage discharge increases. Across Canada the problem of large quantities of untreated or inadequately treated wastewater being discharged into rivers and oceans has been progressing for years. These point source polluters release large quantities of deleterious substances and nutrients to aquatic environments (Murdoch *et al*., 2000). The discharge of excessive ammonia (defined in this dissertation as both ionized ammonium (NH_4^+) and unionized ammonia (NH_3)) leads to eutrophication in receiving water bodies and causes toxic effects on aquatic life. To minimize the adverse impact of released ammonia on aquatic environments, the federal government of Canada has implemented the Wastewater Systems Effluent Regulations (WSER) in January 2015 under the Fisheries Act. The regulations stipulate the limits for five day carbonaceous biochemical oxygen demand ($CBOD_5$), total suspended solids (TSS), total residual chlorine and un-ionized ammonia in the effluent stream of water resource recovery facilities (WRRFs). Specifically, the concentration of un-ionized ammonia as nitrogen (NH_3-N) in wastewater discharges must be less than 1.25 mg/L at 15 ± 1 °C. The speciation and transformation of ionized ammonium (NH_4^+) and un-ionized ammonia (NH_3) in wastewater is temperature and pH sensitive. The fraction of un-ionized ammonia has a positive correlation with increasing temperature and pH in wastewater and can be calculated based on the following Equations (1.1 and 1.2), where pKa is the acid dissociation constant and T is the solution temperature (°C).

$$pKa = 0.09 + \frac{2730}{273+T} \qquad \textbf{Equation 1.1}$$

$$\boldsymbol{NH_3}\% = \frac{1}{1+10^{pKa-pH}} \times 100\% \qquad \textbf{Equation 1.2}$$

As the average ammonia concentration in untreated municipal wastewater in Canada is between 20 to 30 mg-N/L, which equals to approximately 0.13 mg/L NH_3-N at a typical pH of

wastewater effluent of 7.2 (Metcalf and Eddy, 2003) and standard temperature of 15°C, the ammonia concentration of the effluent from WRRFs is unlikely to exceed the limit of 1.25 mg NH_3-N/L. However, WSER also stipulate that the wastewater discharges cannot be acutely lethal according to the median lethal concentration (LC50) assay, where the LC50 defines acute lethality as 50% mortality of rainbow trout after 96 hours in 100% wastewater effluent (Canada Gazette, 2012). Effluent ammonia concentrations between 15-20 mg-N/L have been shown to fail the LC50 test (Di Giulio and Hinton, 2008). Although the LC50 testing under WSER in Canada allows additional testing with increasing sampling frequency for ammonia toxicity, the inclusion of this test in the federal regulations has resulted in upgrade initiatives at many WRRFs across the country to reduce the discharge of ammonia into our natural waters.

Lagoon treatment systems are the most common technology for the treatment of rural municipal wastewaters around the world. There are over 2500 lagoons in operation in France, over 3000 in Germany, over 8000 in the US and approximately 1200 lagoon WRRFs in operation across Canada (Mara, 2009; USEPA, 2011; Statistics Canada, 2016). The latest municipal infrastructure survey in the province of Ontario has identified over 100 lagoon systems that are considered ill-equipped to properly treat wastewater to current standards (National Guide to Sustainable Municipal Infrastructure, 2004).These lagoon systems face the common challenge of achieving consistent ammonia removal due to excess loading and low temperature operation. Replacing these low capital cost along with low operation and maintenance cost rural WRRFs with mechanical treatment facilities is often neither cost-feasible in small communities nor is it sustainable in many remote communities where highly trained operator resources are not easily accessible. In this regard, the BioCord biofilm technology is a promising upgrade solution to achieve efficient and stable ammonia removal at rural, municipal lagoon treatment systems. The BioCord technology as an upgrade solution at current operating lagoon facilities would require lower capital investment along with low

operation and maintenance costs. Furthermore, the upgrade BioCord system would require limited operator training. Recent studies have demonstrated its potential capacity for consistent ammonia removal in rivers, lakes and as an upgrade to a wastewater stabilization pond (Feng *et al.*, 2012, 2015; Xu *et al.*, 2012; Yuan *et al.*, 2012; Yang *et al.*, 2014; Gan *et al.*, 2018). However, there is a knowledge gap with respect to our current fundamental understanding of the biofilm and embedded biomass of this technology and subsequently limited knowledge pertaining to the optimal design and operation of this technology as an upgrade system for ammonia removal.

While in urban and peri-urban areas, although established effective biological treatment systems such as activated sludge systems and advanced attached growth systems have been widely employed, these facilities still face the challenge of achieving enhanced ammonia removal with increases in urban and peri-urban population and increases in urban commercial activities. Further, many peri-urban and urban municipal WRRFs face the key challenge of reducing their carbon and energy footprint while maintaining elevated treatment performance. To achieve net-zero emissions in present and future systems energy-efficient processes are urgently needed, which includes energy efficient ammonia removal processes. The anaerobic ammonium oxidation (anammox) process, which was been discovered in the last decades, has shown great potential in energy saving. As compared to traditional nitrification-denitrification processes, anammox combined with the partial nitrification process largely reduces aeration requirement, produces less sludge and there is no need for the addition of an external carbon source. The anammox process has become an important process in sidestream wastewater treatment and a key research topic due to its potential engineering applications for mainstream wastewater. However, one of the main challenges with the anammox process is that the growth rate of anammox bacteria is slow with an estimated doubling time of 11 days (Strous et al., 1998). This has resulted in the significant limitation of not being able to quickly enrich and

retain anammox bacteria in treatment systems and hence not being able to achieve rapid start-up of anammox treatment (Mulder et al., 1995; van de Graaf et al., 1996). The long required start-up time currently limits the installation of anammox systems at the full-scale and restricts their application at wastewater treatment facilities across the world.

Attached growth anammox technologies have been validated at the full-scale and has been installed for sidestream ammonia removal at several full-scale WRRFs in Europe and the US. The attached growth anammox technology provides energy and cost efficient robust treatment with a smaller land footprint compared to suspended growth anammox systems (Christensson et al., 2013; Hollowed et al., 2013; Lackner et al., 2014; Lu et al., 2016). The ANITA Mox technology is a single-stage deammonification (partial nitrification-anammox) process based on the moving bed biofilm reactor (MBBR) technology that has shown promise and is installed as a sidestream anammox system at Sjölunda and Växjö WRRFs in Sweden along with two more installations at Holbæk and Grindsted WRRFs in Denmark. However, the start-up of these attached growth technologies is more complex than suspended growth technologies; as the system requires the attachment of anammox cells to engineered bio-carriers followed by the growth and development of mature anammox biofilm on the carriers (Veuillet et al., 2014). Few studies and limited data exists with respect to appropriate and feasible start-up strategies for all anammox technologies and specifically for the enhanced attachment of anammox cells to carriers followed by the rapid growth and maturation of anammox biofilms during the start-up of attached growth anammox systems such as the ANITA Mox technology (Christensson et al., 2011; Lemaire et al., 2014; Manonmani and Joseph, 2018) This lack of start-up knowledge restricts the current uptake and installation of this energy and cost-effective technology at numerous wastewater treatment facilities in Canada and around the world.

1.2 Research objectives

The overall research objective is to investigate and optimize the performance of two attached growth technologies to meet the current challenges associated with ammonia removal from rural and peri-urban/urban municipal wastewaters. The specific objectives of this research are related to the development of new knowledge at the macro, meso, micro and molecular scales for the nitrifying attached growth BioCord technology as an economical technology to achieve ammonia removal in rural WRRFs and anammox attached growth technology as an economical technology to achieve ammonia removal in peri-urban and urban WRRFs. In particular, this research will (i) investigate the kinetic performance and microbial response of a post-carbon removal, nitrifying BioCord technology at various operational loading and aeration conditions; and (ii) characterize and compare anammox kinetics, biofilm attachment, growth and maturation along with the maturation of the anammox microbial community of two different seeding strategies and carrier effects across the start-up of the anammox attached biofilm technology.

1.3 Thesis organization

This dissertation is written in the form of a manuscript-based thesis as specified by the school of Graduate and Postdoctoral Studies at the University of Ottawa. The organization of this thesis is as follows:

Chapter 2 presents a literature review on fundamental knowledge regarding attached growth (also referred to as biofilm) technologies for ammonia removal via conventional nitrification and the more recently applied anammox process.

Chapter 3 is a published research article entitled "*Nitrifying BioCord reactor: performance optimization and effects of substratum and air scouring*". This article has been published in Environmental Technology in 2019. In this study, ammonia removal kinetics and solids production performance of the BioCord technology are studied. Three nitrifying reactors

housing different BioCord substratum were operated at five different ammonia loading rates. In addition, air scouring was applied at the highest loading rates to study whether the air scouring of the biofilm can reduce performance fluctuations at elevated loadings.

Chapter 4 is a research article entitled "*Meso and micro-scale effects of loading and air scouring on nitrifying BioCord biofilm*". This article has been published in Environmental Science: Water Research & Technology in 2019. This research investigates the performance of the post-carbon removal, nitrifying BioCord biofilm technology at both the meso and micro-scale. In particular, the study focuses on the effects of ammonia loading, various BioCord substratum and air scouring on biofilm mass detachment, biofilm porosity and morphology along with embedded biomass viability.

Chapter 5 is a research article entitled "*Anammox attachment and biofilm development on surface-modified carriers with planktonic- and biofilm-based inoculation*". This article has been submitted for publication in Bioresource Technology in 2020. In this study, two laboratory reactors housing both non-modified virgin MBBR carriers and pre-seeded denitrifying carriers were started-up using two different inoculation methods: anammox biomass detached from the anammox carriers versus a more conventional means of using pre-seeded anammox carriers. This study investigates the kinetics, anammox biomass attachment, biofilm development and anammox bacteria enrichment of a novel detached anammox biofilm inoculation method on the two carrier types and compares these results to the more common use of attached anammox carriers for anammox inoculation.

Chapter 6 is a research article entitled "*Carrier surface modification for enhanced attachment and growth of anammox biofilm*". This article has been submitted for publication in Chemosphere in 2020. This study investigates and compares the kinetic performance, anammox biomass attachment along with the growth and maturation of the anammox biofilm on different MBBR modified carriers. The surface modifications included: carriers coated with

dextran (chemical enhancement); carriers coated with silica crystals (roughness enhancement); pre-seeded carriers sourced from a denitrification plant (pre-biofilm enhancement); and virgin, unmodified carriers (control).

Chapter 7 summarizes the main conclusions of the research along with a discussion of the contributions and practical implications of the research.

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2 Chapter 2 – Literature review

2.1 Biological wastewater treatment

In the natural environment (soil and water), there are a large number of microbes which have a great capacity to oxidize and decompose the organic matter and transform nutrients. Natural water self-purification is achieved through the life-sustaining activities of these microorganisms. Biological wastewater treatment processes are based on this natural process. The biological treatment of water is achieved by engineering an environment conducive to the vital activities of microorganisms, such as a suitable temperature and pH ranges and sufficient nutrients, resulting in mass reproduction of microorganisms, and hence, increasing the efficiency of degrading organic compounds, nutrients and select pollutants. Biological wastewater treatment systems are widely used for the removal of the soluble and colloidal biodegradable organic materials and to reduce the level of nitrogen, phosphorus and other nutrients from municipal and industrial wastewaters (Metcalfe and Eddy, 2003; Mulkerrins et al., 2004; Wiesmann et al., 2006;).

Biological wastewater treatment processes can be classified into suspended growth systems and attached growth systems. In suspended growth systems, the microorganisms grow in suspension in the reactor, and the wastewater is purified by contact with these suspended microorganisms. While in attached growth systems, microorganisms adhere to certain inert media (carriers) to grow and form a biofilm. The sewage becomes purified when wastewater flows across the surface of the biofilm.

2.2 Biological ammonia removal processes

2.2.1 Nitrification

Nitrification is a natural biological ammonia removal process, where ammonia is decomposed and oxidized to nitrate by nitrifying bacteria under aerobic conditions. It is

currently the most common means utilized in Water Resources Recovery Facilities (WRRFs) worldwide for ammonia removal (Metcalfe and Eddy, 2003). The process of nitrification operates in two-steps: first, ammonia is converted into nitrite (Equation 2.1) through the action of autotrophic, ammonia oxidizing bacteria (AOB), the stoichiometric dissolved oxygen (DO) required in this step is 3.43g O_2/g NH_4^+-N; Then, nitrite is further converted into nitrate (Equation 2.2) through the action of autotrophic, nitrite oxidizing bacteria (NOB) and the stoichiometric DO required in this step is 1.14g O_2/g NH_4^+-N. Thus, the DO requirement is 4.57g O_2/g NH_4^+-N for the overall reaction (Equation 2.3). As the process of nitritation (ammonia to nitrite) produces hydrogen ion (Equation 2.1), alkalinity needs to be added to neutralize the solution. The stoichiometric alkalinity consumption is 7.14g -$CaCO_3$/g NH_4^+-N (Metcalfe and Eddy, 2003).

$$NH_4^+ + 1.5\,O_2 \rightarrow NO_2^- + H_20 + 2H^+ \qquad \textbf{Equation 2.1}$$

$$NO_2^- + 0.5\,O_2 \rightarrow NO_3^- \qquad \textbf{Equation 2.2}$$

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + H_20 + 2H^+ \qquad \textbf{Equation 2.3}$$

The rate of nitrification is environment sensitive. It can be affected by DO concentration, alkalinity, pH, temperature, organic compounds concentration and the presence of metals and other toxic compounds (Grady et al. 2011). To achieve nitrification in WRRFs, the above parameters need to be well controlled.

Oxygen is the electron acceptor in nitrification reaction. Therefore, increasing the DO level can accelerate the nitrification kinetics. However, the energy costs increase with the increasing aeration rates. Researchers have found significant nitrification achieved within the DO range of 2.0 to 3.0 mg/L (Nagel and Haworth, 1969; Gerardi, 2002). It is recommended to maintain the bulk DO level above 2.0 mg/L, and below 0.5 mg/L, nitrification tend to stop (Metcalfe and Eddy, 2003). However, recent studies demonstrated that complete nitrification

could be achieved at the DO concentration of 0.5 mg/L (Perk and Noguera, 2004; Bellucci et al., 2011).

Low pH in wastewater affects nitrification kinetics by inhibiting enzymatic activity of nitrifying bacteria. The inhibition is also related to concentrations of free ammonia, NH_3 (FA) and free nitrous acid, HNO_2 (FNA), which are in the equilibriums of NH_4^+ to FA and NO_2^- to FNA that are affected by the pH of the solution (Anthonisen et al., 1976; Wong-Chong and Loehr, 1978; Groeneweg et al., 1994; Park et al., 2010; Munz et al., 2011). The optimum pH range for nitrification is 7.2 to 8.0, and below a pH of 6.7, nitrification kinetics significantly decline (Gerardi, 2002; Metcalf & Eddy, 2003; Ergas and Aponte-Morales, 2013).

Nitrification occurs over a wide range of temperatures (5 - 35℃). As nitrifying bacteria growth rates increase with temperature increasing, the optimum temperatures for nitrifiers are considered between 25 and 30℃ (Watson et al., 1981; Kors et al., 1998; Rabionowitz et al., 2004). Researchers found that nitrification becomes very limited when the temperature in wastewater is below 10℃ and becomes nearly non-exist below 5℃ (Oleszkiewicz and Berquist, 1988; WEF, 2011; Hurse and Connor, 1999). However, recent studies demonstrated that enhanced nitrification rates are attainable at the wastewater temperature as low as 1℃ (Delatolla et al., 2009; Young et al., 2016; Ahmed et al., 2019).

2.2.2 Anaerobic ammonium oxidation (anammox)

Anammox is another nitrogen removal pathway in which anaerobic ammonia oxidizing bacteria use nitrite as an electron acceptor to convert ammonia nitrogen into nitrogen gas with limited nitrate production under anoxic conditions (Equation 2.4). These anammox bacteria are chemoautotrophic, which take bicarbonate as the carbon source for cell synthesis and obtain energy via oxidizing NO_2^- to NO_3^-. The overall stoichiometry for anammox metabolic reaction is shown in Equation 2.5. The anammox process was first confirmed in a denitrifying fluidized bed reactor in mid-1990s (Mulder et al., 1995).

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad \textbf{Equation 2.4}$$

$$1.32NO_2^- + NH_4^+ + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 2.03H_2O + 0.066CH_2O_{0.5}N_{0.15} \quad \textbf{Equation 2.5}$$

As the NO_2^- utilized by anammox bacteria is produced via nitritation, the first step of nitrification (ammonia to nitrite) that has mentioned in above subsection, the practical applications of anammox process for ammonia removal often combines nitritation and anammox. This combination can be performed either in single-stage system, where nitritation and anammox are combined in one reactor, or in two-stage system, where nitritation and anammox process are separated into two reactors (van der Star et al., 2007; Van Hulle et al., 2010; Christensson et al. 2013; Hu et al., 2014). Anammox process is considered as the most energy-effective process. As compared to nitrification-denitrification processes, anammox combined with partial nitrification process requires much less aeration, produces less sludge and there is no need for the addition of an external carbon source. (Tsushima et al., 2007; Kuenen, 2008; Kartal et al., 2010; Tang et al., 2010).

Anammox activity is also environment sensitive. Factors including temperature, DO, pH, substrate concentration and organic compounds concentrations significantly affect the rate of anammox. The optimum temperature range for anammox reaction is between 30 and 40℃ (Strous et al., 1998). Previous studies demonstrated that anammox activity can be maintained at 20℃, while significant decrease in anammox kinetics was observed when the temperature dropped below 15℃ (Isaka et al., 2008; Awata et al., 2012; Narita et al., 2017).

Anammox bacteria are strictly anaerobic, which can be reversibly inhibited at DO concentration above 0.1 mg/L and irreversible inhibition takes place when DO concentration is higher than 1.6 mg/L (Egli et al., 2001). However, as anammox and nitrifying bacteria usually coexist in the system, anammox bacteria could maintain normal activity at DO level higher than 0.2 mg/L as DO is consumed by nitrifying bacteria. As AOB has higher oxygen

affinity than NOB, researchers found that when DO concentration was maintained at 0.5 mg/L at 25 ℃, AOB activity was barely affected, while NOB activity significantly decreased (Hanaki et al., 1990; Hellinga et al., 1998; Jianlong and Ning, 2004). Hence, it is common to operate the anammox process at the bulk DO concentration no greater than 0.5 mg/L.

NH_4^+ and NO_2^- are the essential substrates for anammox bacteria growth. However, anammox bacteria are inhibited at high levels of substrates. Previous studies demonstrated that anammox activity is less affected by NH_4^+, where inhibition only occurs when ammonia concentration is above 600 mg N/L (Carvajal-Arroyo et al., 2013). While NO_2^- has a greater influence on anammox bacteria. Nitrite inhibition was observed in previous studies at nitrite concentrations between 100 and 400 mg NO_2^--N/L (Kimura et al., 2010; Lotti et al., 2012; Puyol et al., 2014). It is believed that FA and FNA are the actual inhibitors of anammox bacteria, as the chemical equilibriums of NH_4^+ to FA and NO_2^- to FNA are affected by the concentration of ammonia and nitrite, as well as the pH value of the solution. When substrates concentrations (NH_4^+ and NO_2^-) increase, the chemical equilibriums proceed toward the direction of FA and FNA formation (Jin et al., 2012). In addition, when the pH value is greater than 8.0 and less than 5.5, the proportion of FA and FNA in the system increases rapidly (Anthonisen et al., 1976). The optimum pH range for anammox is between 6.7 and 8.3 (Jetten et al., 2009).

Generally, single-stage anammox system is suitable for the treatment of wastewater with low C/N ratio (Gao et al., 2012). Excessive concentrations of organic carbon promote the growth of heterotrophic bacteria and thereby limit the space and substrates for anammox bacteria growth (Lawson et al., 2017). Guven et al. (2005) demonstrated an anammox inhibition when C/N ratio in the influent was higher than 1. However, Lackner et al. (2014) pointed out that the removal rate of total nitrogen did not decrease when the C/N ratio in the influent was increased from 1 to 1.5.

2.3 Biofilms

A biofilm is the aggregate of one or more microbial species that are embedded in a self-produced matrix of extracellular polymeric substances (EPS) and that are attached to either an inert or living surface (Carpentier and Cerf, 1993; Costerton et al., 1999; Flemming and Wingender, 2010). Research has revealed that bacteria cells existing in biofilms (sessile form) exhibit properties, behaviors and survival properties that far exceed their capabilities as individual or freely suspended (planktonic form) cells (Davey and O'toole, 2000; Donlan, 2002; Dunne, 2002). Biofilms can be found everywhere where there are adequate environmental conditions: natural environments, industrial environments and even in human bodies. Biofilms were first recognized in wastewaters in the early 20th century (Zobell, 1943) and has now been believed as a key component of biological reactions in wastewater treatment systems. Biofilm formation is a complex process that involves different mechanisms, therefore, the understanding and management of biofilm development, structure and dynamics have become an important concept in improving water and wastewater treatment (Characklis, 1990; Flemming et al., 2000).

2.3.1 Biofilm development

Biofilm development is not a random process but a series of physical, chemical and biological processes. Generally, there are five distinct stages (Figure 2.1):

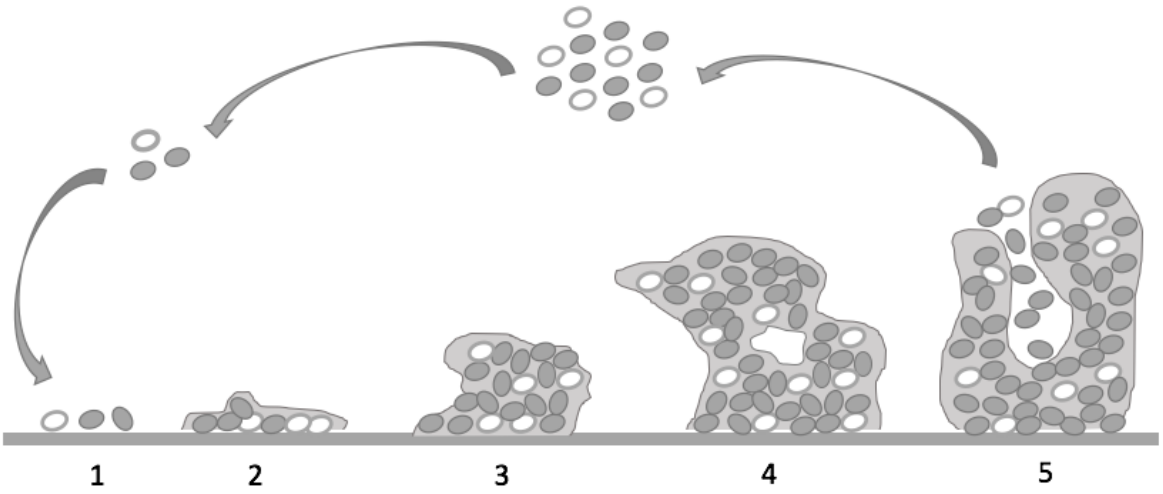

Figure 2.1 Stages of biofilm development: (1) Reversible attachment; (2) Irreversible attachment; (3) Early development of biofilm architecture; (4) Biofilm maturation; (5) Dispersal/detachment. (Adapted from Peg Dirckx and David Davies, 2003. Center for Biofilm Engineering Montana State University).

(1) Reversible attachment. In aquatic environment, the chemical molecules such as proteins and polysaccharides are transported from the bulk liquid phase to a solid surface by hydraulics, a portion of these molecules is adsorbed to the surface quickly forming a conditioning film. This conditioned surface has a higher level of the nutrients compared to the liquid phase and a modified preferential electric charge and, hence attracts bacteria to attach and promote the biofilm formation (Kumar and Anand, 1998). The initial attachment of the planktonic cells to the conditioned surface is referred to as the revisable attachment. The microorganisms are attracted to the conditioned surface through the van der Walls attraction forces. Once the cells drawn to the surface, they must overcome the repulsive forces such as electrostatic forces and hydrophobic interactions. Physical contact between pili, flagella or glycocalyx and the conditioned surface enable the cells to be attached (Donlan, 2001). Some of the microbial cells

may be desorbed due to hydraulic shear, temperature or other environmental factors, whereas the rest become irreversible that remain on the surface (Dunne, 2002; Garrett et al., 2008).

(2) Irreversible attachment. To consolidate the adhesion to the conditioned surface, a permanent attachment mechanism is activated by the primarily attached microorganisms. They produce EPS and become embedded in the polymeric matrix. This permanent attachment of a species consequently promotes adhesion of other species and, microbial aggregates or micro-colonies start forming on the substratum (Watnick and Kolter, 2000; Dunne, 2002).

(3) Early development of biofilm architecture. During this phase, the irreversibly attached microorganisms begin cell reproduction and the micro-colonies become significantly enlarged. These bacteria secrete additional EPS and polysaccharide intercellular adhesion polymers to form strong bonds between cells and a barrier between the community and the extracellular environment and, hence help the colonies stabilize on the surface (Characklis, 1990; Dunne, 2002). At the same time, planktonic cells along with nonliving material such as silt, sand and organics are randomly or specifically recruited to attach to the biofilm from the bulk liquid phase. Specific recruitment is referred to as coaggregation in which genetically distinct bacteria become attached to one another via specific molecules. This process enhances the growth of multi-species biofilms (Rickard et al., 2003).

(4) Biofilm maturation. As the organisms continuously reproduce and the EPS accumulates, the biofilm grows into a three-dimensional structure. The overall density and complexity of the biofilm increases. The growth potential can be limited by the property of the community, the availability of substrate and bulk phase conditions such as temperature, pH and bulk liquid hydraulics. At the end of this phase, a critical mass is reached. Subsequently, dynamic equilibrium is initiated as bacteria located in the outmost layer of growth and, newly generated cells are planktonic and are free to colonize other surfaces (Watnick and Kolter, 2000; Dunne, 2002).

(5) Dispersal/detachment. Dispersal is a detachment process that is initiated once matured growth has occurred and the biofilm is in a state of dynamic equilibrium. The dispersal of planktonic cells or fragments of film is often associated with cells that have the potential to colonize new surfaces. Detachment, then is any mechanism which causes the loss of individual cells or cell masses from the biofilm to the bulk liquid (WEF, 2010). There are four detachment processes including abrasion, erosion, sloughing, and grazing (Metcalf and Eddy, 2003). Abrasion and erosion is the loss of small groups of cells which initiated by particle collision and hydrodynamic shear near the biofilm surface respectively. Both processes commonly occur in well maintained biofilms and have minor changes on biofilm morphology. Whereas sloughing is the loss of large patches of biofilm which usually caused by sudden changes in hydrodynamic stress or a sudden nutrient limitation. Grazing is the biofilm preyed by protozoa or other predators. Sloughing and excessive predation has significant influence on biofilm stability (WEF, 2010).

2.3.2 Mass transfer effects

Diffusion and advection are the most important mechanisms in convective mass transfer. When mass transfer occurs between the fluid and the solid surface, a mass transfer boundary layer (MTBL) with a concentration gradient will be formed around the solid surface (Figure 2.2). In a biofilm reactor, advective transport brings the substrates to the MTBL, where the flow of the bulk liquid is decreased along the interface, and diffusion becomes the major transport mechanism across the MTBL and in the biofilms, which controls the rates of substrates entering and transporting through the biofilm matrix (Rusten et al., 1992; Rittmann and McCarty, 2001; Herrling et al., 2015)

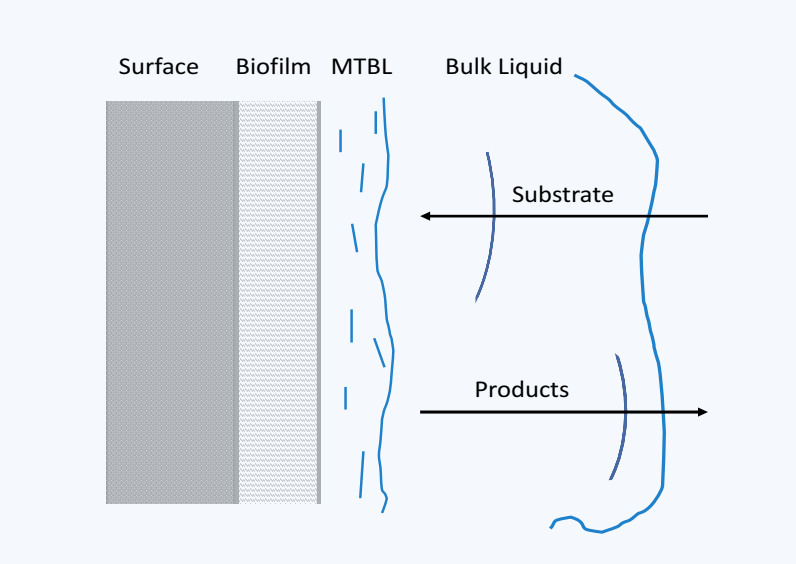

Figure 2.2 Transfer of substrates through the bulk liquid, the mass transfer boundary layer and biofilm.

Each individual substrate diffuses at different rates across the MTBL and into the biofilm, where the diffusion rate is depend on the substrate concentration gradient the concentration gradient between the biofilm and the bulk liquid. Therefore, the treatment efficiency of a biofilm reactor can be limited by the limited diffusion rate which caused by insufficient substrate (i.e. DO, organic carbon, nutrients) concentrations in bulk liquid (Boltz & Daigger, 2010). In addition, the thickness of the MTBL is also critical for the substrate to enter into the biofilm. Minimizing the effective thickness of the MTBL, which can be achieved by increasing the flow velocity, significantly decreases the mass transfer resistance and subsequently increases the mass transfer flux of a substrate through the MTBL (Stewart, 2003; Taherzadeh et al., 2012).

Substrate gradients along with different metabolic rates of various bacteria create different chemical environment in depth and promote a heterogeneous community with stratified biofilm structure (Zhang et al., 1994; Nadell et al., 2009). Taking DO diffusion gradient as an example, aerobic bacteria will grow in the top layers of the biofilm when DO

diffuses into the biofilm, while anoxic and anaerobic bacteria will grow in the deeper layers, where DO diffusion is limited.

2.3.3 Anammox biofilms

The single-stage deammonification process, which combines aerobic nitritation and anoxic ammonia oxidation in a single reactor, is designed based on the theoretical basis of diffusion gradient. As previously mentioned, the nitrite utilized by anammox bacteria is the product of the first step of nitrification, where ammonia oxidized to nitrite via AOB. Therefore, due to the diffusion gradient of oxygen and nitrite, as well as different growth optima for AOB and anammox bacteria, anammox biofilm structures are often stratified (Figure 2.3).

Based on diffusion principles, AOB are located in the top layers of the biofilms, where oxygen and ammonia are not limited. However, the nitrite gradients created by AOB will firstly result in NOB clustering, as oxygen is still available for NOB. Therefore, AOB and NOB often co-exist in anammox biofilms and NOB are more abundant in deeper top layers (Schramm et al., 1996; Okabe et al., ; Kindaichi et al., 2004; Gieseke et al., 2005; Lydmark et al., 2007). Anammox bacteria grow in the inner layers near the surface of the substratum, which is the anoxic zone where the oxygen concentration gradient is down to zero (DO concentration is generally below 0.5 mg/L in anammox systems).

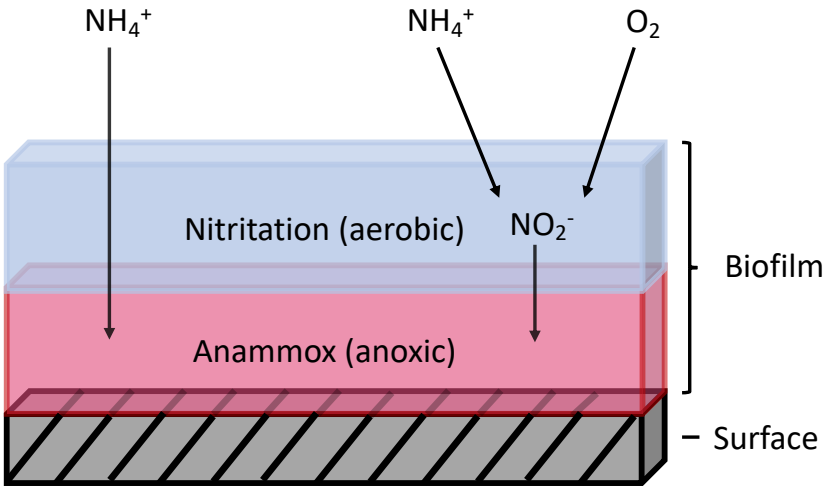

Figure 2.3 Anammox biofilm structure.

2.4 Biofilm technologies for wastewater treatment

Attached growth technologies, also refers to biofilm technologies, for wastewater treatment are designed to promote the growth and maintenance of specific biofilms to achieve targeted wastewater treatment. Wastewater treatment in these systems is achieved with the continuous growth and detachment of the biofilm in the system (Rittmann and McCarty, 2001). Comparing to the conventional activated sludge technology, biofilm technologies shows advantages in their highly effective performance with respect to organic matter and nitrogen removal and strong adaptability to influent fluctuations. In addition, small land footprint and lower quantity of sludge production make biofilm technologies more cost- and energy-effective (Grady et al., 2011). Conventional biofilm processes such as trickling filters and rotating biological contactors (RBC) were studied and constructed in mid 20th century (WEF, 2011; Antonie, 2018). In the past subsequent decades, more advanced biofilm technologies including the BioCord biofilm reactor and the moving bed biofilm reactor (MBBR) have been studied worldwide and have been installed as full-scale WRRFs.

2.4.1 BioCord biofilm reactor

The BioCord or bio-lace is a loop like medium that comprised of a core strand with a number of rings of polymer threads that provides a large surface area for microbial attachment. The BioCord technology is designed to promote the growth of high-density microbial consortia, as biofilm attaches to the housed cord substratum, and hence increases the organic pollutant and nutrient removal rate per reactor volume as comparing to more conventional suspended growth treatment technologies. The BioCord technology has been commonly applied to integrated fixed-film activated sludge (IFAS) systems designed for nitrification and carbon removal (Hamoda et al., 2004; WEF, 2010). The first full-scale pilot installation of the BioCord IFAS system was built in North America in the 1990s (Randall and Sen, 1996; R. Copithorn et

al., 2006; Loupasaki and Diamadopoulos, 2013). In the past decades, biofilm matrixes have been installed in small/medium size rivers and lakes in the eastern countries to promote the purification in water bodies (Xu et al., 2012; Yuan et al., 2012; Feng et al., 2015). The BioCord biofilm reactor has also demonstrated its potential as a standalone system and as an upgrade system to current wastewater treatment facilities (Feng et al., 2012; Yang et al., 2014; Tian and Delatolla, 2019; Tian et al., 2019). However, to date, there is limited application of standalone BioCord system specific for wastewater treatment in North America. Hence few publications on its performance are available. A recent study of the BioCord technology investigated the use of a BioCord reactor as an add-on system installed between two lagoons for carbon and nutrients removal in Canada and demonstrated an outstanding performance of the BioCord system in both carbon and ammonia removal. (Gan et al., 2018)

2.4.2 MBBR

The MBBR is one of the advanced attached growth system that firstly developed in the late 1980s in Norway as engineers and researchers believed this compact biofilm process is the most cost-effective upgrade option to reduce the nitrogen discharge (Ødegaard et al., 1991; Hem et al., 1994) . In the past decades, more than 500 MBBRs have been established worldwide and show great performance on both BOD and nutrients removal (Ødegaard, 2006). In Canada, the MBBR carrier was firstly used in a full-scale wastewater treatment plant as a part of an IFAS nitrification in 1997 to 1998 (WEF, 2011). Recently, other researches have approved a great performance of MBBR as stand-alone system in cold weather (Hoang et al., 2014; Young, Delatolla, et al., 2016).

The key component of this high efficient MBBR system is the pecially designed carriers. These carriers were designed with specific gravity close to water so they can freely float in the reactor and easily keep in a fluidized state by the aeration and water lifting. The carriers also have a large surface area and a protected interior for biofilm growth so as to maintain a high

proportion of active biomass. These two properties ensure a full contact of the attached microorganisms with DO and substrates in the bulk liquid phase, protecting the biofilm from the physical abrasion and enable the carriers to maintain a healthy thickness by constant colliding and fluid shear force in the system. Over the years many manufactures have produced a number of different carriers varied in material, technics and geometrics. The specific surface area available for microbes growth is in a range of 500 - 1200 m^2/m^3. To date, most AnoxKaldnes (an established company) MBBR installations worldwide use K-type carriers which have the bulk specific surface area of 500 m^2/m^3 (Ødegaard, 2006; WEF, 2011).

Many beneficial properties, such as no backwashing, no media clogging, lower head loss and easy operation, make MBBR a favorable alternative to other conventional attached growth system (Andreottola et al., 2000; Metcalf and Eddy, 2003). Furthermore, the MBBR system is easy to expand for future increases in treatment capacity by simply increasing the media percentage fill. The percentage fill is recommended to be between 25 – 70% of the total reactor volume (Rusten et al., 2006).

ANITA Mox – ANITA Mox, by Veolia Water Technologies, is a single-stage deammonification process based on the MBBR technology which conventionally combines aerobic nitritation and anoxic ammonia oxidation (anammox) in a single reactor. The ANITA Mox system may also be operated as a two-stage system with a nitritation reactor followed by an annamox reactor. MBBR carriers with large protective surface area allow anammox bacteria, and in the case of a single reactor system nitrite producing bacteria (AOBs) as well, to develop as a biofilm. The nitritation step oxidzes about 55% of the influent ammonia to nitrite. Subsequently the produced nitrite and the remaining ammonia are utilized by the anammox bacteria and converted to nitrogen gas and a small amount being converted to nitrate (Fux et al., 2004). Specific operation conditions such as controlled mixing and aeration strategies are applied within the Anita Mox reactors to optimize biofilm growth for nitrogen removal. The

ANITA Mox process is often used for treatment of streams highly loaded in ammonia, such as effluents from anaerobic sludge digestion, drying condensates, industrial wastewaters, and landfill leachates (Christensson, et al., 2013) It has been installed at several full-scale water resource recovery facilities (WRRFs) in Europe and the US, with the attached growth ANITA Mox technology providing energy and cost efficient robust treatment with a smaller land footprint compared to suspended growth anammox systems (Christensson et al., 2011; Hollowed et al., 2013; Lemaire et al., 2014; Lu et al., 2016).

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3 Chapter 3 – Nitrifying BioCord reactor: performance optimization and effects of substratum and air scouring

3.1 Setting the context

Chapter 3 presents the published research article entitled *Nitrifying BioCord reactor: performance optimization and effects of substratum and air scouring* by Tian, X., Ahmed, W. and Delatolla, R. (Environmental Technology, 2019. 40(4), 480–488). It investigates the effects of ammonia loading, three BioCord substrata and the influence of air scouring on ammonia removal kinetics and solids production of the post-carbon removal nitrification BioCord reactors.

3.2 Abstract

Ammonia removal kinetics and solids production performance of the BioCord technology are studied in this research. Three nitrifying reactors housing different BioCord substratum were operated at five different ammonia loading rates. All of the BioCord substrata demonstrated stable and high ammonia nitrogen removal efficiencies of 96.8 ± 0.9, 97.0 ± 0.6and 92.0 ± 0.4% at loading rates of 0.8, 1.6 and 1.8 g NH_4^+-N/m^2·d, respectively. At these same loading rates, the BioCord reactors housing the three substrata also showed low solids production rates of 0.19 ± 0.03, 0.23 ± 0.02, 0.25 ± 0.03 g TSS/d. A reduction of system stability, identified via fluctuating ammonia removal rates was however observed for all substrata at loading rates of 2.1 and 2.4 g NH_4^+-N/m^2·d. Further, the solids production rates at these higher loading conditions were also observed to fluctuate for all substrata, likely indicating intermediate sloughing events. The effects of enhancing the air scouring of the BioCord on the ammonia removal rate was shown to be dependent upon the substratum, while enhanced air scouring of the BioCord was shown to stabilize the production of solids for all

substrata. This study represents the first performance and optimization study of the BioCord technology for tertiary, low carbon nitrification and shows that air scouring of the substratum reduces sloughing events at elevated loading rates and that the BioCord technology operated with various substratum achieves stable nitrification kinetics above conventional rates of 1 g NH_4^+-N/ $m^2 \cdot d$ to values of 1.8 g NH_4^+-N/ $m^2 \cdot d$.

3.3 Introduction

The wastewater industry is facing challenges of increased loading, aging infrastructure, constricted land availability and financial constraints (Novotny, 2010; Elshafie *et al.*, 2012). These challenges have directly resulted in the need for new, compact, economical biological wastewater treatment technologies. Further, to preserve water supply, various countries are implementing more stringent wastewater effluent regulations with respect to ammonia release into natural waters (EEC, 1991; Canada Gazette, 2012; USEPA, 2014). Ammonia removal is conventionally removed from municipal wastewaters using nitrification, the biologically mediated oxidation of ammonia to nitrate via nitrite (USEPA, 2014).

Within the past two decades, biofilm attached growth treatment technologies have demonstrated small land footprints and less solids production as comparing to conventional activated sludge (CAS) treatment (Hamoda *et al*., 2004; Elshafie *et al.*, 2012; Loupasaki and Diamadopoulos, 2013). An important component of biofilm treatment technologies is the substratum that is used for the attachment and growth of the biofilm. The BioCord or bio-lace technology is comprised of a core strand with a number of rings of polymer threads that provides a large surface area for microbial attachment. BioCord has been used for nitrification and organic pollutant removal, with the most common applications being its incorporation into fixed-film activated sludge systems (IFAS) (WEF, 2010). The first full-scale installations of the BioCord IFAS system were built in North America during the 1990s (Sen, 1995; Randall and Sen, 1996). BioCord also shows promise for nitrogen removal as a standalone system and

as an upgrade systems to current wastewater treatment facilities (Metcalf and Eddy, 2003; Feng *et al.*, 2012; Yuan *et al.*, 2012; Yang *et al.*, 2014). However, as the BioCord system is limited in its installations as a standalone wastewater treatment technology, there are few publications on its nitrifying performance. In particular, the limited number of studies reported on nitrifying BioCord systems are largely related to the treatment of polluted river water, with ammonia concentrations of approximately 4 mg NH_4^+-N/L being treated (Feng et al., 2015; Xu et al., 2012; Yuan et al., 2012). The ammonia removal efficiencies are reported to range between 55% to 80% removal for the treatment of polluted river waters. Therefore, a knowledge gap currently exists with respect to performance and optimization studies of the BioCord technology for wastewater nitrification.

Further studies on the BioCord system are hence required to understand its potential as an economical, small footprint, nitrifying upgrade technology to current wastewater treatment facilities. In particular, the effects of loading, various types of substratum and air scouring on the system's performance has been shown to affect the removal rate and solids production of other biofilm technologies (Ødegaard *et al*., 2000; Ivanovic *et al*., 2006; Aygun *et al.*, 2008; Delatolla *et al.*, 2012; Zhang *et al.*, 2013; Karizmeh *et al*., 2014; Zupančič *et al.*, 2014; Forrest *et al.*, 2015; Young *et al.*, 2016). As such, the main objective of this study is to optimise the performance of the BioCord technology through investigating the effects of ammonia loading, three BioCord substrata and the influence of air scouring on both kinetics and solids production. This study represents the first performance and optimization study of the BioCord technology for nitrification. The study is performed at the laboratory-scale using synthetic wastewater in order to isolate the effects of the above listed design and operating parameters on the performance of a low-carbon, nitrifying BioCord system.

3.4 Materials and methods

3.4.1 Experimental set-up

Three laboratory scale continuous stirred tank reactors (CSTRs) with an effective volume of 3.17 L were used to house various types of BioCord substratum. In particular, 0.24 m of PP+K-45 BioCord (vinylon and polypropylene; specific surface area: 1.6 m^2/m, Bishop Water Technologies, Canada), 0.24 m of MK-PP50 BioCord (polypropylene; specific surface area: 1.6 m^2/m, Bishop Water Technologies, Canada) and 0.16 m of SP-100 BioCord (vinylon and polypropylene; specific surface area 2.4 m^2/m, Bishop Water Technologies, Canada) were fixed to stationary stainless steel bars in reactor 1 (R1), reactor 2 (R2) and reactor 3 (R3), respectively. The total surface area of the BioCord readily available for microbial attachment in each reactor was hence set at 0.384 m^2 in each reactor.

The laboratory set-up of the BioCord reactors included a reservoir feed tank, two peristaltic pumps, three aerated cylindrical reactors with air diffusers fixed at the bottom used for oxygen supply and mixing, and an effluent collection tank (Figure 3.1). All three reactors were operated under consistent hydraulic retention time (HRT), dissolved oxygen (DO), pH and temperature conditions throughout the experiments. HRT was maintained constant at 3 hours; DO concentration was maintained at 7.0 ± 0.8 mg/L; pH was 7.1 ± 0.2; and the temperature was 23.0 ± 1.0°C. The three BioCord reactors with various substratum were operated at five surface area loading rates and with various aeration configurations to optimize these design parameters and hence enhance the technology's performance. In particular, the three reactors were operated at surface area loading rates (SALRs) of 0.8, 1.6, 1.8, 2.1 and 2.4 g NH_4^+-N/m^2·d. In addition, the three reactors were operated at an SALR of 2.4 g NH_4^+-N/m^2·d with enhanced air scouring; where enhanced air scouring was achieved by positioning the air bubble diffuser directly under the BioCord substratum in the BioCord reactor. Each of the SALR conditions, in addition to the enhanced air scouring condition at 2.4 g NH_4^+-N/m^2·d,

was operated for a minimum of three weeks and until steady state conditions were achieved; with steady state conditions being defined as less than 10% fluctuation of effluent ammonia-nitrogen (NH_4^+/NH_3-N), nitrite-nitrogen (NO_2^--N), nitrate-nitrogen (NO_3^--N) and soluble chemical oxygen demand (sCOD) concentrations. Following steady state operation, the reactors were operated for an additional 12 to 15 days to characterise kinetics and solids production of the reactors.

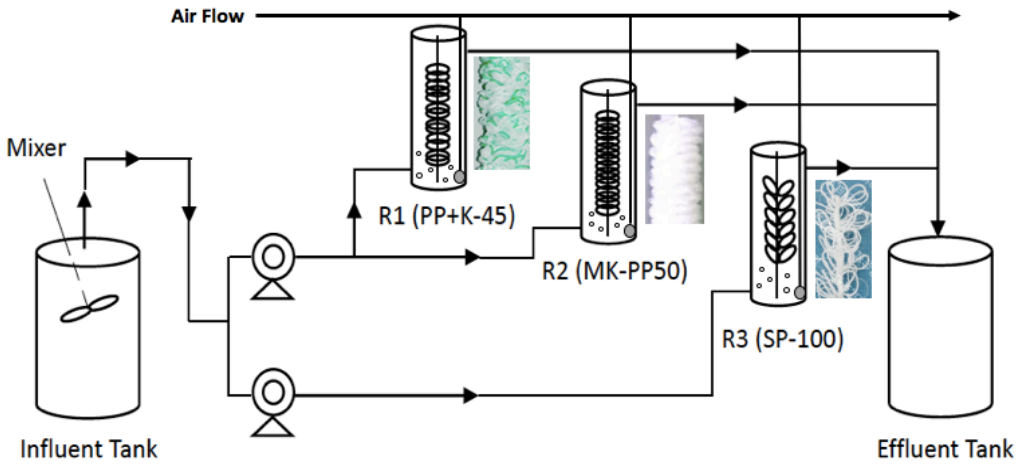

Figure 3.1 Experimental set-up and BioCord images of PP+K-45, MK-PP50 and SP-100.

3.4.2 Wastewater source

The laboratory reactors were fed with synthetic wastewater, which simulate conventional domestic wastewaters following secondary carbon removal without nitrification. The recipe used in this study is a modified version of Delatolla et al.(2009) and has been more recently used in subsequent publications that have demonstrated comparative ammonia removal rates with real domestic wastewater (Almomani *et al.*, 2014; Hoang *et al.*, 2014; Forrest *et al.*, 2015; Young, *et al.*, 2016). The specific composition of the synthetic wastewater at the ammonia SALR of 1.8 g NH_4^+-N/m^2·d is as follows: $(NH_4)_2SO_4$: 128.55 mg/L, $NaHCO_3$: 354.32 mg/L, $MgSO_4 \cdot 7H_2O$: 70.98 mg/L, $CaCl_2 \cdot 2H_2O$: 29.34 mg/L, KH_2PO_4: 79.09 mg/L, $FeSO_4 \cdot 7H_2O$: 4.98 mg/L. Trace nutrients: $MnCl_2 \cdot 4H_2O$: 0.100 mg/L, $Na_2MoO_4 \cdot 2H_2O$: 0.025

mg/L, $CuSO_4 \cdot 5H_2O$: 0.103 mg/L, $CoCl_2 \cdot 6H_2O$: 0.001 mg/L, $ZnSO_4 \cdot 7H_2O$: 0.030 mg/L, and carbon source: glucose: 3.87 mg/L, sodium acetate 2.06 mg/L, peptone 3.87 mg/L. As the carbon source is biodegradable, the COD: BOD ratio of the synthetic wastewater is 1:1. The calculated sCOD concentration was kept as 10 mg/L constantly when adjusting the ammonia loading. The wastewater composition was adjusted at SALRs of 0.8, 1.6, 2.1 and 2.4 g NH_4^+-N/$m^2 \cdot$d.

3.4.3 Biofilm inoculation of BioCord

The inoculum source of the BioCord reactors was collected from an attached growth nitrifying reactor. The total suspended solids (TSS) of the inoculum source wastewater was concentrated to 1500 mg TSS/L prior to being added to the BioCord reactors. The reactors were inoculated with 50 ml of concentrated nitrifying sludge (1500 mg TSS/L) each day for four consecutive days, while being fed at an ammonia loading rate of 0.4 g NH_4^+-N/$m^2 \cdot$d. After the four days of the inoculation phase biofilm growth was observed on the three BioCords. Following the fourth day of inoculation, the reactors were operated at a low ammonia loading rate of 0.4 g NH_4^+-N/$m^2 \cdot$d for approximate three weeks until 90% NH_4^+-N removal efficiency was achieved; and subsequently the ammonia loading rate of the reactors was increased to the experimental loading conditions.

3.4.4 Analytical methods

The following parameters were triplicate tested in the reactors throughout the study: NH_4^+-N, NO_2^--N, NO_3^--N, sCOD, TSS, volatile suspended solids (VSS), DO, pH and temperature. All nitrogen constituents and solids were measured in accordance with standard methods (APHA, 1995). In particular, the following methods were used in this study: 4500-NH_3, 4500-NO_3^- B, 4500-NO_2^-, 2540 D-Total Suspended Solids (TSS dried at 103-105˚C) and 2540 E-Volatile Solids and Volatile Suspended Solids (fixed and volatile solids ignited at 550˚C). The sCOD was analyzed using a DR 5000 spectrophotometer (HACH, Loveland, CO)

after 0.45 μm filtration using a Millipore G filter. DO, pH and temperature were measured using a Multi Parameter Meter with an attached DO probe and pH probe (VWR, Canada, Ontario), and a mercury glass thermometer, respectively.

3.4.5 Statistical methods

The t-test was used to validate significant statistical differences of ammonia concentrations, ammonia removal rates, ammonia removal efficiencies, solids concentrations and solids production rates, with a *p*-value less than 0.05 indicating significance in this study.

3.5 Results and Discussion

3.5.1 SALR and BioCord substratum effects on surface area removal rate (SARR)

The ammonia removal kinetics were investigated at SALRs of 0.8, 1.6, 1.8, 2.1 and 2.4 g NH_4^+-N/m^2·d, respectively. The conversion from ammonia to nitrate was almost complete at all experimental conditions, with a maximum nitrite concentration of 0.42 ± 0.10 mg of NO_2^--N/L being observed at an SALR of 2.4 g NH_4^+-N/m^2·d. Nitrogen mass balance errors of less than ± 10% were observed at all loading conditions of the study.

During the first two loading phases of 0.8 and 1.6 g NH_4^+-N/m^2·d, all three reactors performed similarly and demonstrated an elevated and stable surface area removal rate (SARR) and elevated and stable removal efficiencies of approximately 97% with small variations (Table 3.1 and Figure 3.2). It should be noted that the discrepancy between the averaged SALR and SARR values shown in Table 3.1 compared to the percent removal values shown in the table are due to the fact that the SARR and percent removal efficiency were independently calculated at each time of sampling. The effluent ammonia concentrations from all three reactors housing different BioCord substratum were 0.45 mg NH_4^+-N/L and 0.61 mg NH_4^+-N/L for SALR 0.8 g NH_4^+-N/m^2·d and 1.6 g NH_4^+-N/m^2·d, respectively. As demonstrated by the measured SARR, percent removal efficiency and effluent ammonia concentrations, the

various BioCord substrata demonstrated no statistical difference in operational kinetics at SALRs of 0.8 g NH_4^+-N/m^2·d and 1.6 g NH_4^+-N/m^2·d.

Table 3.1 SARR and percent removal efficiency at various SALRs; averages and 95% confidence intervals.

SALR (g NH_4^+-N/m^2·d)	Reactor (substratum)	SARR (g NH_4^+-N/m^2·d)			Percent Removal Efficiency (%)
		Average	Minimum	Maximum	
0.8 ± 0.02	R1 (PP+K-45)	0.79 ± 0.02	0.78	0.81	96.8 ± 1.9
	R2 (MK-PP50)	0.80 ± 0.01	0.80	0.81	98.1 ± 1.2
	R3 (SP-100)	0.78 ± 0.01	0.77	0.79	95.5 ± 1.8
1.6 ± 0.01	R1 (PP+K-45)	1.57 ± 0.03	1.54	1.59	96.8 ± 2.1
	R2 (MK-PP50)	1.56 ± 0.02	1.55	1.57	96.8 ± 1.2
	R3 (SP-100)	1.57 ± 0.03	1.55	1.59	97.2 ± 1.6
1.8 ± 0.03	R1 (PP+K-45)	1.66 ± 0.03	1.59	1.72	92.6 ± 0.6
	R2 (MK-PP50)	1.64 ± 0.03	1.60	1.73	91.6 ± 0.9
	R3 (SP-100)	1.65 ± 0.02	1.59	1.69	91.9 ± 1.1
2.1 ± 0.12	R1 (PP+K-45)	1.96 ± 0.14	1.65	2.20	92.2 ± 5.2
	R2 (MK-PP50)	1.75 ± 0.20	1.36	2.08	81.6 ± 10.6
	R3 (SP-100)	1.46 ± 0.32	0.99	2.07	69.5 ± 15.2
	R1 (PP+K-45)	2.11 ± 0.17	1.69	2.39	86.6 ± 7.7
2.4 ± 0.04	R2 (MK-PP50)	2.05 ± 0.24	1.39	2.40	84.3 ± 10.1
	R3 (SP-100)	1.93 ± 0.23	1.48	2.38	79.3 ± 10.1
Aeration effects: by air scouring					
2.4 ± 0.03	R1 (PP+K-45)	2.10 ± 0.15	1.65	2.36	86.4 ± 5.7
	R2 (MK-PP50)	2.19 ± 0.09	1.87	2.32	90.3 ± 3.5
	R3 (SP-100)	1.45 ± 0.43	0.45	2.21	59.7 ± 17.4

During the third operational SALR of 1.8 g NH_4^+-N/m^2·d, the reactors again demonstrated an ability to maintain elevated SARRs. However, the removal efficiencies were shown to decline compared to the lower loading conditions, with a statistically significant decrease in the removal efficiency being shown compared to the removal efficiencies measured at SALRs of 0.8 and 1.6 g NH_4^+-N/m^2·d (Table 3.1). The effluent ammonia concentrations from all reactors at an SALR of 1.8 g NH_4^+-N/m^2·d increased to an average of 2.15 ± 0.36 mg NH_4^+-N/L as compared to 0.45 ± 0.11 mg NH_4^+-N/L and 0.61 ± 0.12 mg NH_4^+-N/L for SALR 0.8 g NH_4^+-N/m^2·d and 1.6 g NH_4^+-N/m^2·d, respectively. At an SALR of 1.8 g NH_4^+-N/m^2·d the various BioCord substrata again demonstrated no statistical differences in SALR, percent ammonia removal efficiency or effluent ammonia concentrations. The statistically significant decrease in percent ammonia removal efficiency at an ammonia-nitrogen SALR of 1.8 g NH_4^+-N/m^2·d and the transition from a first order kinetic relationship between SARR and SALR to a zero-order kinetic relationship at loadings greater than 1.8 g NH_4^+-N/m^2·d is indicative of the transition from an ammonia mass transfer rate limited biofilm to an oxygen mass transfer rate limited biofilm (Figure 3.2) (Ødegaard, 2006). Hence to increase the ammonia removal rate at an ammonia loading rate of 1.8 g NH_4^+-N/m^2·d or above, an increase in DO is necessary.

The highest SALRs studied in this research of 2.1 g NH_4^+-N/m^2·d and 2.4 g NH_4^+-N/m^2·d were tested to quantify the maximum ammonia SARR of the BioCord technology and develop an understanding of the performance of the BioCord reactor under elevated loadings and DO mass transfer rate limited operation. As the tests performed at SALRs of 2.1 g NH_4^+-N/m^2·d and 2.4 g NH_4^+-N/m^2·d did not show a statistically significant decrease in SARR or ammonia percent removal efficiency compared to the performance of the reactors at an SALR of 1.8 g NH_4^+-N/m^2·d, the design curve in Figure 3.2 is set at a maximum SARR of 1.65 g NH_4^+-N/m^2·d (the maximum statistically significant quantity measured in this study). It should be noted however, that above a loading of 1.8 g NH_4^+-N/m^2·d the three substrata show high

variance in operation that prevent statistical distinction at various SALRs and between various BioCord substrata. This variance in the SARR of the reactors is indicative of system instability at elevated loading rates.

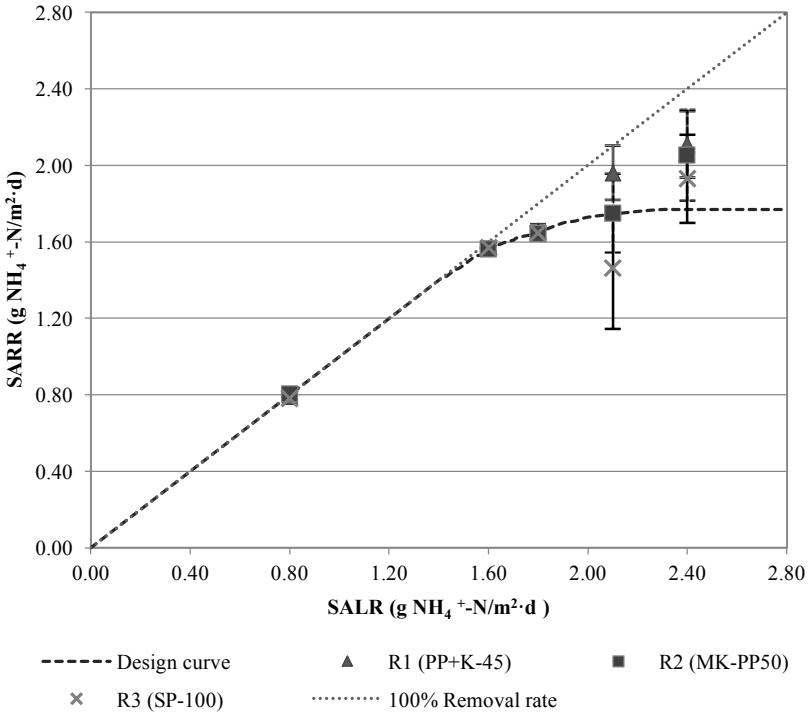

Figure 3.2 Average and 95% confidence intervals of SARR versus SALR; influent sCOD = 10 mg/L, DO = 7.0 ± 0.8 mg/L, pH = 7.1 ± 0.2, temperature = 23.0 ± 1.0°C.

The operational instability of the three BioCord reactors housing the three distinct substrata can be clearly identified in Table 3.1 and Figure 3.3 by the minimum, maximum and variance of the average SARRs at elevated loadings. The unstable operation resulted in minimum ammonia-nitrogen effluent concentrations of 0.91 and 0.76 mg NH_4^+-N/L at SALRs of 2.1 and 2.4 g NH_4^+-N/m^2·d and maximum ammonia-nitrogen effluent concentrations of 17.58 and 15.15 mg NH_4^+-N/L, respectively. This instability is further illustrated in Figure 3.2, which compares the SARR of the three reactors at SALRs of 1.8 and 2.1 g NH_4^+-N/m^2·d. The increase in SARR fluctuation at elevated SALR is observed for all BioCord substrata. The instability in SARR may be associated with sloughing events becoming increasing prevalent at

elevated loading conditions. The sloughing characteristic of higher loaded BioCord systems results in varying biofilm thicknesses. Higher SARR values are likely associated with thinner biofilms conditions that promote more efficient mass transfer while lower SARR values are likely associated with thicker biofilms that occur prior to sloughing. Further, the SARR variance measured for the three substrata is not consistent at the elevated SALRs of 2.1 g NH_4^+-N/m^2·d and 2.4 g NH_4^+-N/m^2·d. Hence, a distinction between the BioCord substrata is observed with the SP-100 (R3) substratum showing the largest variance in performance and hence the greatest operational instability at elevated loadings (Figure 3.3).

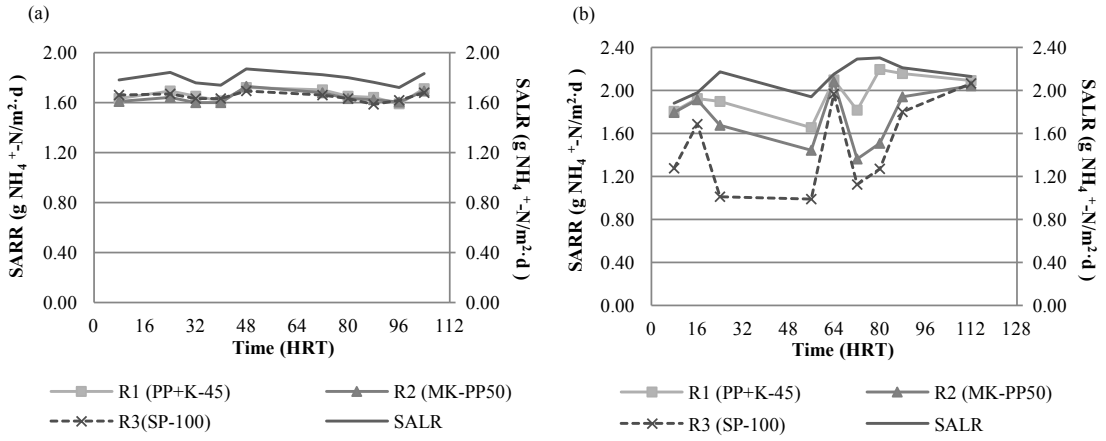

Figure 3.3 SARR across HRT, (a) SALR = 1.8 g NH_4^+-N/m^2·d; (b) SALR = 2.1 g NH_4^+-N/m^2·d.

3.5.2 Air scouring effects on SARR

The effects of air scouring the biofilm on the nitrifying removal rate was determined in this study by changing the position of the aerator in the reactors from against the sidewall of the reactors to directly under the BioCord substratum (Figure 3.1). The ammonia loading rate during the air scouring experiments was maintained at the highest SALR tested in this study, 2.4 g NH_4^+-N/m^2·d, to investigate the potential of air scouring to stabilize ammonia removal efficiencies and reduce the variation in the removal rates. Table 3.1 and Figure 3.4 demonstrate that the effects of air scouring of the biofilm at an elevated loading of 2.4 g NH_4^+-N/m^2·d did not show a statistically significant improvement in removal due to the high variation in the

removal efficiencies at elevated loading conditions. This finding is supported by Elenter (2007), who showed that biofilm sloughing had no significant influence on organic substrate removal or ammonia oxidation.

Although no statistically significant improvement in the averaged SARR during air scouring was observed in this study, it was observed that an improved SARR stability and a reduction in SARR variance was BioCord substratum dependent. R1 with substratum PP+K-45 demonstrated the least variation in SARR at elevated loading conditions prior to applying scouring. Hence the applied effects of scouring did not demonstrate a significant improvement for substratum PP+K-45, possibly due to the limited instability of this substratum even at elevated loadings. R2 with substratum MK-PP50 did not show a statistically significant change in SARR, however this substratum did demonstrate a 66% reduction in performance viability demonstrating the potential of scouring to improve the operation of highly loaded nitrifying BioCord systems housing the MK-PP50 substratum. While R3 with substratum SP-100 also did not show a statistically significant change in SARR, contrarily this substratum demonstrated a 73% reduction in performance viability. Hence, the study demonstrates that scouring is potentially detrimental to the operation of highly loaded nitrifying BioCord systems housing the SP-100 substratum. These contradictory results are indicative that further information regarding the morphology and structure of the biofilm attached to the various substrata may be required to understand the effects of air scouring on BioCord systems.

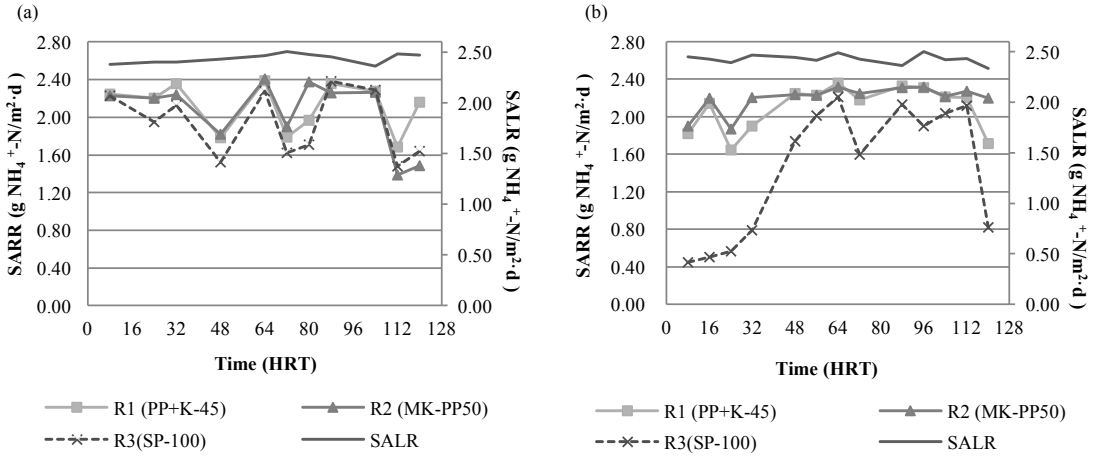

Figure 3.4 SARR of each reactor at (a) SALR = 2.4 g NH_4^+-N/m^2·d without air scouring; (b) SALR = 2.4 g NH_4^+-N/m^2·d with air scouring.

3.5.3 SALR effect on solids production

This study used synthetic wastewater void of suspended solids to feed the laboratory reactors in order to eliminate the masking effects of influent solids and accurately measure the solids production of different BioCord substrata. Hence the measured solids in the effluent of each reactor were biologically produced by the nitrifying biofilm attached to the various BioCords. The average and 95% confidence interval effluent TSS concentration of each reactor slightly increased from 8 8.1 ± 1.7 to 10.6 ± 2.3 mg/L for R1 (PP+K-45), from 8.4 ± 1.8 to 10.2 ± 1.9 mg/L for R2 (MK-PP50), and from 5.6 ± 0. to 9.3 ± 1.7 mg/L for R3 (SP-100) as the SALR increased from 0.8 to 1.8 g NH_4^+-N/m^2·d (Figure 3.5). This increase in TSS concentration at higher loadings is expected due to increased microbial activity and is in agreement with the findings of Forrest (2015) on tertiary nitrogen removal moving bed biofilm reactor (MBBR) systems. However, it should be noted that the average effluent TSS concentration of the BioCord reactors was approximately 40% less than MBBR solids production rates reported at similar loading conditions. The lower loading rates of 0.8 to 1.6 and 1.8 g NH_4^+-N/m^2·d demonstrated limited variance in solids production rate of 0.19 ± 0.03, 0.23 ± 0.02, 0.25 ± 0.03 g TSS/d, respectively across time as is demonstrated by the small

variance in the data sets. The steady production of solids at lower loading rates coincided with stable removal rates, which supports the finding of steady performance of the BioCord systems at these lower loading conditions irrespective of substratum.

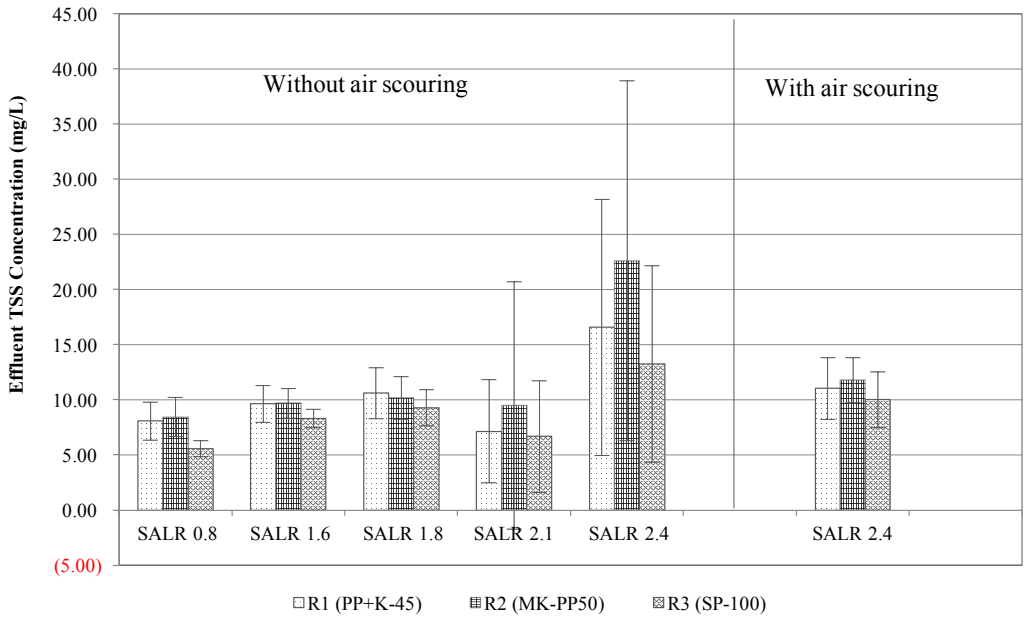

Figure 3.5 Average and 95% confidence intervals of effluent TSS concentration at different SALRs.

The detachment of the biomass was shown to increase linearly with increased nitrogen loading at lower loading conditions (Figure C-3.1). This linear relationship between TSS production and loading rate observed at loading rates of 0.8 to 1.6 and 1.8 g NH_4^+-N/ m^2·d is in agreement with previous studies on COD removal MBBR systems (Orantes *et al*., 2003; Aygun *et al*., 2008). Hence the nitrifying detached biofilm yield for R1, R2 and R3 are 0.51 ± 0.13, 0.52 ± 0.16 and 0.40 ± 0.05 g TSS/g-N, respectively, with the effluent solids VSS to TSS ratio being 0.78 ± 0.12.

As the SALR increased to 2.1 and 2.4 g NH_4^+-N/m^2·d, the measured effluent TSS concentrations demonstrated fluctuations in concentration (Figure 3.5). The larger 95% confidence intervals at loadings of 2.1 and 2.4 g NH_4^+-N/m^2·d are indicative of the unstable

performance of all three reactors at these higher loading conditions. In particular, the effluent TSS concentrations measured at a loading of 2.1 NH_4^+-N/m^2·d ranged between 1.0 - 20.2, 1.4 - 47.6 and 1.6 - 18.6 mg/L for R1, R2 and R3, respectively. During the subsequent higher loading condition of 2.4 g NH_4^+-N/m^2·d, the effluent TSS concentrations ranged between 8.5 - 36.8, 6.3 - 42.0, 7.0 - 28.9 mg/L for R1, R2 and R3, respectively. The instability in the solids production at higher loading conditions in this study coincides with instability in the SARR performance of the reactors (Figure 3.3). The reason for such instability is likely due to sloughing events. In particular, measured peaks in SARR values are likely associated with sloughed biofilms that are thinner and promote more efficient mass transfer during an incremental time of operation. Previous studies have demonstrated that high surface substrate loading rates can result in higher growth rates, thicker biofilms and weaker biofilms that trigger an instability in biofilm accumulation and result in a large biomass loss (Characklis, 1990; Peyton and Characklis, 1993; Tijhuis *et al.*, 1996; Picioreanu *et al*., 2001; Telgmann *et al*., 2004). Fluctuations in solids production often presents difficulties in downstream sedimentation units.

3.5.4 Air scouring effects on solids production

Comparing the solids production of the three substrata operated at an SALR of 2.4 g NH_4^+-N/m^2·d without enhanced air scouring (position of the aerator against the sidewall) and with air scouring (position of the aerator directly under the BioCord substratum) demonstrated an improved stability in solids production for all three substrata (Figure 3.5). Stability in solids production is indicated by a reduced fluctuation in TSS concentration (Figure 3.5). The stabilized effluent TSS concentrations indicate that sloughing events were minimized due to air scouring at elevated operation. This finding is supported by previous work demonstrating that air scouring enhances biofilm detachment forces and accelerates the detachment of ageing biofilms while maintaining thinner biofilms that are less susceptible to sloughing (Kwok *et al.*,

1998). In this study, no evident statistical change was observed in the average SARR or TSS production with air scouring. However, sloughing events along with subsequent variances in removal rates and effluent solids concentrations were shown to be reduced for specific BioCord substratum operated at elevated loading conditions while exposed to enhanced air scouring.

3.6 Conclusion

The study focuses on the performance of stand-alone BioCord biofilm reactors for tertiary, post carbon removal nitrification. The work isolated the effects of ammonia SALR, various BioCord substrata and air scouring on nitrification kinetics and solids production in order to evaluate the performance and optimization strategies for the BioCord technology. The application of various SALRs demonstrate that BioCord substrata PP+K-45, MK-PP50 and SP-100 may be efficiently operated at SALRs up to 1.8 g NH_4^+-N/ m^2·d, providing steady ammonia nitrogen removal efficiencies greater than 90%. The efficient and steady operational rate of 1.8 g NH_4^+-N/ m^2·d demonstrates a recommended design rate above conventional nitrification attached growth design rates of 1 g NH_4^+-N/ m^2·d. The BioCord reactors housing the three substrata showed low solids production rates of 0.19 ± 0.03, 0.23 ± 0.02, 0.25 ± 0.03 g TSS/d, at 0.8, 1.6 and 1.8 g NH_4^+-N/m^2·d, respectively. A loss of system stability, identified as fluctuating ammonia removal rates and solids production rates, were however observed for all substrata at elevated loading rates of 2.1 and 2.4 g NH_4^+-N/m^2·d. The fluctuation in solids production is likely indicative of sloughing events. The effects of air scouring of the BioCord on the ammonia removal rate was shown to be dependent upon the substratum. Enhanced air scouring of the BioCord as an operational optimization strategy was shown to stabilize the production of solids at elevated SALR for all substrata through significantly reduced fluctuations in biofilm detachment and effluent solids concentrations.

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4 Chapter 4 – Meso and micro-scale effects of loading and air scouring on nitrifying BioCord biofilm

4.1 Setting the context

Chapter 4 presents the published article entitled *Meso and micro-scale effects of loading and air scouring on nitrifying BioCord biofilm* by Tian, X. and Delatolla, R. (Environmental Science: Water Research & Technology, 2019. 5(6) 1183-1190). This study investigates the effects of various ammonia SARR, air diffusion modification and specific substratum types on kinetics performance and studies the response of the biofilm porosity, the biofilm morphology and the biomass viability to these design and operational parameters.

4.2 Abstract

The municipal wastewater treatment industry is currently facing the challenges of increased loading, restricted land availability and stringent discharge regulations. This results in the need for new, compact and cost-effective biological wastewater treatment technologies. The study focuses on the performance of the stand-alone BioCord biofilm reactors for post carbon removal nitrification at both the meso and micro-scale. In particular, it investigates the effects of ammonia loading, various BioCord substratum and air scouring on nitrifying kinetics, biofilm mass detachment, biofilm porosity and morphology along with embedded biomass viability. At low to moderate loadings of 0.8 g NH_4^+-N/m^2·d and 1.6 g NH_4^+-N/m^2·d, the various substratum show stable and high ammonia removal efficiencies of approximately 97%, while unstable performance was observed at a high ammonia loading of 2.4 g NH_4^+-N/m^2·d for all substratum. Air scouring of the substratum as a means of counteracting instability did not stabilize the performance of the system. Biofilm detachment rates were observed to increase with increasing

loading rate. Improved detachment stability was observed with applied air scouring of the substratum. Biofilm porosity was shown to decrease with increasing ammonia loading rates for all substratum, with air scouring of the substratum showing an increase in the biofilm porosity. Embedded biomass viability testing shows that the activity of the bacterial cells and not cell coverage is related to kinetic performance.

4.3 Introduction

Municipal wastewater treatment plants are point source polluters that release large quantities of deleterious substances and nutrients to aquatic environments. The excessive quantity of nutrients can cause eutrophication and various other detrimental impacts in surface waters. Particularly, free ammonia-nitrogen is toxic to fish and numerous other aquatic organisms. Therefore, various countries are implementing more stringent wastewater effluent regulations with respect to the discharge of ammonia (Canada Gazette, 2012; EEC, 1991; Murdoch *et al*., 2000; USEPA, 2014). Hence, new, compact and cost-effective biological wastewater treatment technologies are required to meet these stringent regulations at locations with limited land and capital.

Within the past few decades, attached growth, biofilm technologies have demonstrated smaller land footprints and reduced solids production as comparing to conventional, suspended growth systems (Elshafie *et al.*, 2012; Hamoda *et al*., 2004; Loupasaki and Diamadopoulos, 2013). These technologies have shown to have a potential as nitrifying upgrade systems to passive lagoon treatment systems (Almomani *et al.*, 2014; Delatolla *et al.*, 2010). The BioCord or bio-lace technology, an attached growth technology housing cord substratum that supports microbial growth, has been applied to integrated fixed-film activated sludge (IFAS) systems designed for nitrification and carbon removal (Hamoda *et al.*, 2004; WEF, 2010). The first full-scale pilot

installation of the BioCord IFAS system was built in North America in 1993 (Loupasaki and Diamadopoulos, 2013; Copithorn *et al*., 2006; Randall and Sen, 1996; Sen, 1995). The BioCord technology is designed to promote the growth of high-density microbial consortia, as biofilm attaches to the housed cord substratum, and hence increases the organic pollutant and nutrient removal rate per reactor volume as comparing to more conventional suspended growth treatment technologies. The BioCord biofilm reactor has demonstrated its potential as a standalone system and as an upgrade system to current wastewater treatment facilities (Feng *et al*., 2012; Yang *et al.*, 2014; Yuan *et al.*, 2012). However, to date, there is no standalone BioCord system application specific for wastewater treatment in North America. Hence few publications on its nitrifying performance are available. In particular, Yuan et al. (2012), Xu et al. (2012) and Feng et al. (2015) have simulated BioCord reactors to treat river waters and have shown that the BioCord substratum provides a high porosity surface area for microbial growth and that the system provides efficient chemical oxygen demand (COD) and total ammonia nitrogen (TAN) removal. Xu et al. (2012) demonstrated distinct COD and TAN removal performances related to type of cord substratum. A recent study of the BioCord technology investigated the use of a BioCord reactor as an add-on system installed between two lagoons for carbon and nutrients removal (Gan *et al*.,2018). The results demonstrate that the BioCord system provided approximately 77% of carbon and 69% of TAN removal, respectively. Further, the effects of loading rate, type of substratum and air scouring on the BioCord system has recently been shown to effect the rate of nitrification and solids production of the technology (Tian *et al.*, 2019). Although the potential of this technology and the correlation between specific design and operational parameters and the system's performance has been recently demonstrated, there are currently no studies to date that provide fundamental information on the effects of design and operational parameters at the meso and micro-scale of the

biofilm. Hence there is a current gap in knowledge with respect to the technology's design, operation and performance and the meso and micro-scale response of the biofilm attached to the cord substratum.

The main objective of this study is to investigate the meso and micro-scale characteristics of BioCord biofilm and the response of the nitrifying BioCord technology to ammonia loading conditions, three different types of BioCord substrata and the influence of air scouring. In particular, this research will study the response of the biofilm porosity, the biofilm morphology and the biomass viability to these design and operational parameters. This research represents the first study of the biofilm response of the nitrifying BioCord technology.

4.4 Materials and Methods

4.4.1 Experimental set-up

This research was conducted using three 3.17L laboratory scale continues flow reactors housing three various types of BioCord substratum: PP+K-45 in reactor 1 (R1), MK-PP50 in reactor 2 (R2) and SP-100 in reactor 3 (R3). Each BioCord substratum is described in Table 4.1. The total surface area of each BioCord in each of the reactors was 0.384 m^2. The experimental set-up consisted of a feed and effluent collection tanks, peristaltic pumps pumping the wastewater to the reactors, three aerated reactors with an air diffuser fixed at the bottom of each reactor (Tian *et al.*, 2019). The experiments were conducted at three surface area loading rates (SALR) of 0.8 g NH_4^+-N/m^2·d, 1.6 g NH_4^+-N/m^2·d and 2.4 g NH_4^+-N/m^2·d. The loading and removal rates used in this study are normalized per surface area of biofilm attachment as is conventional for attached growth treatment technologies. Furthermore, the effects of air scouring were tested at the high SALR of 2.4 g NH_4^+-N/m^2·d in this study by changing the position of the aerator in the reactors from along the sidewall of the reactors to being placed directly centre in the reactors, where the air

bubbles were in full contact with the BioCord substratum for the duration of the experiments. The effects of air scouring were measured after two weeks of operation to avoid shock effects of scouring on the BioCord. The reactors were operated for a minimum 50 days at each loading phase for acclimation and subsequently, constituent characterization and microbial analysis. The following operational conditions were maintained throughout the experiments: hydraulic retention time (HRT) of 3 hours; dissolved oxygen (DO) concentration of approximately 7 ± 0.8 mg/L; pH of 7.1 ± 0.2; and temperature of 23.0 ± 1.0°C (Tian, Ahmed and Delatolla, 2019). The aeration rate used in this study, and hence the subsequent DO concentration, was employed to ensure that air scouring effects could be tested at the laboratory scale.

Table 4.1 Characteristics of BioCord substrata.

BioCord	Specific Surface Area	Material	Photograph
PP+K-45	1.6 m^2/m	Vinylon, Polypropylene	
MK-PP50	1.6 m^2/m	Polypropylene	
SP-100	2.4 m^2/m	Vinylon, Polypropylene	

4.4.2 Wastewater source

Synthetic wastewater simulating post-carbon removal municipal wastewater treatment, without ammonia removal, was used in this study. The specific composition of the synthetic wastewater at the low ammonia SALR of 0.8 g NH_4^+-N/m^2·d is as follows: $(NH_4)_2SO_4$: 57.13 mg/L, $NaHCO_3$: 157.48 mg/L, $MgSO_4 \cdot 7H_2O$: 70.98 mg/L, $CaCl_2 \cdot 2H_2O$: 29.34 mg/L, KH_2PO_4:

79.09 mg/L, $FeSO_4 \cdot 7H_2O$: 4.98 mg/L. Trace nutrients: $MnCl_2 \cdot 4H_2O$: 0.100 mg/L, $Na_2MoO_4 \cdot 2H_2O$: 0.025 mg/L, $CuSO_4 \cdot 5H_2O$: 0.103 mg/L, $CoCl_2 \cdot 6H_2O$: 0.001 mg/L, $ZnSO_4 \cdot 7H_2O$: 0.030 mg/L, and carbon source: glucose: 3.87 mg/L, sodium acetate 2.06 mg/L, peptone 3.87 mg/L. The wastewater composition was proportionally adjusted at moderate and high SALRs of 1.6 and 2.4 g NH_4^+-N/$m^2 \cdot$d, respectively, by augmenting the ammonium sulfate concentration proportionally. The calculated sCOD concentration was maintained at 10 mg/L when adjusting the ammonia loading to mimic post carbon effluent (Delatolla *et al*., 2009).

4.4.3 Constituent analysis

Standard methods 4500-NH_3, 4500-NO_3^- B, 4500-NO_2^-, 2540 D-total suspended solids (TSS dried at 103-105˚C) and 2540 E-volatile solids and volatile suspended solids (VSS) (fixed and volatile solids ignited at 550˚C) were used to test influent and effluent concentrations of TAN, nitrate, nitrite and TSS/VSS, respectively (Eaton *et al.*, 2005). The biofilm detachment rate is normalized in this study per surface area of substratum. As the feed wastewater was synthetic without solids, the detachment rate was simply calculated based on the effluent TSS concentration of the reactors, the volumetric flow rate of the reactors and the surface area of substratum in the reactors. DO, pH and temperature measurements were acquired using a Multi Parameter Meter with an attached DO probe and pH probe (VWR, Canada, Ontario). All wastewater samples were collected and measured in triplicate.

4.4.4 Microbial analysis

Biofilm morphology and porosity Variable pressure scanning electron microscope (VPSEM) imaging was used to observe the biofilm morphology and porosity of the BioCord samples at various experimental conditions. Sample preparation is not necessary for VPSEM imagine as comparing to the regular SEM, therefore minimizing sample destruction prior to

analysis (Flemming and Wingender, 2010). Images were acquired at a pressure of 40 Pa using a Vega II-XMU SEM (Tescan USA Inc., Cranberry, PA). Five images of four replicate samples (20 total images) were taken at random locations across the surface of the BioCord sample at ×60 magnification (Delatolla *et al.*, 2012; Hoang *et al.*, 2014; Young *et al.*, 2016). Biofilm porosity was quantified in the acquired VPSEM images using Nikon NI Vision Assistant (National Instruments, LabView, 8.0) to calculate the percent coverage of the pore spaces within the extracellular polymeric substances (EPS) of the biofilm (Figure C-4.1).

Cell viability The viability of embedded cells in the biofilm samples were observed using a 510/AxioImager confocal laser scanning microscope (CLSM) (Zeiss, US, VA) with a ×63 water objective. FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (Life Technologies, US, CA) was used to fluoresce the cells and Calcofluor White Stain (Life Technologies, CA, US) was used to fluoresce the biofilm EPS (Chen *et al.*, 2007). The CLSM was used to acquire five stacks of four images (20 images in total) per sample at random locations (Young *et al.*, 2017). Cell viability was analyzed and quantified using Nikon NI Vision Assistant (National Instruments, LabView, 8.0). The biofilm area (cell coverage) of each image was outlined by tracing the Calcofluor White Stain. The viable and nonviable cells were identified and their numbers per biofilm area were obtained by measuring the green and red fluorescence, respectively.

4.4.5 Statistical methods

Statistical significance of TAN removal efficiencies, biofilm detachment rate, biofilm porosity and cell viability were determined using the student t-test with a p-value of 0.05 identifying statistical significance in this study.

4.5 Results and Discussion

4.5.1 Nitrifying kinetics

The nitrification kinetics of the BioCord reactors are presented as the surface area removal rate (SARR) in this study. At both, low loading (SALR = 0.8 g NH_4^+-N/m^2·d) and moderate loading conditions (SALR = 1.6 g NH_4^+-N/m^2·d), steady and high removal efficiencies of approximately 97% with very small variations in performance were maintained by all three substrata with the average and 95% confidence interval of the SARR being 0.79 ± 0.01 g NH_4^+-N/m^2·d and 1.57 ± 0.01 g NH_4^+-N/m^2·d, respectively (Figure 4.1). As demonstrated by the similar measured SARR at the low and moderate loading conditions, no significant statistical difference in operational kinetics is observed for the three reactors housing three distinct BioCord substrata.

During the high loading phase (SALR = 2.4 g NH_4^+-N/m^2·d), a statistically significant reduction in the percent TAN removal efficiency is observed for all three substrata compared to the performance at low and moderate loadings. The trend of elevated loading resulting in decreases in removal efficiency is expected in biological and biofilm treatment technologies (Ødegaard, 2006; Rusten *et al.*,2006). Although a decrease in removal efficiency was expected, the system also demonstrated an inherent instability in the system's kinetics at the high loading condition, which is clearly indicated in Figure 4.1 by the large variance in the SARR measurements. The effluent TAN concentration increased from below 1 mg NH_4^+-N/L for all reactors at both low and moderate loading phases to a maximum effluent TAN concentration of 14.5 ± 2.3 mg NH_4^+-N/L. The effluent TAN concentration showed large variations as it was unstable at elevated loading conditions. The various BioCord substratum tested in this research again demonstrated no statistical difference in SARR at a high loading SALR of 2.4 g NH_4^+-N/m^2·d, possibly due to the high variance masking significant differences between the data sets.

The effects of air scouring were tested at the high SALR of 2.4 g NH_4^+-N/m^2·d by changing the position of the aerator in the reactors from along the sidewall of the reactors to being placed directly centre in the reactors, where the air bubbles were in full contact with the BioCord substratum for the duration of the experiments. Air scouring did not improve the system stability as it did not demonstrate a statistically significant improvement in SARR. Again, the lack of statistical significance may be due to the high variation in the SARR measurements at the high loading condition (Figure 4.1).

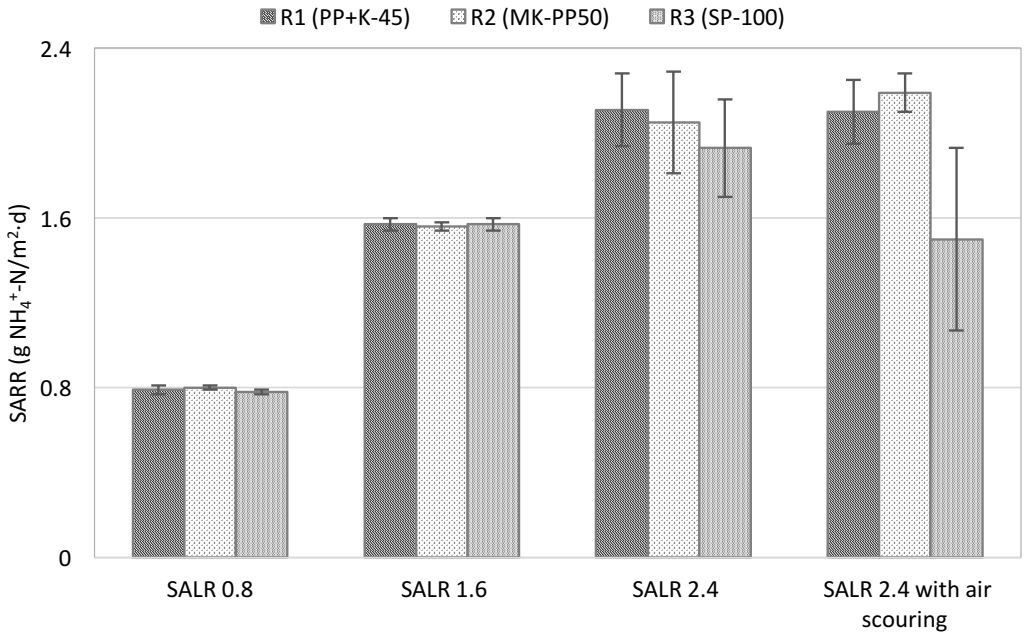

Figure 4.1 Average and 95% confidence intervals of SARR at various SALR.

4.5.2 Biofilm detachment rate

The biofilm detachment rate is calculated in this study based on the difference between the influent and effluent TSS concentrations. No solids were added to the influent synthetic wastewater to ensure the accuracy of the detachment measurements. The results demonstrated no statistical difference in detachment rate between the various BioCord substratum types at all three

loading rates and at the high loading rate with air scouring (Table 4.2). However, a significant increase in biofilm detachment rate was shown with increasing SALR, both with and without air scouring. This increase in biofilm detachment rate at higher loadings is expected due to increased microbial activity and is seen in carbon and nitrogen biological treatment studies on (Aygun *et al*., 2008; Forrest *et al.*, 2015; Karizmeh *et al.*, 2014).

The fluctuations in biofilm detachment indicated by the large 95% confidence intervals observed at a high loading of 2.4 g NH_4^+-N/m^2·d again demonstrate unstable performance. This instability in biofilm detachment coincides with the observed instability in the ammonia SARR at these same high loading conditions (Table 4.2). In particular, this instability in biofilm detachment rate resulted in maximum effluent TSS concentrations of 36.8, 42.0 and 28.9 mg/L for substrata PP+K-45, MK-PP50 and SP-100, respectively, which exceeds common discharge regulations. Furthermore, such fluctuations may cause difficulties in the operation of downstream sedimentation units. This biofilm detachment instability is indicative of intermittent sloughing events that are often triggered by thicker and weaker biofilms that may be formed in attached growth technologies under high surface substrate loading conditions (Characklis, 1990; Peyton and Characklis, 1993; Picioreanu *et al*., 2001; Telgmann *et al*., 2004; Tijhuis *et al*., 1996).

Although there is no statistical difference in biofilm detachment rates at an SALR of 2.4 g g NH_4^+-N/m^2·d with and without applying air scouring for each of the substrata studied in this research, an improved stability was observed when air scouring was applied to all three substrata (Table 4.2). This improved stability in operation is indicated by the reduced variation in the in the detachment rate values in Table 4.2 and hence the reduced fluctuations in the detachment rate for all three substrata with air scouring. Air scouring may enhance erosion detachment as opposed to sloughing detachment without air scouring. This finding is in agreement with previous studies

demonstrating that air scouring provides sheer forces that enhance biofilm detachment and results in thinner biofilms that are less susceptible to sloughing events (Kwok *et al.*, 1998). Hence, the stabilized biofilm detachment rates observed with air scouring are indicative that the sloughing events were minimized.

Table 4.2 Biofilm detachment rate across substrata.

SALR ($g\ NH_4^+$-$N/m^2 \cdot d$)	Reactor (substratum)	Biofilm detachment rate ($g\ TSS/m^2 \cdot d$)		
		Average	Minimum	Maximum
0.8	R1 (PP+K-45)	0.53 ± 0.11	0.42	0.68
	R2 (MK-PP50)	0.56 ± 0.13	0.42	0.70
	R3 (SP-100)	0.37 ± 0.05	0.29	0.39
1.6	R1 (PP+K-45)	0.64 ± 0.11	0.55	0.78
	R2 (MK-PP50)	0.64 ± 0.09	0.55	0.76
	R3 (SP-100)	0.55 ± 0.05	0.49	0.60
2.4	R1 (PP+K-45)	1.09 ± 0.77	0.57	2.42
	R2 (MK-PP50)	1.49 ± 1.08	0.42	2.79
	R3 (SP-100)	0.88 ± 0.59	0.47	1.90
2.4 with air	R1 (PP+K-45)	0.73 ± 0.18	0.60	0.99
	R2 (MK-PP50)	0.78 ± 0.13	0.60	0.96
	R3 (SP-100)	0.66 ± 0.17	0.52	0.94

4.5.3 Biofilm porosity and morphology

Acquired VPSEM images were used to measure the porosity and characterize the morphology of the biofilm. The acquired images show no statistical difference in biofilm porosity for the three substrata at all experimental conditions without air scouring. However, a significant decrease in biofilm porosity was observed for each substratum with increasing loading rates. The

average porosity measurements decreased from 47% to 36.3% and to 20.8% at SALR values of 0.8, 1.6 and 2.4 g NH_4^+-N/m^2·d, respectively (Figure 4.2). This observation is in agreement with Young et al. (2016) study that observed thicker nitrifying moving bed biofilm reactor (MBBR) biofilm a at higher loadings conditions. It is expected that higher substrate concentrations promote deeper substrate penetration into the biofilm, greater cell growth at depth in the biofilm and hence a thicker biofilm and/or a biofilm with a lower porosity (Gerardi, 2002). However, previous studies have also demonstrated that system efficiency can be repressed by limitations in substrate transport within thick biofilm or biofilm with lower porosities (Beyenal, 2000; Stewart, 2003). Therefore, the reduction in porosity with increased loadings and decreased TAN removal efficiency that was observed in this study implies that the excessive growth of biofilm restricts substrate mass transfer through the biofilm under high loading condition, which significantly reduced the nitrifying kinetics and the system stability. Moreover, as indicated by the large fluctuations observed in this research in biofilm detachment at elevated loadings, the excessive biofilm growth causes biofilm sloughing and increased the risk of breakthrough effluent TSS and VSS (Lee and Rittmann, 2002).

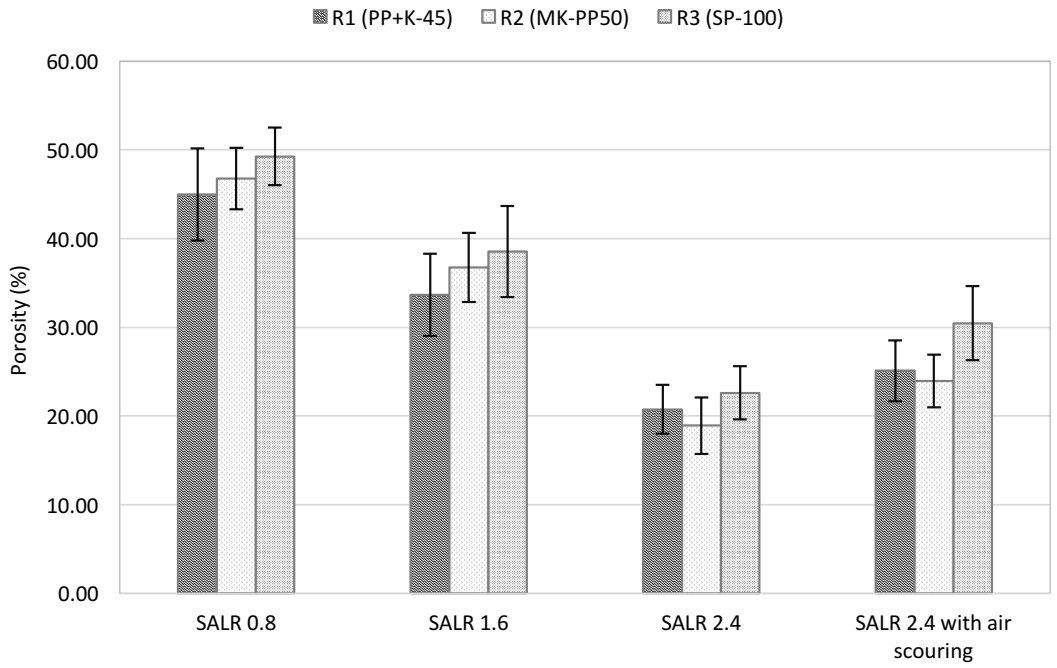

Figure 4.2 Average and 95% confidence intervals of biofilm porosity.

A statistically significant increase in biofilm porosity at an SALR of 2.4 g NH_4^+-N/m^2·d with air scouring was observed compared to the same loading condition without air scouring, with *p*-values equal to 0.046, 0.020 and 0.003 for PP+K-45, MK-PP50 and SP-100, respectively. This increase in biofilm porosity is possibly due to the additional shear forces provided by air scouring and subsequently the erosion of the biofilm and the prevention of clogged pores. The upper layers of the biofilm that are near the bulk water interface may also be strengthened by the erosion and less easily detached (Coufort *et al.*, 2007; Picioreanu *et al.*, 2001). The increased biofilm porosity along with the stabilized biofilm detachment rate at an SALR of 2.4 g NH_4^+-N/m^2·d with air scouring again demonstrates that air scouring promotes a thinner and stronger biofilm which prevents biofilm sloughing (Kwok *et al.*, 1998).

It should also be noted that a distinction between the BioCord substrata is observed in this study with respect to the porosity of the attached biofilm during air scouring operation. The SP-

100 (R3) substratum showed a statistically larger biofilm porosity compared to the PP+K-45 and MK-PP50 substrata. The manufactural specific surface area of the SP-100 substratum (2.4 m^2/m) was larger than the other two BioCord substrata (1.6 m^2/m). The larger surface area per length of bio-card resulted in a smaller length of the SP-100 BioCord being housed in R3 as compared to the length of BioCord housed in R1 and R2. Hence the SP-100 bio-card received a larger shear force per unit length due to its larger surface area. Previous studies in both carbon loading systems and nitrifying systems have shown distinct differences in biofilm morphology at various loading conditions with a decrease in biofilm density in thick biofilms being attributed to filamentous biofilm morphology (Jang *et al.*, 2003; Karizmeh *et al*., 2014; Morgenroth *et al.*, 1997). This filamentous morphology is not observed in this study at lower loadings of 0.8 and 1.6 g NH_4^+-$N/m^2 \cdot d$ (Figure 4.3b and c), while it is observed at high loadings of SALR of 2.4 g NH_4^+-$N/m^2 \cdot d$ without and with air scouring (Figure 4.3d and e). The acquired VPSEM images also highlight differences in the protozoa, water mite and rotifer communities across loading conditions and across air scouring effect. At low loading condition the VPSEM images of all substrata show the absence of water mites, rotifers and demonstrate that few protozoa exist at the surface of the biofilm (Figure 4.3b). While with the increasing loading rate, testate amoebae (Szelecz *et al.*, 2014) (Figure 4.3f) and water mites (Figure 4.3g and h) tend to be more dominant (Figure 4.3c and d). Further, Figure 4.3d and e indicate that air scouring effects results in more diverse advanced microorganisms such as rotifers (Figure 4.3i) living at the surface of the biofilm. These results demonstrate that the operational conditions directly affect the meso-scale structure of the biofilm and the micro-animal populations.

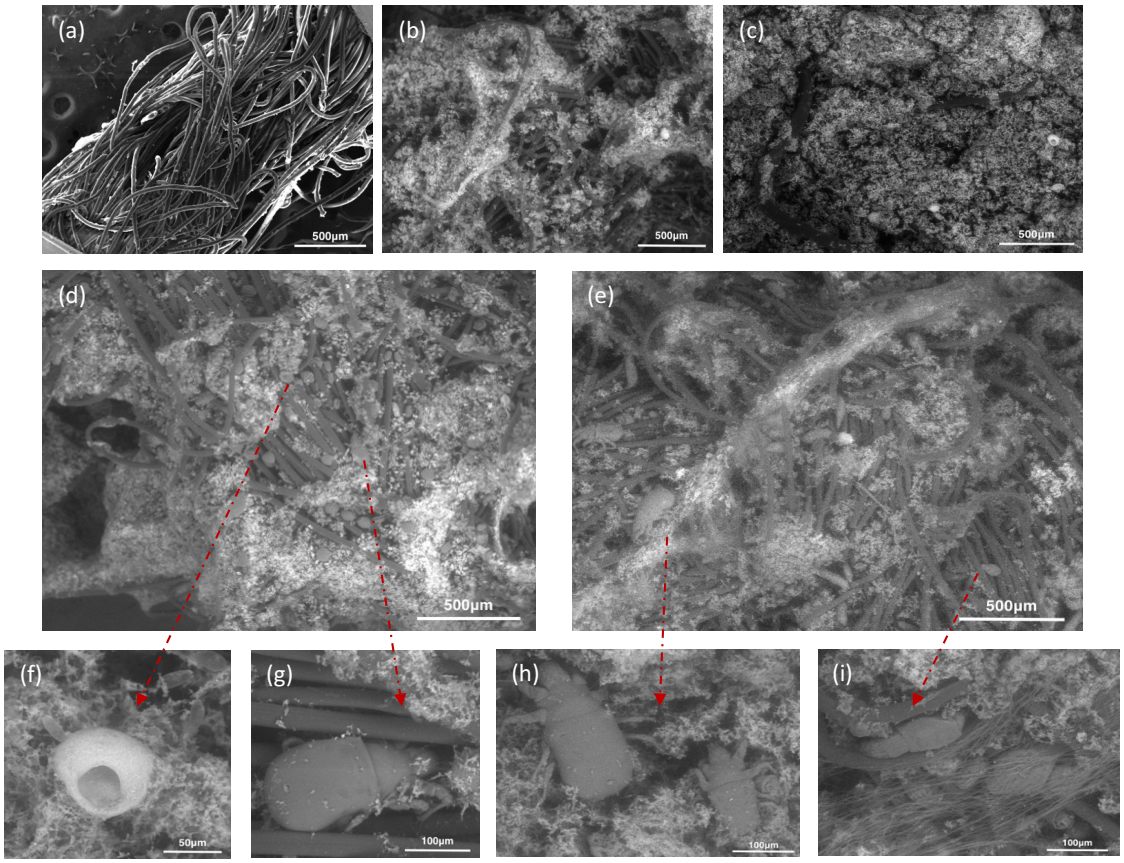

Figure 4.3 Representative VPSEM images of biofilm attached to the PP+K-45 substratum at different loading conditions: (a) Clean BioCord; (b) SALR of 0.8 g NH_4^+-N/m^2·d; (c) SALR of 1.6 g NH_4^+-N/m^2·d; (d) SALR of 2.4 g NH_4^+-N/m^2·d; (e) SALR of 2.4 g NH_4^+-N/m^2·d with air scouring; (f) testate amoebae; (g) water mite; (h) water mites; (i) rotifers.

4.5.4 Biomass viability

The viability test illuminates viable (live) cells in green and non-viable (dead) cells in red in images captured at depth in the biofilm by CLSM. The percent of live and dead cells embedded in the biofilm were quantified in the upper 100 µm of the biofilm. Viability analysis was limited to a depth of 100 µm, where the upper 100 µm of the biofilm is likely the range of the most active aerobic activity of the biofilm. To ensure that when analyzing locations of thinner biofilm

(approximately 100 µm) the same depth and number of images acquired were consistent for all locations analyzed. No significant difference was observed at low, moderate or high loading conditions with respect to the percent biofilm coverage, percent of live cells or percent of dead cells. Furthermore, the percent biofilm coverage, percent of live cells or percent of dead cells, at the high loaded condition was shown to not be statistically different with or without air scouring (Figure 4.4). Hence, the lack of observed statistical difference in biofilm coverage or live cell percentage suggests that the activity of the cells themselves and not cell coverage is responsible for the varying to the kinetic performance of the system (Young *et al.*, 2016).

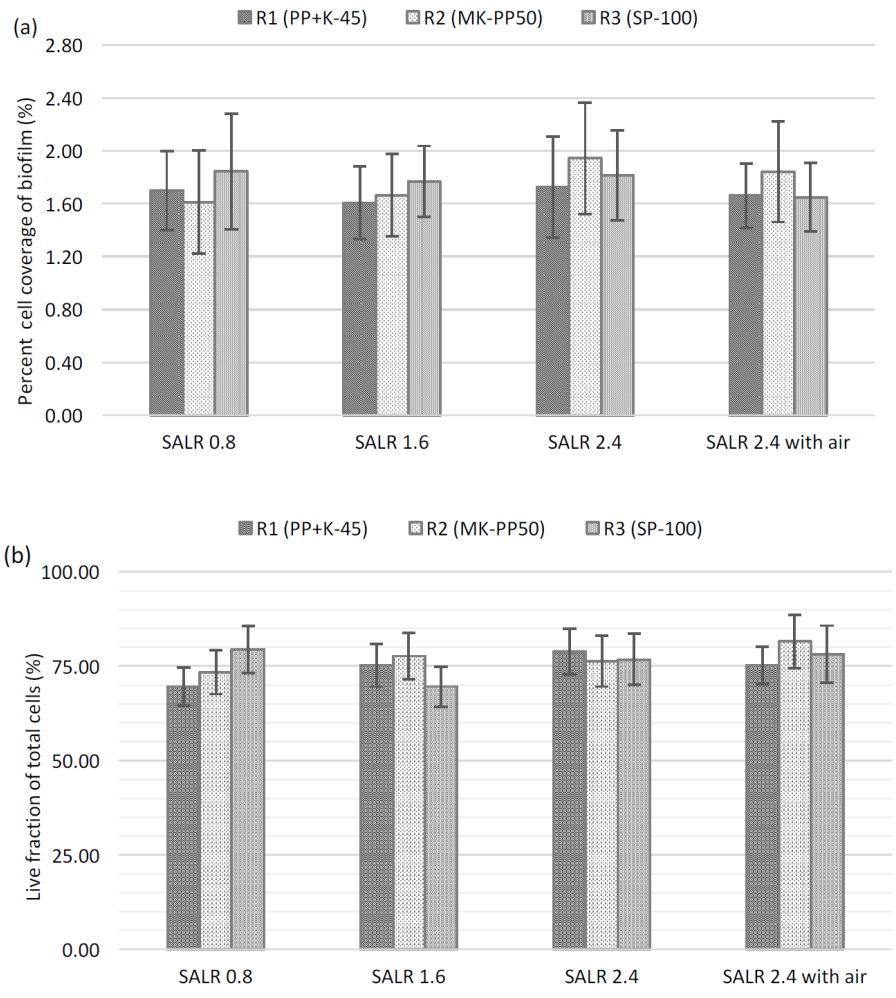

Figure 4.4 CLSM viability of embedded cells across SALR, (a) percent cell coverage of total cells and (b) live fraction of total cells of each substratum.

4.6 Conclusions

This study focuses on the performance of stand-alone BioCord biofilm reactors for low carbon, tertiary nitrification and the link between the performance of this system and the meso and micro-scale characteristics of the biofilm. It investigates the effects of low, moderate and high ammonia SALR, various BioCord substratum and air scouring on nitrifying kinetics, biofilm detachment rate, biofilm porosity and morphology and biomass viability. At low loading (SALR = 0.8 g NH_4^+-N/m^2·d) and moderate loading conditions (SALR = 1.6 g NH_4^+-N/m^2·d), the substratum PP+K-45, MK-PP50 and SP-100 all performed steadily and showed high removal efficiencies of approximately 97%. An unstable performance was observed at the high loading SALR of 2.4 g NH_4^+-N/m^2·d, with air scouring not demonstrating an improvement in stability. Biofilm detachment rates were shown to increase with increasing SALR and an improved stability was observed with applied air scouring for all three substrata. Biofilm porosity was shown to decrease with increasing SALR for all three substrata and was shown to increase with enhanced air scouring. Hence air scouring was shown as a potential strategy to prevent the clogging of biofilm pores in the BioCord technology. With respect to biomass viability, as there is no statistical difference in biofilm coverage and live cell percentage, the activity of the cells themselves and not cell coverage was shown to be related to kinetic performance.

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5 Chapter 5 – Anammox attachment and biofilm development on surface-modified carriers with planktonic- and biofilm-based inoculation

5.1 Setting the context

Chapter 5 presents the research article entitled *Anammox attachment and biofilm development on surface-modified carriers with planktonic- and biofilm-based inoculation* that has been submitted to Bioresource Technology, 2020. This study investigates the kinetics, anammox biomass attachment, biofilm development and anammox bacteria enrichment of a novel detached anammox biofilm inoculation method on the two carrier types (non-modified virgin MBBR carriers and pre-seeded denitrifying carriers) and compares these results to the more common use of attached anammox carriers for anammox inoculation during the start-up of the attached growth anammox systems

5.2 Abstract

This study investigates the kinetics, attachment, biofilm development and anammox bacteria enrichment of a novel detached anammox biofilm inoculation method on non-modified virgin MBBR carriers and pre-seeded denitrifying carriers. In addition, the study compares these results to the more common use of attached anammox carriers for anammox MBBR inoculation. The anammox bacteria specific attachment-growth rates for virgin carriers inoculated with detached anammox biofilm mass were 38.1% greater for the first 25 days, leading to approximately 30% less time required to achieve complete biofilm coverage than those measured in attached biofilm carrier inoculated systems during the attachment and early biofilm growth stages. The

biofilm thickness increase rate was also 52.3% higher for virgin carriers with detached biofilm inoculum. Further, inoculation using pre-seeded denitrifying carriers compared to virgin carriers demonstrated a 13.8% preferential increase in anammox bacteria specific attachment-growth rate and a corresponding 47.2% higher NH_4^+-N removal rate at the time of biofilm maturation.

5.3 Introduction

Anaerobic ammonium oxidation (anammox), a pathway for nitrogen removal, is mediated by anaerobic autotrophic organisms, anammox bacteria that directly oxidize ammonia to nitrogen gas with limited nitrate production (van de Graaf et al., 1996). Compared to conventional nitrification-denitrification processes, the anammox process is advantageous in that it requires a lower oxygen demand, produces less sludge and does not require the addition of an external carbon source. (Tsushima et al., 2007; Kuenen, 2008; Kartal et al., 2010; Tang et al., 2010). However, the slow growth rate of anammox bacteria (with an estimated doubling time of 11 days) limits the installation of anammox wastewater treatment units as it is difficult to quickly enrich and retain anammox bacteria in the treatment reactor during the start-up period (Strous et al., 1998; Abma et al., 2007; van der Star et al., 2007; Cho et al., 2010).

Technologies for nitrogen removal via anammox ranges from suspended growth to attached growth systems that are operated as batch or continuous feed systems (Lackner et al., 2014; Ali and Okabe, 2015). Comparing to suspended growth systems, attached biofilm technologies such as the moving bed biofilm reactor (MBBR) system demonstrate higher relative abundances of anammox bacteria due to their ability to retain elevated quantities of anammox biomass within the biofilm (Guo et al., 2016). Furthermore, due to diffusion gradients intrinsic to biofilm technologies, anammox bacteria can grow in biofilm niches and the biomass embedded in the biofilm are less susceptible to changes in influent characteristics such as temperature, nitrite

accumulation, toxic compounds and substrate loading; which can all lead to loss of biomass in suspended growth systems (Gilbert et al., 2015; Morgan-Sagastume, 2018).

The start-up of anammox attached-growth technologies require the attachment of anammox cells to engineered bio-carriers followed by the growth and maturation of anammox biofilm on the carriers (Veuillet et al., 2014). However, few studies and limited data exist with respect to appropriate and feasible start-up strategies for the enhanced attachment of anammox cells to carriers followed by the rapid growth and maturation of anammox biofilms during the start-up of attached growth systems (Christensson et al., 2011; Lemaire et al., 2014; Manonmani and Joseph, 2018). Recent studies have focused on seeding anammox systems with inoculum including denitrification sludge, mature granules and anammox enriched sludge to reduce the start-up period (Tsushima et al., 2007; van der Star et al., 2007; Hu et al., 2011; Ni et al., 2011; Chen and Jin, 2017; Kowalski et al., 2018; Li et al., 2018). In particular, the use of heterotrophic, biofilm pre-seeded carriers for the start-up of attached growth anammox systems has demonstrated increasing rates of anammox attachment (Klaus et al., 2016; Kowalski et al., 2019). Previous studies also have demonstrated that heterotrophic biofilm-detached cells as inoculum show a propensity for attachment and recolonization of surfaces to form biofilms when compared to planktonic cells (Bester et al., 2005; Nadell et al., 2009; Khelissa et al., 2017). However, little is known about the influence of biofilm-detached anammox cells on the recolonization and subsequently the biofilm development. To the best of the authors' knowledge, no previous studies have evaluated potential of detached biofilm as inoculum for attachment and growth of anammox bacteria on carriers.

Thus, the aim of this study is to investigate and compare the attachment, activity and growth rates of anammox biofilms grown on two types of carriers: (a) pre-seeded denitrifying

carriers and (b) non-modified virgin carriers, when implementing two different inoculation methods using: (i) detached anammox biofilm mass, or (ii) attached anammox biofilm on carriers.

5.4 Methodology

5.4.1 Experimental setup and approach

The experimental setup includes two 18 L laboratory moving bed biofilm reactors (MBBRs) with installed mixers and four stainless steel cages outfitted in each reactor. In each reactor, only two cages were utilized in this research, while the other two cages housed different carriers that were used for other research purposes. To investigate the potential of pre-seeded denitrifying carriers to enhance the attachment, growth and maturation of anammox biofilm in the reactor, each reactor was filled with virgin AnoxK^{TM}5 carriers (AnoxKaldnes, Lund, Sweden) in one cage and same amount of the pre-seeded denitrifying biofilm AnoxK^{TM}5 carriers in the other (Figure 1). The manufactured protected surface area for AnoxK^{TM}5 carrier is 800 m^2/m^3. To investigate the inoculation of using detached biofilm mass in place of attached biofilm mass for start-up of the attached growth anammox system, one of the reactors (denoted as R1) was seeded with anammox biofilm that was mechanically detached from anammox biofilm pre-existed AnoxK^{TM}5 carriers. The second reactor (denoted as R2) used common method for inoculation of anammox MBBR systems, where 10% of the carriers (in each of the two cages) were replaced by anammox biofilm pre-existed AnoxK^{TM}5 carriers.

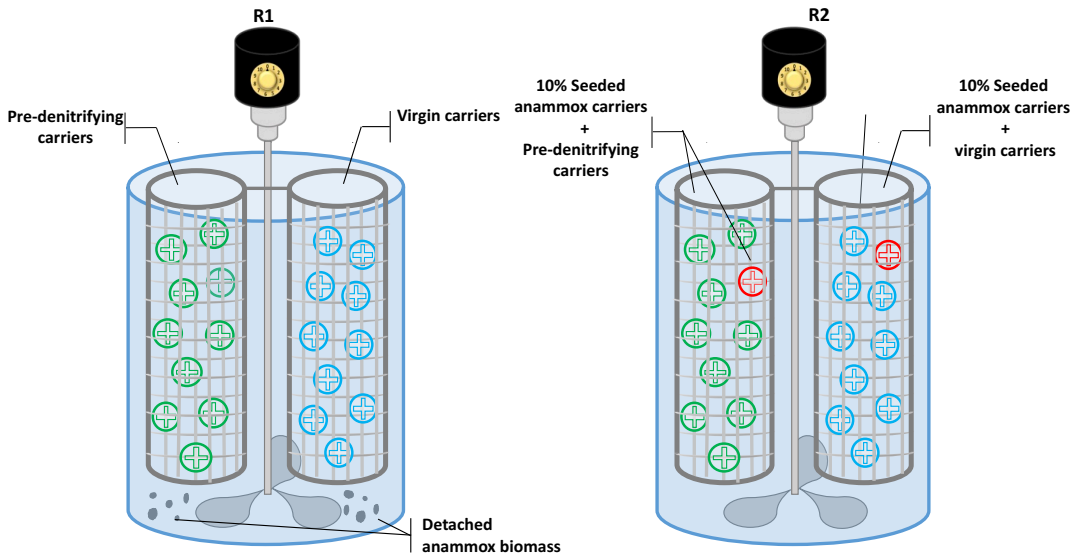

Figure 5.1 Schematic of the cages in laboratory reactors R1 (detached biofilm inoculation) and R2 (attached carrier inoculation). Each reactor contains two cages. The first cage houses non-modified virgin carriers and the second cage houses pre-seeded denitrifying carriers.

R1 and R2 were operated in parallel at a temperature of 30°C in the temperature control room and fed with diluted harvested centrate (characterization of centrate shown below), pH was controlled between 7.5 to 7.8, dissolved oxygen (DO) was below 0.5 mg/L. Both R1 and R2 were operated as sequencing batch reactor (SBR) systems for approximately two months until sufficient biofilm mass was observed on the various carriers. Following sequencing batch operation, R1 and R2 were operated as continuous feed reactors for approximately an additional three months with HRT of 24 hours. The designated start-up of the reactors in this study to be first operated in SBR mode operation followed by continuous feed operation mimics the full-scale start-up of anammox MBBR technologies.

Anammox kinetic assays were used to isolate the anammox activity of the carriers in each of the individual four cages in R1 and R2. The kinetic assays were performed by operating 500 mL batch reactors with 75 carriers in each reactor over an 8-hour period. The kinetic assay reactors

were stirred using a magnetic stirrer and were operated at a temperature of 30°C. The reactors were fed with 400 mL of diluted centrate from the same feed source as R1 and R2. However, the initial concentrations of the kinetic assay feed were set to 40 mg NH_4^+-N/L, 40 mg NO_2^--N/L, and 250 mg as $CaCO_3$/L. These constituent concentrations were targeted based on the kinetics observed in R1 and R2 along with expected removal rates of anammox systems to ensure a minimum significant change in concentration (2 mg/L). DO concentration was reduced below 0.5 mg O_2/L with nitrogen gas air stripping and pH was adjusted to a target range of 7.5 – 7.8 prior to starting the kinetic assay testing. Ammonia, nitrite, nitrate and alkalinity were measured in triplicate every 30 minutes for the first 2 hours, and every 2 hours thereafter.

5.4.2 Reactor inoculation

All mature anammox AnoxK^{TM}5 carriers used to inoculate the reactors were collected from a full-scale, sidestream anammox ANITA Mox system (South Durham WRRF, Durham, NC, US). The total number of mature anammox carriers (70 carriers) from which the biofilm mass was detached to inoculate R1 was equal to the number of mature anammox carriers added to R2. The biofilm mass was removed from thex anammox AnoxK^{TM}5 carriers, to be used as seed for R1, by vortexing the carriers using a Vortex Mixer (VWR, Canada, Ontario) and verifying that the biofilm was sufficiently removed by the carriers with variable pressure scanning electron microscope (VPSEM) (Tescan USA Inc., Cranberry, PA). Confocal laser scanning microscopy (Zeiss, US, VA) in combination with viability staining (Life Technologies, US, CA) was performed on the attached biofilm of the anammox AnoxK^{TM}5 carriers that were used to seed R2 and to the detached biofilm mass from the same sample set of the anmmox AnoxK^{TM}5 carriers used to seed R1 (Forrest et al., 2016; Tian and Delatolla, 2019). The viability of attached anammox biofilm (percent viable cells = 72.2 ± 8.6%) and detached biofilm mass (percent viable cells = 79.7 ± 10.1%) was not

statistically distinct ($p > 0.5$). Hence the cell detachment process was verified as not being destructive to the attached cells.

5.4.3 Centrate feed characteristics

The anammox reactors were fed with diluted anaerobic digestion centrate that was augmented with nitrite to simulate anammox conditions following partial nitrification. The centrate was harvested from the municipal WRRF located in Cornwall, Ontario, Canada every two to four weeks and stored at 4°C until brought to a temperature of 30°C before being fed to the reactors. Typical concentrations in the centrate ranged from 530 – 976 mg NH_4^+-N/L for ammonia and 1530 – 1690 mg/L for soluble chemical oxygen demand (sCOD), respectively. The centrate was diluted with distilled water to target ammonia influent concentrations of 150 – 200 mg NH_4^+-N/L. Due to inherently low nitrite concentrations in the centrate (~ 0 mg NO_2^--N/L), the MBBR feed was augmented with a stock sodium nitrite solution to mimic centrate following partial nitrification.

5.4.4 Reactor operation

The two reactors, R1 and R2, were operated as SBR for a period of 65 days and followed by continuous feed operation until the end of the research. SBR operation during inoculation of attached growth anammox technologies is common. The influent and effluent nitrogen concentration during SBR and continuous feed operation was shown in Appendix A. Between days 1 to 10, the target feed concentration of NH_4^+-N and NO_2^--N were 200 mg/L each to allow the reactors to start-up without substrate limitation. On day 11 the target feed concentrations were modified to 150 mg/L of NH_4^+-N and 50 mg/L NO_2^--N based on the measured performance of the reactors.

On day 65, the start-up operation of R1 and R2 shifted to continuous feed operation. A shift to continuous feed following SBR operation is common during start-up as the steady operation of these systems operate as continuous flow systems. Between day 65 to day 98, the target influent concentration for both reactors were approximately 150 mg/L of NH_4^+-N and 75 mg/L of NO_2^--N. Based on the performance of reactors, the target feed nitrite concentration was augmented to approximately 150 mg/L of NO_2^--N from day 99 in R1(Figure C-5.1a). While in R2, nitrite augmentation started on day 106 to a target nitrite feed concentration of 125 mg/L of NO_2^--N (Figure C5.1b). On day 128, a new batch of centrate was collected for the feed. However, this batch of centrate showed different characteristics to previous centrate batches collected causing system fluctuation. In particular, the C/N ratio was significantly higher for this batch.

5.4.5 Constituent analysis

The following parameters were triplicate monitored throughout the study: $N\text{-}NH_4^+$, $N\text{-}NO_2^-$, $N\text{-}NO_3^-$, sCOD, DO, pH and temperature. All nitrogen constituents and solids analyses were conducted in accordance with standard methods (APHA, 2005); methods 4500-NH_3, 4500-NO_3^- B and 4500-NO_2^-, sCOD was analyzed using a DR 5000 spectrophotometer (HACH, Loveland, CO) after filtration with a 0.45 μm, Millipore G filter. DO, pH and temperature were measured using a symphony Multi Parameter Meter with an attached DO probe and pH probe (VWR, Canada, Ontario).

5.4.6 Analysis of biofilm coverage and thickness

A Vega II-XMU SEM (Tescan USA Inc., Cranberry, PA) variable pressure scanning electron microscope (VPSEM) was used to observe the biofilm attachment, biofilm coverage and to measure the in-situ biofilm thickness on the carriers (Delatolla et al., 2015; Young et al., 2017). 3 carriers were harvested from the reactors to measure biofilm thickness. For biofilm thickness,

the acquired images were processed with Atlas imaging processing software (Tescan USA Inc., Cranberry, PA). This imaging protocol resulted in 25 thickness measurements per carrier, for a total of 75 images processed per set of 3 carriers.

5.4.7 Droplet digital polymerase chain reaction analysis

Three carriers from each reactor were harvested (three biological replicates) and analyzed using droplet digital PCR (ddPCR). DNA of the biofilm from harvested carriers was extracted using a FastDNA® Spin Kit (MP Biomedicals, US, CA), with the extracted DNA stored at -20°C until ddPCR analysis. Hydrazine oxidoreductase (hzo) mRNA was selected as a candidate biomarker of anammox bacteria activity of the extracted DNA. The set of hzo primers (forward:CATGGTCAATTGAAAGRCCACC and reverse: GCCATCGACATACCCATACTS) was designed based on conserved regions of known hzo sequences in three different anammox bacterial strains: KSU-1, Candidatus "Brocadia anammoxidans", and Candidatus "Kuenenia stuttgartiensis" (Park et al., 2010). The ddPCR reaction mixture was prepared using 5 μL of sample and 15 μL of QX200™ ddPCR™ EvaGreen® Supermix (Bio-Rad, Hercules, CA), including 0.1 μM of primers. The 20 μL reaction mixture was mixed with 60 μL droplet generate oil through the droplet generator (Bio-Rad, Hercules, CA). After droplet generation, 40 μL of the generated droplet emulsion was transferred to a 96-well PCR plate (Eppendorf, Hamburg, Germany) and amplified in a T100™ Thermal Cycler (Bio-Rad, Hercules, CA). The amplification conditions consisted of denaturation at 95 °C for 5 min, 50 cycles of 30 s at 95 °C, 60 s at 57 °C and 30 s at 72 °C followed by 5 min cooldown at 4 °C, 5 min at 90 °C for droplet stabilization and hold at 12 °C. The plate was transferred to a QX200 droplet reader (Bio-Rad, Hercules, CA). Data acquisition and analysis were performed using the QuantaSoft analysis software (Bio-Rad, version 1.7.4, Hercules, CA). Droplets were identified as positive or negative based on the fluorescent

signals being above or below a background threshold. The number of molecules of target DNA present in the 20 µL reaction mixture was determined from the ratio of positive/total droplets. The quality controls criteria employed in this study included that the number of total droplets quantified were above 10,000 and no more than 5 positive droplets were identifiable in the negative controls.

5.4.8 Anammox bacteria doubling time and growth rate calculation

The doubling time (t_d) and the specific growth rate of anammox bacteria was approximated according to Equation 5.1 and Equation 5.2 (Park et al., 2010), respectively. In this study, doubling time is calculated based on the copies of attached and/or grown anammox DNA amplicons on the carriers as distinction between attachment and growth is not possible. As such, specific attachment-growth rate is used in this study in place of a conventional specific growth rate.

$$\boldsymbol{t_d = \frac{\ln(2)(t-t_0)}{ln\frac{C}{C_0}}} \qquad \textbf{Equation 5.1}$$

$$\boldsymbol{\ln C = \ln C_0 + \mu(t - t_0)} \qquad \textbf{Equation 5.2}$$

Where:

$t - t_0$: time interval, days

C: equivalence concentration of anammox genes, copies/carrier

C_0: initial concentration of anammox genes, copies/carrier

μ: specific attachment-growth rate, d^{-1}

5.4.9 Statistical analyses

Statistical analyses of constituents, live/dead cell percentage, biofilm thickness, anammox bacteria doubling time and specific growth rate were tested using a t-test with a *p*-value less than 0.05 being considered significant. Statistical analysis for bacterial gene copies was tested using t-test with a *p*-value less than 0.10 being considered significant.

5.5 Results and discussion

5.5.1 Anammox kinetic

The kinetic assay NH_4^+-N surface are removal rate (SARR) values of the virgin and pre-seeded denitrifying carriers are shown to significantly increase across the time of operation of R1 and R2 (Table 1). As virgin and pre-seeded denitrifying carriers were collected from the cages of R1 and R2 during operation of these reactors to undergo kinetic assay testing of the distinct carriers from each reactor, the observed increase in kinetic assay rates performed across time supports attachment, growth and maturation of anammox biofilm on the virgin and pre-seeded denitrifying carriers of both R1 and R2. In R1, the kinetic assay shows 79.5% and 47.2% higher NH_4^+-N SARR for the pre-seeded denitrifying carriers compared to the virgin carriers on days 43 and 64, respectively. Previous studies have inferred that the heterotrophic denitrifying biofilm provides a matrix of extracellular polymeric substances (EPS) that promotes the attachment of suspended anammox bacteria (Kowalski et al., 2019). While in R2, which was seeded with attached anammox biofilm carriers, no statistical difference in the kinetic assay NH_4^+-N SARR was observed between the virgin and pre-seeded denitrifying carriers during this period. As individual cells are dispersed/detached from the biofilm to recolonize on new surfaces, the lack of distinction between the kinetics of the R2 virgin carriers and the R2 pre-seeded denitrifying carriers indicates that the detachment rate of anammox cells from the seeding anammox carriers of R2 may be the limiting factor in the attachment process of anammox cells to both the virgin and the pre-seeded denitrifying carriers in R2. The results hence demonstrate the preferential attachment of anammox cells to the pre-seeded denitrifying carriers.

Table 5.1 Kinetic assay NH_4^+-N SARR with the 95% confidence intervals for virgin and pre-seeded denitrifying carriers in R1– detached biofilm inoculation and R2 – attached carrier inoculation. Variance indicates 95% confidence interval.

		Kinetic assay NH_4^+-N SARR (g NH_4^+-N/m^2·d)			
		Day 43	Day 64	Day 106	Day 156
Detached biofilm inoculation (R1)	Virgin	0.15 ± 0.02	0.20 ± 0.02	0.68 ± 0.04	0.41 ± 0.08
	Pre-seeded denitrifying	0.26 ± 0.04	0.29 ± 0.03	0.67 ± 0.04	0.61 ± 0.12
Attached carrier inoculation (R2)	Virgin	0.04 ± 0.01	0.12 ± 0.01	0.60 ± 0.10	0.40 ± 0.08
	Pre-seeded denitrifying	0.06 ± 0.01	0.11 ± 0.01	0.75 ± 0.11	0.63 ± 0.12

Comparing the kinetic assay NH_4^+-N SARR for both virgin and pre-seeded denitrifying carriers in R1 and R2 during SBR operation, the results demonstrate that the NH_4^+-N SARR for both virgin carriers and pre-seeded denitrifying carriers in R1 on day 43 were 2.7 and 3.5 times higher than those in R2, respectively. The results also demonstrates 68.1% higher NH_4^+-N SARR for virgin carriers and 1.7 times higher NH_4^+-N SARR for pre-seeded denitrifying carriers in R1 than those in R2 on day 64. This demonstrates that adding detached biofilm mass as inoculum to the reactor enhanced the biofilm attachment and possibly growth on both the virgin carriers and on pre-seeded denitrifying carriers within 64 days of SBR operation. Moreover, the significant difference in the kinetic assay NH_4^+-N SARR of the pre-seeded denitrifying carriers of R1 compared to R2 is indicative that the elevated anammox kinetics observed in R1 are due to the attachment of anammox cells followed by growth rather than growth of anammox cells pre-existing in the denitrifying biofilm.

After 64 days of operation and subsequent to reactors R1 and R2 being changed to continuous operation, the kinetic assay NH_4^+-N SARR measurements of the virgin and pre-seeded denitrifying carriers of R1 on days 106 and 156 of operation were no longer distinct from the kinetic assay rates measured of the virgin and pre-seeded denitrifying carriers of R2 on the same days. This lack of distinction in the kinetic assay measurements after longer operation times are indicative of the carriers of both reactors having achieved biofilm maturation and hence no longer demonstrating statistically significant increases in kinetic performances across operation time.

5.5.2 Biofilm coverage

VPSEM imaging was used to quantify cell attachment and biofilm coverage of the virgin carriers in both reactors. Non-uniform biomass attachment and very limited coverage of the virgin carriers were observed on day 3 of operation of both reactors, with greater than 50% biofilm coverage of the virgin carriers being observed on day 26 in both reactors. Full biofilm coverage was achieved between day 32 and 57 for virgin carriers that were exposed to detached biofilm inoculum (R1). Whereas full biofilm coverage for virgin carriers exposed to attached biofilm inoculum (R2) was observed between days 57 and 71 (Figure C-5.2). The trend in biofilm attachment over time indicates that seeding with detached anammox biofilm mass reduces approximately 30% time required for virgin carriers than those seeded with attached anammox biofilm to achieve complete biofilm coverage and promotes the biofilm development subsequently.

5.5.3 Biofilm thickness

The biofilm thickness measurements of the virgin and the pre-seeded denitrifying carriers were quantified in this study via VPSEM starting on day 32 of operation. Day 32 VPSEM images were used to start quantifying biofilm thickness in this study as the biofilm coverage measurements corresponding to this day of operation demonstrated that the virgin carriers began to show elevated

biofilm coverage. The measurements of the biofilm thickness for the pre-seeded denitrifying carriers in both R1 and R2 were significantly higher than the virgin carriers during the early stages of the study due to the pre-existing denitrifying biofilm on carriers. It was also shown in this study that the pre-seeded denitrifying carriers maintained statistically significantly higher biofilm thicknesses in comparison to the virgin carriers throughout the entire experiment (Figure 5.2). The distinction between biofilm thickness of the virgin and pre-seeded denitrifying biofilm carriers is likely indicative that the biofilm morphology remains related to the origins of the carriers for longer operational times compared to the anammox activity of the biofilm.

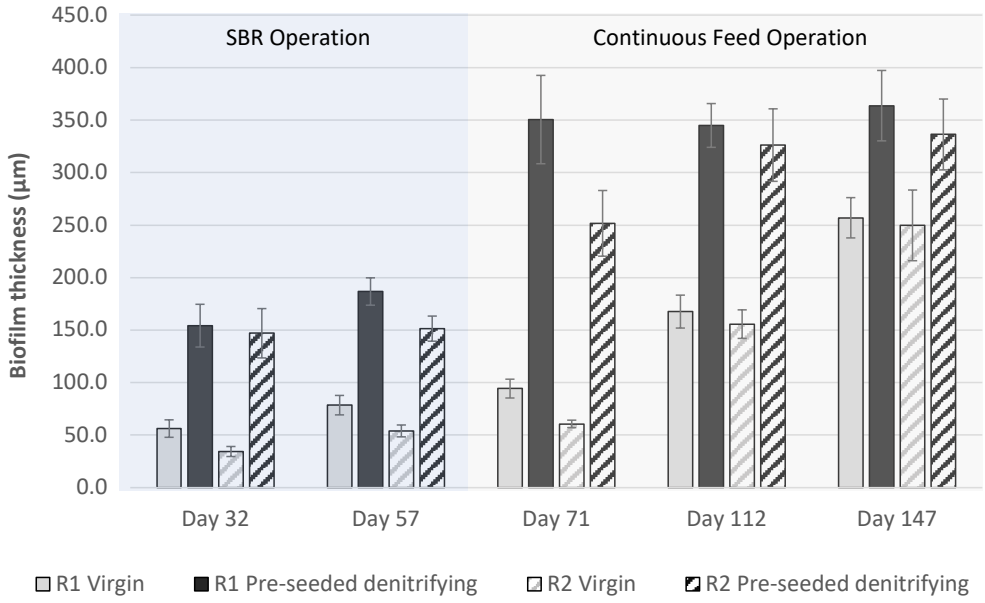

Figure 5.2 Biofilm thickness on the virgin and pre-seeded denitrifying carriers in R1– detached biofilm inoculation and R2 – attached carrier inoculation. Error bars indicate the 95% confidence intervals.

In R1, the biofilm thickness of virgin carriers statistically increased from 56.2 ± 8.3 µm on day 32 to 78.5 ± 9.3 µm on day 57 and 94.3 ± 9.0 µm on day 71 during SBR operation and the

transition period from SBR to continuous feed operation. This operational period demonstrated an average rate of biofilm thickness increase of 0.99 μm/d. As the reactor was continued to be operated as a continuous feed system, the biofilm thickness on the virgin carriers showed a significant increase to 167.6 ± 15.7 μm on day 112 and 257.0 ± 19.2 μm on day 147 and an average rate of biofilm thickness increase of 2.16 μm/d. This rapid increase in biofilm thickness coincides with an observed significant increase in the kinetic assay NH_4^+-N SARR of the virgin carriers measured on day 106 of operation.

The pre-seeded denitrifying carriers of R1 showed a high biofilm initial thickness of 154.2 ± 20.3 μm on day 32 and an increase in thickness to 350.5 ± 42.1 μm by day 71 with an average biofilm thickness increase rate of 6.00 μm/d, which is approximately 6 times greater than the rate observed for virgin carriers. No subsequent changes in biofilm thickness were observed for the pre-seeded denitrifying carriers, which indicates that the pre-seeded denitrifying carriers achieved stable biofilm thickness prior to or on day 71 of operation. As such, the pre-seeded denitrifying carriers likely demonstrated a stable biofilm thickness prior to achieving stable anammox kinetics, as indicated by the kinetic assay NH_4^+-N SARR increase between days 64 and 106 of operation.

In R2, the biofilm thickness of the virgin carriers increased from 34.4 ± 4.8 μm on day 32 to 54.0 ± 5.7 μm on day 57 and 60.7 ± 3.6 μm on day 71 with the average rate of biofilm thickness increase of 0.65 μm/d during SBR operation and the transition period from SBR to continuous feed operation. As the reactor was continued to be operated as a continuous feed system, the biofilm thickness on the virgin carriers showed a significant increase to 155.7 ± 13.6 μm on day 112 and 249.8 ± 33.7 μm on day 147 and an average rate of biofilm thickness increase of 2.49 μm/d. Consistent with the results of R1, this rapid increase in biofilm thickness again coincides with an observed significant increase in the kinetic assay NH_4^+-N SARR of the virgin carriers

measured on day 106 of operation. It has been noticed that the biofilm thickness of the virgin carriers in both R1 and R2 kept increasing after 106 days of operation while the anammox kinetic decreased on day 156 (Table 1). This is probably due to the significantly high C/N ratio in the new batch of centrate that was collected for the feed on day 128, which cause the anammox inhibition and the growth of heterotrophic biofilms.

The biofilm thickness of the pre-seeded denitrifying carriers in R2 did not statistically change from 147.0 ± 23.5 μm on day 32 to 151.4 ± 12.0 μm on day 57 and significantly increased to 251.7 ± 31.3 μm on day 71 and 326.2 ± 34.5 μm on day 112. The average biofilm thickness increase rates for the pre-seeded denitrifying carriers were 2.68 μm/d between days 32 to 71, with a continual rate of thickness increase of 1.82 μm/d between days 71 to 112. No further change observed in biofilm thickness after day 112, which indicates that the pre-seeded denitrifying carriers achieved stable biofilm thickness prior to or on day 112. The trend of biofilm thickness growth on the pre-seeded denitrifying carrier coincides with the increase pattern in kinetic assay NH_4^+-N SARR of the pre-seeded denitrifying carrier which increased and stabled prior to or on day 106.

Comparing the biofilm thickness of each carrier type in both reactors indicates that the biofilm on both virgin and pre-seeded denitrifying carriers in R1 were significantly thicker than those in R2 on day 32, day 57 and day 71 with the exception of the pre-seeded denitrifying carriers on day 32. In particular, the virgin carriers in R1 were 63.4%, 45.4% and 55.4% thicker than the virgin carriers in R2 on days 32, 57 and 71, respectively. In addition, the virgin carrier in R1 showed a 52.3% greater rate of biofilm thickness increase compared to R2 between days 32 and 71 of operation. The pre-seeded denitrifying carriers in R1 were 23.4% and 39.3% thicker than the pre-seeded denitrifying carriers in R2 on days 57 and 71, respectively. The pre-seeded denitrifying

carrier in R1 showed a 1.2 times greater rate of biofilm thickness increase compared to R2 between days 32 and 71 of operation. The biofilm thickness of the R2 pre-seeded denitrifying carriers is not statistically greater than the initial biofilm thickness of the pre-seeded denitrifying carriers during the SBR operation period, which indicates a general delay in biofilm maturation of the carriers in R2 that were operated with attached biofilm inoculation. Following the same trend shown by the kinetic assay NH_4^+-N SARR, the biofilm thickness of both the virgin and pre-seeded denitrifying carriers in R2 demonstrate a significant increase by day 112 after the system transit from SBR to continuous feed operation and no statistical difference with those in R1 thereafter. The differences observed in biofilm attachment, growth and maturation rates between the carriers exposed to detached biofilm mass (R1) and attached biofilm carriers (R2) may have resulted in improved biofilm development and subsequently enhanced reactor performance (Rollet et al., 2008; Zhang et al., 2013; Wang et al., 2018).

5.5.4 Anammox bacteria enrichment

The quantity of anammox bacteria enriched across operation time on the virgin and pre-seeded denitrifying carriers seeded with detached biofilm inoculum (R1) and attached biofilm inoculum (R2) were analyzed using ddPCR to quantify the anammox gene that encodes the hzo mRNA, a biomarker of anammox bacteria activity (Figure 5.3). During the SBR operation (day 1 to 64), the anammox bacteria gene counts of the virgin and pre-seeded denitrifying carriers in R1 significantly increased from $2.25 \pm 0.96 \times 10^6$ and $1.35 \pm 0.61 \times 10^7$ copies/carrier at day 25 to $2.08 \pm 0.76 \times 10^7$ and $7.21 \pm 4.64 \times 10^7$ copies/carrier at day 39 and $3.94 \pm 3.5 \times 10^8$ and $1.07 \pm 0.65 \times 10^9$ copies/carrier at day 64, respectively. The anammox bacteria abundance attached to pre-seeded denitrifying carriers was significantly higher than the virgin carriers in R1. From day 105 onward, the increase in attached anammox gene copies stabilized on both carriers in R1, which

is consistent with the observed attachment, biofilm thickness and ammonia removal kinetic assay data.

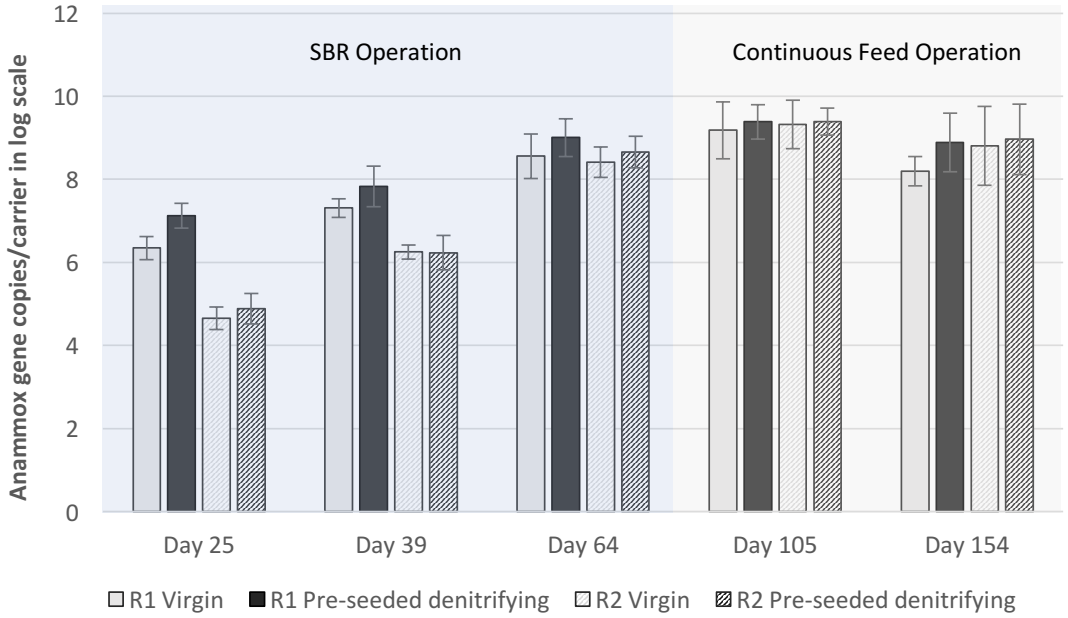

Figure 5.3 Comparison of the ddPCR results with operation time for the virgin and pre-seeded denitrifying carriers seeded with detached biofilm inoculum (R1) and attached biofilm inoculum (R2). Error bars indicate 95% confidence intervals.

In R2, the trend in attached anammox bacteria abundance suggests that both types of carriers demonstrated similar incremental increases in attachment of anammox bacteria across time. This similar increase observed in both carrier types may be a result of the inoculation method and hence the resultant pathway of attachment of anammox bacteria that occurred in this reactor. In particular, the similar attachment rate of anammox bacteria to both types of carriers may indicate that the rate of dispersal and detachment of anammox bacteria from the seeded anammox carriers in R2 is the limiting factor and hence controls the rate of attachment of anammox bacteria on all carriers.

Comparing the two anammox inoculation methods, the anammox gene copy numbers attached to the virgin and pre-seeded denitrifying carriers in R2, seeded with attached biofilm inoculum, were $4.60 \pm 2.06 \times 10^4$ and $7.97 \pm 4.13 \times 10^4$ copies/carrier at day 25 and increased to $1.79 \pm 0.45 \times 10^6$ and $1.79 \pm 1.21 \times 10^6$ copies/carrier at day 39, respectively. The results indicate 47.9 and 10.6 times less abundance for virgin carriers as well as 168.4 and 39.3 times less abundance for pre-seeded denitrifying carriers in R2 at days 25 and 39, respectively, compared to R1 that was seeded with detached biomass inoculum. There were no statistical differences measured between the two inoculation methods after day 64 of operation. This indicates that the detached biofilm seeding outperformed the attached biofilm carrier seeding during the early stages of start-up, where seeding with carriers may be rate limited by the dispersal and detachment rate of the attached biofilm from the seeded anammox carriers in the reactor. The observed decline in anammox microbial populations at day 154 corresponds to the declined kinetic assay NH_4^+-N SARR measured at day 156, which are likely related to a change in feed characteristics.

5.5.5 Anammox bacteria doubling time

The assessment of the doubling time and growth rate may be used as key indicators of growth status of the anammox bacteria on the various carriers exposed to the different inoculation methods. As mentioned above, the doubling time in this study is in regards to bacteria attachment and growth. The operational period between days 1 and 25 is likely an attachment phase based on the elapsed time estimated for achieving elevated biofilm coverage on the virgin carriers in both reactors (Figure C-5.2) along with the limited kinetic performances of R1 and R2 during this period of operation. The anammox bacteria average doubling time and the specific attachment-growth rate of the virgin and pre-seeded denitrifying carriers of the study are showed in Table 5.2. The doubling times of the carriers in R1 between days 1 and 25 of this study are lower than the 11 day

values reported in previous studies by Strous et al. (1998) and van der Star et al. (2007). This may be due to this period being a period of attachment and growth as the biofilm coverage for the virgin carriers in R1 only achieved over 50% coverage on day 26. The measured doubling times of anammox bacteria on the carriers in R1 are similar to the findings published by Bae et Al. (2010), who reported a doubling time of 1.18 days for activated sludge in anammox bacteria enrichment cultivation using SBR, and also similar to the doubling time of 1.8 days reported by Isaka et. al (2006) who tested an anaerobic biological filtrated reactor between day 14 to day 21of operation. However, the growth rate is not comparable to the reported values of Bae et al. (2010) or Isaka et al. (2006) as the cultivation conditions such as substrate concentration and reactor type were quite different. The anammox bacteria specific attachment-growth rates of pre-seeded denitrifying carriers indicates 13.8% greater than the virgin carriers in R1. In addition, the anammox bacteria specific attachment-growth rates of the virgin and pre-seeded denitrifying carriers in R1 between days 1 and 25 indicates 38.1% and 46.7% greater than those in R2, respectively. This demonstrates that pre-seeded denitrifying carriers inoculated with detached biofilm promotes anammox bacterial attachment and growth at early start-up stage of attached growth anammox systems.

Table 5.2 Specific attachment-growth rate and doubling time of anammox bacteria attached on virgin and pre-seeded denitrifying carriers in different time periods. R1, detached biofilm inoculation, R2, attached carrier inoculation. Variance indicates 95% confidence intervals.

		R1		R2	
		Virgin	Pre-seeded denitrifying	Virgin	Pre-seeded denitrifying
Day 1 to Day 25	Average Doubling Time (d)	1.19 ± 0.05	1.06 ± 0.04	1.62 ± 0.09	1.54 ± 0.12
	Average Specific Attachment-growth Rate (d^{-1})	0.58 ± 0.03	0.66 ± 0.03	0.42 ± 0.03	0.45 ± 0.03

Day 25 to Day 64	Average Doubling Time (d)	5.31 ± 0.81	6.21 ± 0.36	3.09 ± 0.31	3.14 ± 0.23
	Average Specific Attachment-growth Rate (d^{-1})	0.13 ± 0.02	0.11 ± 0.01	0.23 ± 0.02	0.22 ± 0.01
Day 64 to Day 105	Average Doubling Time (d)	20.28 ± 7.52	32.85 ± 8.94	13.69 ± 2.29	16.75 ± 1.48
	Average Specific Attachment-growth Rate (d^{-1})	0.035 ± 0.012	0.021 ± 0.005	0.051 ± 0.008	0.041 ± 0.004

Between days 25 and 64 of operation the doubling times of the virgin and pre-seeded denitrifying carriers of R2 were 41.5% and 50% less than those measured for the virgin and pre-seeded denitrifying carriers of R1, respectively. In accordance with the changes in doubling time, the specific attachment-growth rates were approximately two times greater than R1 (Table 5.2). Correlation analyses between anammox gene copies across operational time for the two carrier types in R1 and R2 all showed positive exponential regressions of $R^2 > 0.97$. This exponential relation is indicative of exponential growth pattern for the anammox community on the two carrier types in both reactors between days 25 and 64 of operation. Although exponential growth is observed for both reactors during this time period, the lower attachment-growth kinetics for the carriers seeded with detached biofilm (R1) compared to those seeded with attached carriers (R2) are indicative that the anammox communities of R1 have further matured and are nearing the end of their growth phase. Where the attached biofilm seeded (R2) carriers again may be rate limited by the dispersal and detachment rate of the attached biofilm from the seeded anammox carriers in the reactor.

The long doubling times along with slow specific attachment-growth rates between days 64 and 105 of operation along with the maximum kinetic assay NH_4^+-N SARR observed at day 105 confirms that the anammox community attached to the virgin and pre-seeded denitrifying carriers (R1), i.e., the anammox biofilm, reached maturation before or on day 105. The trend

towards longer doubling times and slower specific attachment-growth rates between days 64 and 105 of operation are also observed in R2, again indicating progression of the virgin and pre-seeded denitrifying carriers in this reactor towards maturation. The difference in the doubling time and specific attachment-growth rates between R1 and R2 are again indicative of a delay in the time towards anammox biofilm maturation in R2, likely related to the rates of detachment and dispersion of anammox seeding cells from the seeded biofilm carriers in R2.

5.6 Conclusions

The findings of this study show for the first time that the use of a detached anammox biofilm inoculation with pre-seeded denitrifying carriers has the potential to accelerate the anammox biofilm attachment, growth and maturation. Comparing to attached biofilm carrier seeded systems, the start-up activity of systems housing pre-seeded denitrifying carriers inoculated with detached anammox biofilm mass shows 46.7% greater anammox bacteria specific attachment-growth rate and approximately 168 times higher abundance during the attachment and early growth stages. It also shows 1.2 times greater biofilm thickness increase rate and 3.5 times higher NH_4^+-N SARR at early stages of the start-up.

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6 Chapter 6 – Carrier surface modification for enhanced attachment and growth of anammox biofilm

6.1 Setting the context

Chapter 6 presents the research article entitled *Carrier surface modification for enhanced attachment and growth of anammox biofilm*. This article has been submitted for publication in Chemosphere in 2020. This study investigates and compares the kinetic performance, anammox biomass attachment along with the growth and maturation of the anammox biofilm on different MBBR modified carriers. The surface modifications included: carriers coated with dextran (chemical enhancement); carriers coated with silica crystals (roughness enhancement); pre-seeded carriers sourced from a denitrification plant (pre-biofilm enhancement); and virgin, unmodified carriers (control).

6.2 Abstract

This study investigates and compares the kinetics of nitrogen removal?, attachment, biofilm development and anammox bacteria enrichment on various surface modified carriers throughout the start-up of an MBBR system: virgin, dextran-functionalized carriers, silica-functionalized and pre-seeded denitrifying carriers. Silica-functionalized carriers along with pre-seeded denitrifying carriers induced significant higher kinetics, faster biofilm growth and greater anammox bacteria enrichment during the 64 days of operation compared to non-modified virgin and dextran-functionalized carriers. The elevated anammox bacteria counts along with the elevated kinetics of all carriers measured at day 106 indicated that of the carriers completing biofilm growth and achieving biofilm maturation prior to or at day 106 of start-up. An average NH_4^+-N removal

rate of 0.679 ± 0.030 g NH_4^+-N/d·m^2 and an overall ammonia removal efficiency of 74.1% ± 4.9% was achieved for all carriers following the start-up period of 106 days or less. The anammox gene counts for virgin, dextran-functionalized, silica-functionalized and pre-seeded denitrifying carriers were 1.74 ± 1.08 × 10^9, 7.84 ± 4.58 × 10^8, 1.39 ± 0.37 × 10^9 and 2.55 ± 1.02 × 10^9 copies/carrier, respectively. The results demonstrate that the silica-functionalized and pre-seeded denitrifying carriers offer advantages during the early stage of start-up while the dextran-functionalized carriers did not reduce the start-up period for anammox biofilm.

6.3 Introduction

Anaerobic ammonia oxidation (anammox) is a cost-efficient alternative in terms of energy consumption and chemical addition to conventional nitrification/denitrification for the treatment of high-strength ammonia waste streams (Abma et al., 2010; Kartal et al., 2010; Ali, 2015; Li et al., 2018). Anammox systems have grown in popularity over the last two decades, with over 100 full-scale treatment facilities operating worldwide (Ali and Okabe, 2015; Metcalf & Eddy, 2014). Although anammox technologies have demonstrated key advantages, many specific anammox technologies still face challenges such as the slow growth rate of anammox bacteria and difficulties in anammox bacteria retainment in treatment reactors (Strous et al., 1998; Abma et al., 2007; van der Star et al., 2007; Cho et al., 2010). As such, many studies have focused on attached biofilm technologies such as the moving bed biofilm reactor (MBBR) system due to their ability to retain elevated quantities of anammox biomass within the biofilm (Christensson et al., 2011; Gilbert et al., 2015; Guo et al., 2016; Kowalski et al., 2018). However, a knowledge gap exists with respect to fundamental understanding of how to best achieve enhanced attachment of anammox cells to carriers followed by the rapid growth and maturation of anammox biofilms during the start-up of attached growth systems (Lemaire et al., 2014; Manonmani and Joseph, 2018).

In recent years, carrier surface modifications have been explored as a means of accelerating attachment of microorganisms. These techniques include film-coating and pre-seeded biofilm (Morgan-Sagastume, 2018). For example, film coatings of carriers using molecules similar to extracellular polymeric substance (EPS), such as D-glucoronic acid and dextran, have been successful in enhancing attachment of nitrifiers and *Bacillus subtilis*, respectively (Duanis-Assaf et al., 2018; Morgan-Sagastume, 2018). Pre-seeding carriers with biofilm has also demonstrated the potential of what? as a precursor for attachment of specific microorganisms. In particular, Kowalski et al. (2019) reported faster attachment of anammox bacteria on carriers pre-treated with heterotrophic biofilm. Furthermore, the topography of the carrier was demonstrated to impact biofilm adhesion and formation (Janjaroen et al., 2013; Lagree et al., 2018). Coatings with high roughness, such as granular activated charcoal and silica have also been studied as a means of increasing surface area for the attachment of anammox bacteria and *Candida albicans*, respectively (Kowalski et al., 2019; Lagree et al., 2018). Thus far, the use of pre-seeded biofilm and film coatings that have been studied with respect to anammox attachment has shown variable results (Morgan-Sagastume, 2018).

While the body of research on carrier surface enhancement to promote attachment of bacterial cells to substratum is expanding, very few studies have compared multiple carrier modification methods in the same study with the carriers exposed to the same operating conditions to assess the potential of carrier surface modifications for anammox bacterial attachment, growth and maturation. The purpose of this study was to compare various carrier surface modifications under the same operational conditions on anammox kinetics, biofilm attachment and growth on MBBR carriers throughout the start-up of an MBBR system. The carrier surface modifications studied include: carriers coated with dextran (chemical enhancement); carriers coated with silica

crystals (roughness enhancement); pre-seeded carriers sourced from a denitrification system (pre-biofilm enhancement); and virgin, unmodified carriers (control).

6.4 Materials and methods

6.4.1 Reactor set-up and operation

An 18 L anammox laboratory MBBR system was operated for 163 days at a controlled temperature of 30°C, to simulate conventional sidestream municipal wastewater treatment. Operational pH was maintained within the range of 7.5 to 7.8 via the addition of alkalinity and dissolved oxygen (DO) concentration was maintained below 0.5 mg O_2/L to promote microbial anoxic conditions in the system. Mixing was achieved using a benchtop lab mixer at low rotation to gently mix the carriers and to keep the carriers in motion in the reactor while minimizing the entrainment of air into the reactor. The reactor was sealed with a fitted Styrofoam cover to further prevent oxygen entrainment.

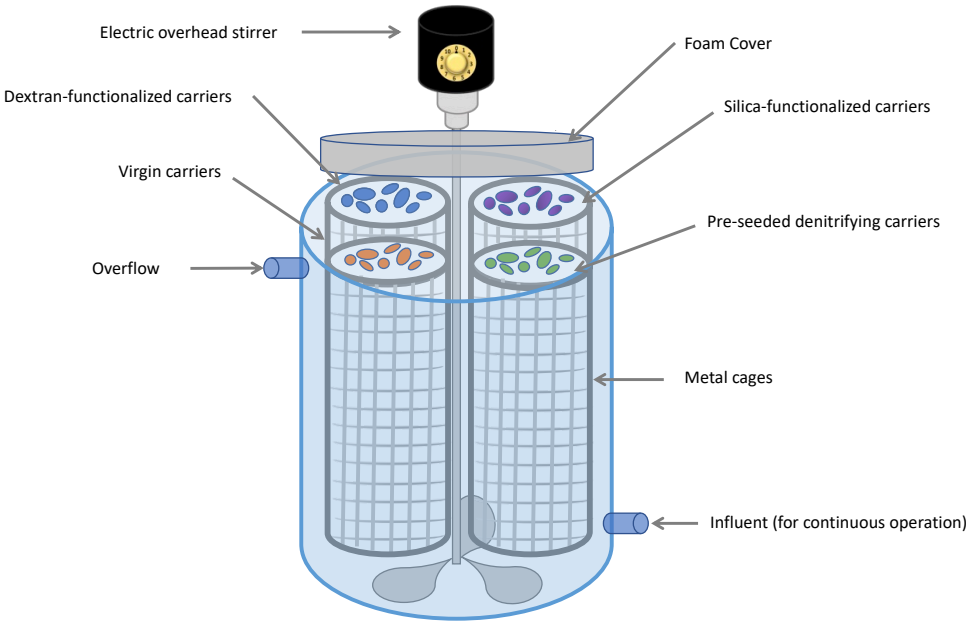

Figure 6.1 Schematic diagram of the laboratory anammox MBBR.

To investigate the carrier modification effects, four stainless steel wire cages were placed inside a single reactor to maintain exposure of all carriers to the same bulk liquid conditions. Each cage (volume of 2.2 L) accommodated 350 AnoxK^{TM}5 bio-carriers (AnoxKaldnes - Veolia Water Technologies, Lund, Sweden) with the carriers in the different cages having various surface modifications (virgin, dextran-functionalized, silica-functionalized and pre-seeded denitrifying biofilm), for a total of 1400 carriers in the reactor (Figure 6.1). The reactor was inoculated with anammox biofilm that was harvested from a full-scale, ANITA^{TM} Mox system operating in the South Durham WRRF, Durham, NC, US. The harvested anammox biofilm was mechanically detached from carriers prior to being added to the reactor as inoculum (Tian et al., 2020). In the start-up of attached growth anammox systems, it is common to seed the reactor with anammox biofilm carriers at a concentration of 3-15% of the design carrier fill percentage (Kowalski et al., 2019). To recreate these conditions with detached biofilm, the detached biomass inoculum was removed from 140 carriers, which is equivalent to 10% of the total carriers added to the reactor. Sufficient biofilm mass was removed from the AnoxK^{TM}5 anammox carriers by vortexing the carriers using a vortex mixer (VWR, Canada, Ontario). Confocal laser scanning microscopy (Zeiss, US, VA) in combination with viability staining (Life Technologies, US, CA) was performed, pre-removal and post-removal, to confirm that the detached cells remained viable after removal of the anammox biofilm from the inoculum carriers.

The system was operated in sequencing batch reactor (SBR) mode for the first 65 days to prevent washout of the detached biomass seed, allow sufficient time for the detached biomass seed to attach to the various carriers. In the second stage of the study, the system was operated as a continuous feed reactor for a period of an additional 98 days with a hydraulic retention time (HRT)

of 24 hours. The experimental phase was ended when steady anammox activity was achieved and mature biofilm was evident on all carrier types in the system.

6.4.2 Centrate feed characteristics

The reactor was fed with diluted anaerobic digestion reject centrate obtained from a municipal WRRF located in Cornwall, Ontario, Canada. Batches of centrate were collected over the 163-day operation period, with collected batches showing some variation in characteristics (Table 6.1). The centrate was stored at 4°C for a maximum of 4 weeks prior to feeding the reactor. Prior to addition to the reactors, the centrate was diluted with distilled water to target ammonia influent concentrations of 150 – 200 mg NH_4^+-N/L. As this study is focussed on the attachment of anammox bacteria, and hence replicates the operation of a post partial nitritation system, nitrite was augmented in the feed to simulate effluent from an upstream partial nitritation unit. As such, the reactor feed was augmented with a stock nitrite solution.

Table 6.1 Centrate feed characteristics.

Constituent	**Raw Concentration** (mg/L)
Ammonia, NH_4^+-N	530- 976
Nitrate, NO_3^-N	~ 0
Nitrite, NO_2^-N	~ 0
sCOD	1530-1690
Alkalinity (as $CaCO_3$)	210-1150

6.4.3 Carrier surface modifications

AnoxK^{TM}5 carriers with four different surface conditions were used in this study (Figure 6.2). The dextran-functionalized carriers were treated with dextran using oxygen plasma activation. The surface-activated carriers were incubated in a 1 g/mL dextran solution for 20 hours with

magnetic stirring. The silica-functionalized carriers were heat-treated with silica beads to adhere silica beads to the surface of the carriers. The pre-seeded denitrifying carriers were harvested from a pilot study denitrification plant achieving 1 g $N/m^2/d$ of removal as a post-denitrification unit. The virgin, unmodified carriers served as a control group in the experiment.

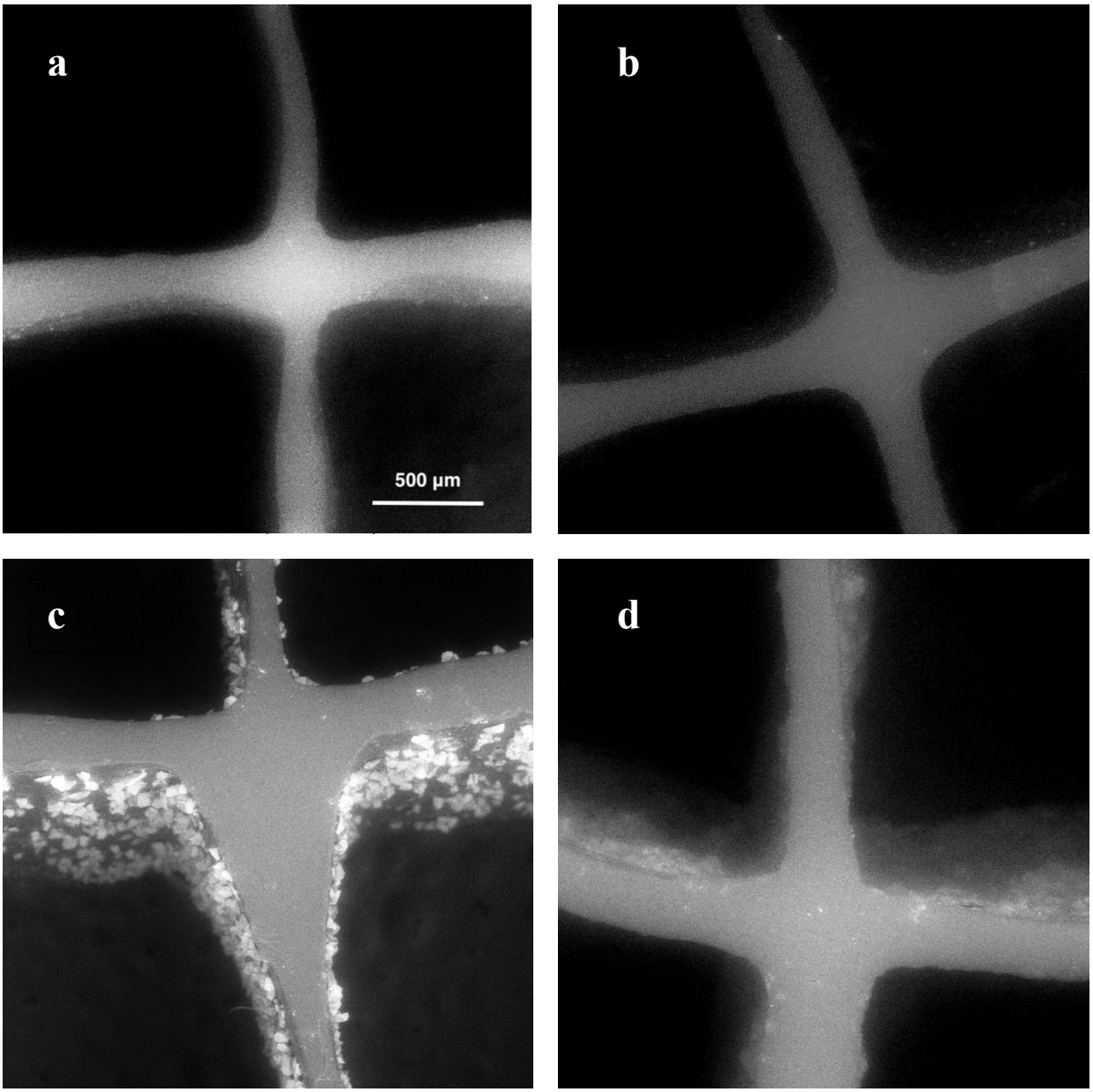

Figure 6.2 VPSEM images of carrier surface conditions at 60x magnification: (a) non-modified virgin carrier; (b) dextran-functionalized carrier; (c) silica-functionalized carrier; (d) pre-seeded denitrifying carrier.

6.4.4 Batch kinetic assays

500-mL batch reactor assays were run to isolate the kinetics of the various carrier types in the reactor at different time throughout the study. Each batch assay vessel contained 75 carriers that were maintained in motion in the vessel using a magnetic stirrer. The batch assay vessels were operated over an 8-hour period at a temperature of 30ºC. The vessels were fed with 400 mL of the same diluted centrate that was fed to the main reactor, from where the carriers were harvested. Initial concentrations of 40 mg NH_4^+-N/L, 40 mg NO_2^--N/L, and 250 mg as $CaCO_3$/L were targeted for the feed to the batch assay vessels. These constituent concentrations were targeted based on the expected removal rates of the carriers within a period of 8 hours while achieving a minimum significant change in NH_4^+-N/ and NO_2^--N concentrations of 2 mg/L. DO concentrations and pH values were adjusted to the same values as the main reactor. Ammonia, nitrite, nitrate and alkalinity were measured every 30 minutes for the first 2 hours, and every 2 hours thereafter. Total suspended solids (TSS), volatile suspended solids (VSS) and soluble chemical oxygen demand (sCOD) were measured at time 0 and 8 hours.

6.4.5 Constituent analytical methods

Operational parameters such as pH, temperature and DO were monitored in the reactor and the batch assay vessels using a symphony Multi Parameter Meter with an attached DO probe and pH probe (VWR, Canada, Ontario). Concentrations of ammonia, nitrite, nitrate, alkalinity, sCOD were triplicate measured in the kinetic assay vessels to quantify sampling and analytical error. Ammonia (TNT 830/832), nitrate (TNT 840), alkalinity (TNT 870) and sCOD (HACH 8000)

concentrations were determined via a DR 5000 spectrophotometry (HACH, Loveland, CO) using HACH vial test kits. The colorimetric method was used to determine nitrite concentration in accordance with Standard Method 4500-NO_2^- (APHA, 2005).

6.4.6 Biofilm coverage and thickness

Biofilm attachment, biofilm coverage and the in-situ biofilm thickness on the carriers were observed and measured from acquired Vega II-XMU SEM (Tescan USA Inc., Cranberry, PA) variable pressure scanning electron microscope (VPSEM) images (Delatolla et al., 2009; Young et al., 2017). To quantify biofilm thickness, 25 thickness measurements per carrier were processed from the acquired images using the Atlas imaging processing software (Tescan USA Inc., Cranberry, PA).

6.4.7 Droplet digital polymerase chain reaction analysis

DNA of the biofilm from harvested carriers was extracted using a FastDNA® Spin Kit (MP Biomedicals, US, CA), with the extracted DNA stored at -20°C until droplet digital polymerase chain reaction (ddPCR) analysis. The ddPCR reaction mixture contained 5 µL of sample and 15 µL of QX200™ ddPCR™ EvaGreen® Supermix (Bio-Rad, Hercules, CA), including 0.1 µM of primers (Tian et al., 2020). The generated droplet amplified in a T100™ Thermal Cycler (Bio-Rad, Hercules, CA). The amplification conditions consisted of denaturation at 95 °C for 5 min, 50 cycles of 30 s at 95 °C, 60 s at 57 °C and 30 s at 72 °C followed by 5 min cooldown at 4 °C, 5 min at 90 °C for droplet stabilization and hold at 12 °C. The plate was transferred to a QX200 droplet reader (Bio-Rad, Hercules, CA). Data acquisition and analysis were performed using the QuantaSoft analysis software (Bio-Rad, version 1.7.4, Hercules, CA). The quality controls criteria employed in this study included that the number of total droplets quantified were above 10,000 and no more than 5 positive droplets were identifiable in the negative controls.

6.4.8 Statistical analyses

Statistical analyses of constituents, biofilm thickness and anammox bacterial gene copies were tested using t-test and ANOVA with a *p*-value less than 0.05 being considered significant.

6.5 Results and discussion

6.5.1 MBBR reactor operation

The MBBR reactor was initially operated in SBR mode for the first 64 days and shifted to continuous feed operation mode for another 98 days (Table 6.2). Between days 1 to 10, during the SBR operation, the target feed concentration of NH_4^+-N and NO_2^--N were 200 mg/L each to allow the reactor to start-up without substrate limitation. However, the low kinetic activity during this early period and the lack of nitrite uptake was deemed a risk to exposing the microbial population to nitrite inhibition. Nitrite inhibition was observed in previous studies at nitrite concentrations between 100 and 400 mg NO_2^--N/L (Kimura et al., 2010; Lotti et al., 2012; Puyol et al., 2014). Therefore, the target feed concentrations were modified to 150 mg/L of NH_4^+-N and 50 mg/L NO_2^--N on day 11 to allow the microbial population to acclimate and initiate significant anammox kinetics prior to augmenting the nitrite concentration. Ammonia surface area removal rates (SARR) were? found to increase steadily between days 11 and 64 at target nitrite concentrations between 15 and 50 mg NO_2^--N/L.

Table 6.2 Ammonia removal kinetics under SBR and continuous feed operations across time.

SBR Operation		**Target Feed Concentrations**	
Batch Period	SARR ± 95% CI (g NH_4^+-N/d·m^2)	Ammonia (mg NH_4^+-N/L)	Nitrite (mg NO_2^--N/L)
Day 1 to 10	~ 0	200	200
Day 11 to 22	0.05 ± 0.01	150	50
Day 23 to 31	0.08 ± 0.01	150	15

Day 32 to 38	0.13 ± 0.01	150	15 to 50
Day 39 to 64	0.26 ± 0.04	150	50
Continuous Feed Operation		**Target Feed Concentrations**	
Day 65 to 71	0.12 ± 0.12	150	75
Day 72 to 98	0.43 ± 0.04	150	75
Day 99 to 126	0.68 ± 0.03	150	150
Day 128 to 158	0.60 ± 0.12	150	150

The MBBR reactor shifted to continuous feed operation on day 65. The target influent concentration was approximately 150 mg/L of NH_4^+-N and 75 mg/L of NO_2^--N between days 65 and 98. The reactor took approximately 6 days to acclimate from SBR to continuous feed operation; with acclimatization to continuous feed across these 6 days of operation being identified by a significant fluctuation in NH_4^+-N SARR (Table 6.2). After the transition period, the average NH_4^+-N SARR was 0.43 ± 0.04 g NH_4^+-N/m^2·d with an average ammonia removal efficiency of 64.4% ± 5.5% and an average NO_2^--N consumption of 97.1% ± 0.7%. The feed nitrite concentration target was augmented to approximately 150 mg/L of NO_2^--N on day 99 in response to the high NO_2^--N consumption rate and limited nitrite availability. The reactor performed stable anammox removal with an average NH_4^+-N SARR of 0.68 ± 0.03 g NH_4^+-N/m^2·d from day 99 to 126, along with an ammonia removal efficiency of 74.1% ± 4.9% and a nitrite consumption of 94.1% ± 2.6%. On day 128, a new batch of centrate was collected for the feed, with this collected centrate showing significantly different characteristics to previous centrate batches collected. In particular, the C/N ratio was significantly elevated for this collected batch, with a C/N ratio of 3.1 compared to previous values of on average 1.1. The feed change corresponded to a decrease in the performance with an average NH_4^+-N SARR of 0.60 ± 0.12 g NH_4^+-N/m^2·d and removal efficiency of 49.7% ± 6.7% and nitrite removal efficiency of 75.6 ± 8.5%.

6.5.2 Ammonia removal kinetics

Kinetic assays were used to isolate the anammox activity of the carriers in each of the individual four cages in the reactor (Figure 6.3). At day 43, the NH_4^+-N SARR was 0.24 ± 0.04 g NH_4^+-N/m^2/d for silica-functionalized carriers and 0.26 ± 0.04 g NH_4^+-N/m^2/d for pre-seeded denitrifying carriers, which were significantly higher than the SARR of 0.15 ± 0.02 g NH_4^+-N/m^2/d for virgin carriers and 0.13 ± 0.02 g NH_4^+-N/m^2/d for dextran-functionalized carriers. There was no statistical difference in SARR between virgin and dextran-functionalized carriers; as well as between silica-functionalized and pre-seeded denitrifying carriers. At day 64, the kinetic activities for the dextran-functionalized, silica-functionalized and pre-seeded denitrifying carriers were consistent with the kinetics measured on day 43; with the exception of the virgin carriers that showed a significant increase in SARR to 0.20 ± 0.02 g NH_4^+-N/m^2/d. As such on day 64, the SARR of silica-functionalized carriers and pre-seeded denitrifying carriers were again significantly higher than those of virgin carriers and dextran-functionalized carriers. These results suggest that silica coating and denitrifying pre-seeded biofilm carriers can reduce the start-up period for anammox attached growth systems by achieving elevated ammonia removal kinetics earlier than virgin carriers and dextran-functionalized carriers.

It is likely that the roughness of the silica coated carriers increased the protected surface area for anammox bacteria (Kowalski et al., 2019; Morgan-Sagastume, 2018). Similarly, the denitrifying pre-seeded biofilm carriers and associated hydrophobic EPS provided an ideal attachment surface for anammox bacteria (Ding et al., 2017; Kowalski et al., 2019). The pre-seeded denitrifying carriers may have also initiated growth of already existing anammox population in the embedded existing biofilm. Dextran coating did not appear to improve ammonia removal kinetics during the MBBR reactor start-up period, contrary to previous studies (Morgan-Sagastume, 2018).

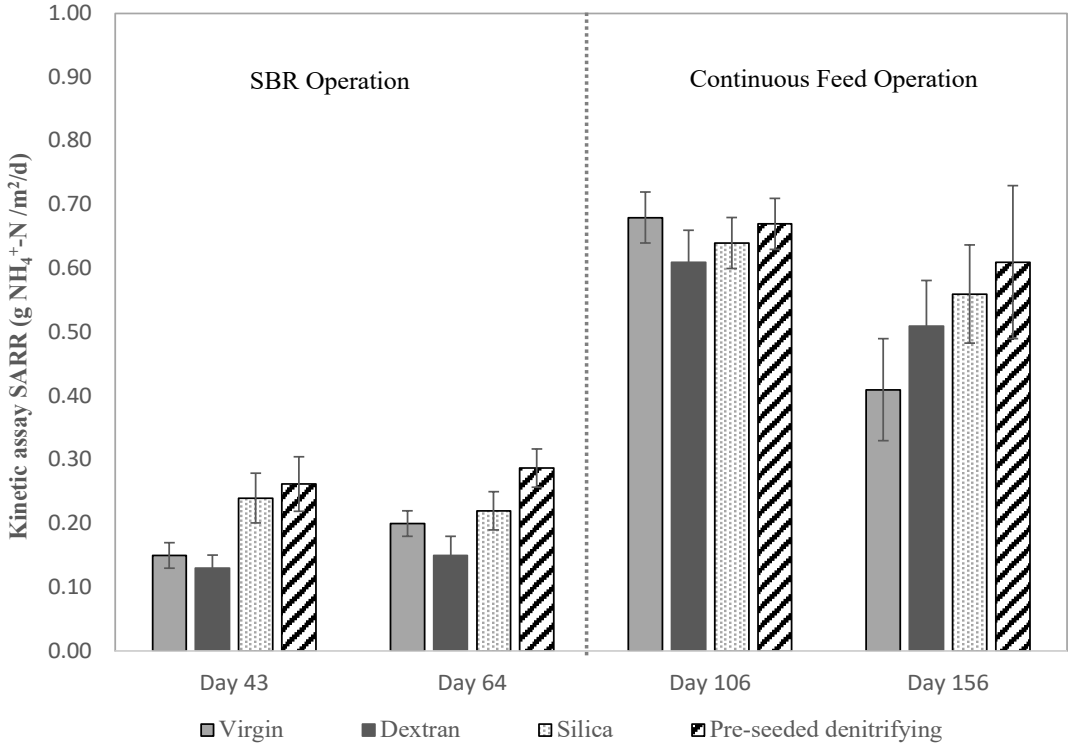

Figure 6.3 NH_4^+-N SARR of kinetic assays of the various carrier types, error bars indicate the 95% confidence intervals.

At day 106 (continuous feed operation mode), the SARR for all carrier types showed a significant increase compared to the SARR on day 64. Specifically, the SARR for the virgin carriers, dextran-functionalized carriers, silica-functionalized carriers and pre-seeded denitrifying carriers increased to 0.68 ± 0.04 g NH_4^+-N/m^2/d, 0.61 ± 0.05 g NH_4^+-N/m^2/d, 0.64 ± 0.04 g NH_4^+-N/m^2/d and 0.67 ± 0.04 g NH_4^+-N/m^2/d, respectively. The SARR associated with the various carriers were all similar and not statistically distinct. The elevated rates measured at day 106 are indicative of the carriers reaching kinetic equilibrium, and likely completing biofilm growth and achieving biofilm maturation prior to or at day 106 of start-up. At day 157, a significant decrease in kinetic activity was observed for all carrier types. This SARR decrease was likely caused by the significantly higher sCOD concentration of the new batch of centrate harvested and fed to the

carriers starting on day 128. Previous studies have demonstrated inhibitory effects of elevated C/N ratio on anammox kinetics (Wang et al., 2018; Zhang et al., 2013).

6.5.3 Biofilm attachment

Biofilm attachment is defined in this study as identifiable biological material, cells and/or EPS that is attached to the various types of carriers prior to the complete coverage of the carriers with biofilm. VPSEM imaging was used to quantify cell attachment and biofilm coverage of the virgin, dextran-functionalized and silica-functionalized carriers; with the pre-seeded denitrifying carriers not being evaluated for biofilm attachment as these carriers already had a mature denitrifying biofilm at the start of the study. Attachment was first observed on day 3 for all three types of carriers, with the carriers exhibiting similar trends in percentage of the imaged carriers being covered by biofilm across time. Over 50% biofilm coverage was observed for all three carriers on day 26 and full biofilm coverage was observed between days 32 and 57 (Figure C-6.1).

6.5.4 Biofilm thickness

The biofilm thickness measurements of all carriers were quantified via VPSEM starting on day 32 of operation as full biofilm coverage began to be observed for the virgin, dextran-functionalized and silica-functionalized carriers at this time (Figure 6.4). On days 32 and 57, during SBR operation, no statistical difference was observed in biofilm thickness for the virgin, dextran-functionalized and silica-functionalized carriers. On day 71 of operation, just following the MBBR reactor acclimatization to continuous feed operation, the virgin and silica-functionalized carriers demonstrated similar biofilm thickness, while the dextran-functionalized carriers showed a significantly lower thickness.

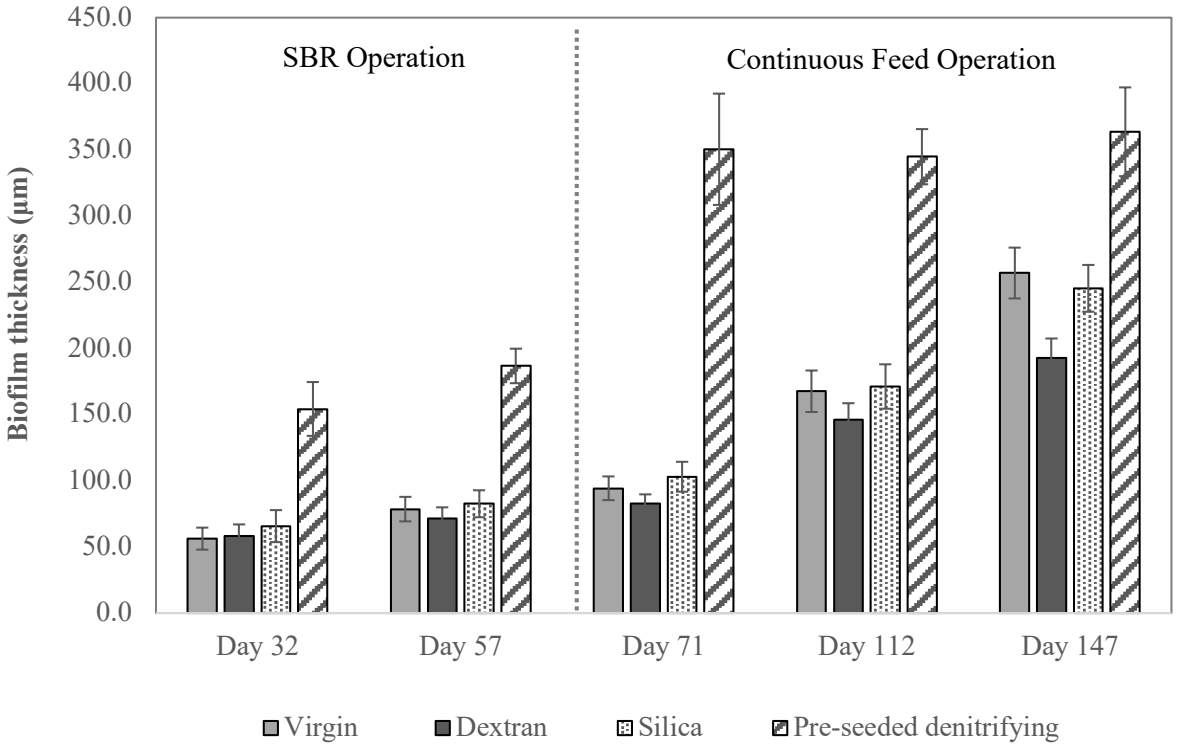

Figure 6.4 Biofilm thickness for various carrier types, error bars indicate the 95% confidence intervals.

Both virgin and silica-functionalized carriers showed a similar average biofilm growth rate of 1.0 µm/d between days 32 to 71; where the biofilm thickness of virgin carriers increased from 56.2 ± 8.3 µm on day 32 to 78.5 ± 9.3 µm on day 57 and 94.3 ± 9.0 µm on day 71, and the biofilm thickness of silica-functionalized carriers increased from 65.7 ± 12.1 µm on day 32 to 82.6 ± 10.2 µm on day 57 and 103.0 ± 11.3 µm on day 71. The dextran-functionalized carriers however demonstrated a lower biofilm growth rate of 0.7 µm/d with a biofilm thickness of 58.1 ± 8.9 µm initially measured on day 32 increasing to 71.5 ± 8.4 µm on day 57 to 82.8 ± 6.9 µm on day 71. There is no statistical difference between the thickness of dextran-functionalized carriers on day 71 to the thickness of virgin and silica-functionalized carriers on day 57, which indicates a delay of biofilm development of the dextran-functionalized carriers. This delay of biofilm development

is likely the cause of the lowest NH_4^+-N SARR kinetic assay performance that was observed for dextran-functionalized carriers on day 64.

After the reactor shifted to continuous operation mode, significant increases in biofilm thickness were observed for all three carrier types: virgin, dextran-functionalized and silica-functionalized carriers. This rapid increase in biofilm thickness coincides with an observed significant increase in NH_4^+-N SARR of these three carriers types measured on day 106 of operation. In particular, the biofilm thickness of virgin carriers was 167.6 ± 15.7 μm on day 112 and 257 ±19.2 μm on day 147, with the highest average biofilm development rate of 2.1 μm/d. The biofilm thickness of silica-functionalized carriers was171.2 ±16.8 μm on day 112 and 245.4 ± 17.7 μm on day 147, with an average biofilm development rate of 1.8 μm/d. While dextran-functionalized carriers showed the lowest biofilm development rate of 1.4 μm/d with the biofilm thickness increased from 146.1 ± 12.5 μm on day 112 to 193.0 ± 14.5 μm on day 147.

As the pre-seeded denitrifying carriers already had a mature biofilm at the start of the experiment, the biofilm thickness of the pre-seeded denitrifying carriers was 154.2 ± 20.3 μm on day 32, 186.8 ± 13.0 μm on day 57 and 350.5 ± 42.1 μm on day 71; demonstrating a biofilm growth rate of 6.0 μm/d. From day 71 to 147, no statistical changes in biofilm thickness was observed on the pre-seeded denitrifying carriers, which likely indicates that the pre-seeded denitrifying carriers have achieved at this time a fully acclimatized and mature biofilm. It is noted that the fully achieved mature and acclimatized biofilm thickness of the pre-seeded denitrifying carriers occurred prior to achieving their maximum anammox kinetics; which was observed in the kinetic assay NH_4^+-N SARR rates measured between days 64 and 106 of operation.

6.5.5 Anammox populations

The number of anammox gene copies enriched on each carrier type over time shows an increase in gene enrichment from day 25 to day 105 of the study (Figure 6.5). This increase in anammox gene enrichment corresponds to increases in anammox kinetics and biofilm thickness; hence demonstrating the attachment and growth of anammox communities during the initial days of operation of the system. The subsequent decline in anammox microbial populations observed on day 154 of operation probably can be attributed to a change in the properties of the centrate feed. As such it is likely that this decline in anammox gene copies attached to the carriers is due to the inhibitory effects of the new batch of centrate that also resulted in an observed decrease in kinetics (Figure 6.3). Microbial sensitivity of anammox communities have been documented in previous findings (Wang et al., 2018; Zhang et al., 2013).

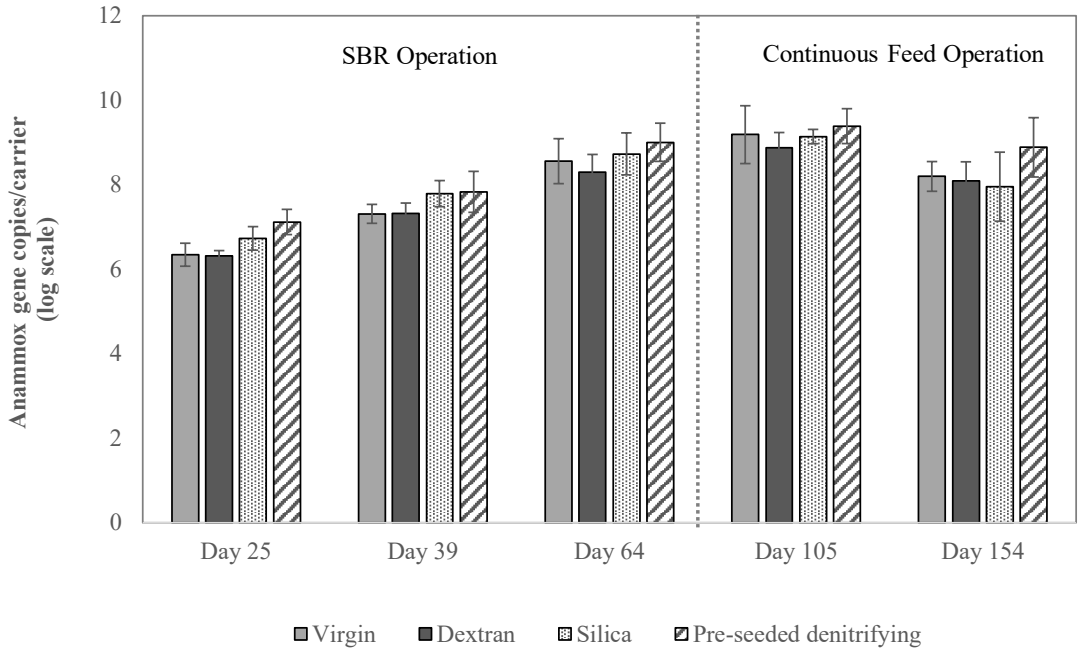

Figure 6.5 Anammox bacteria gene copy counts in log scale for various carrier types. Error bars indicate the 95% confidence intervals.

At day 25, during the biofilm attachment phase, the anammox gene copy counts were 1.35 ± 0.61 × 10^7 copies/carrier and 5.49 ± 2.29 × 10^6 copies/carrier for pre-seeded denitrifying and silica-functionalized carriers, respectively, which were significantly higher compared to 2.25 ± 0.96 × 10^6 copies/carrier for virgin carriers and 2.08 ± 0.42 × 10^6 copies/carrier for dextran-functionalized carriers. At day 39, the early biofilm growth phase, the pre-seeded denitrifying and silica-functionalized carriers again demonstrated higher anammox gene copies. The higher anammox gene copies attached to pre-seeded denitrifying and silica-functionalized carriers supports the kinetic assay NH_4^+-N SARR results and further confirms pre-seeded denitrifying carriers and silica coating as effective carrier surface modification strategies that promote the attachment and subsequent biofilm growth of the anammox populations during the start-up of attached growth systems. It is likely that the pre-seeded denitrifying carriers provides mature EPS structures for attachment and preservation of the anammox bacteria. Silica crystals, on the other hand, provide enhanced roughness and an increased protected surface area for anammox bacteria to attach.

At day 105, during the continuous feed operation mode, the anammox population peaked for all carrier types. The anammox gene counts for virgin, dextran-functionalized, silica-functionalized and pre-seeded denitrifying carriers were 1.74 ± 1.08 × 10^9, 7.84 ± 4.58 × 10^8, 1.39 ± 0.37 × 10^9 and 2.55 ± 1.02 × 10^9 copies/carrier, respectively. The anammox abundance analyzed in this study is measured a similar counts to previous studies with systems achieving elevated ammonia removal kinetics (Isaka et al. 2006; Bae et al. 2010). The elevated anammox bacteria counts measured on day 105 occur during the highest measured kinetics of the carries, which supports the finding that biofilm maturation occurred in the carriers prior to or at day 105 of start-up.

6.6 Conclusions

This study compares multiple carrier modifications in a single study with the carriers exposed to the same operating conditions to assess the potential of carrier surface modifications for anammox bacterial attachment, growth and maturation. Ammonia removal kinetics, biofilm attachment and growth as well as anammox bacteria enrichment for dextran-functionalized carriers (chemically enhanced), silica-functionalized carriers (roughness enhanced), and pre-seeded denitrifying carriers (biologically enhanced) were compared to non-modified virgin carriers (control). Kinetic batch assays showed significant higher ammonia removal rates for silica-functionalized and pre-seeded denitrifying carriers compared to virgin dextran-functionalized carriers at days 43 and 65. Biofilm thickness increase rates were measured significantly higher for the virgin, silica-functionalized and pre-seeded denitrifying carriers compared to dextran-functionalized carriers. At days 25 and 39, the cell attachment and early growth phase, anammox gene copy counts were also significantly higher for silica-functionalized and pre-seeded denitrifying carriers compared to dextran-functionalized and virgin carriers. The results suggest that surface roughness and biofilm enhancements can effectively improve the rate of attachment and growth for anammox bacteria in attached growth systems.

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7 Chapter 7– Discussion and conclusion

This dissertation advances current practice of municipal, biological wastewater treatment by advancing the design and operation along with the start-up of two attached growth treatment technologies for ammonia removal. In particular, the research investigates critical challenges facing attached growth ammonia removal technologies for rural and urban municipal wastewater treatment applications. It develops fundamental new knowledge necessary to design and efficiently operate the nitrifying BioCord technology as a post-carbon removal, tertiary nitrification system for rural municipal wastewater treatment systems. In addition, this research contributes new knowledge on inoculation and carrier modification start-up strategies for reducing the onerous start-up time of an anammox, attached growth, moving bed biofilm reactor (MBBR) technology.

7.1 Design and optimization of nitrifying biocord technology for rural lagoon nitrification

Chapter 3 and 4 provide the findings on the performance of the BioCord attached growth technology for low carbon, tertiary nitrification. The work isolates the effects of ammonia nitrogen surface area loading rate (SALR), various BioCord substratum and air scouring on nitrification kinetics and solids production in order to evaluate the performance and optimization strategies for the BioCord technology. The three types of BioCord substrate, PP + K-45, MK-PP50 and SP-100, are manufactured with different loop structures, which provide different specific surface areas. In addition, the work investigates the microbial response of the attached growth system, including the biofilm porosity and morphology and embedded biomass viability at key operational conditions.

At the macro-scale, the three BioCord substrata did not demonstrate any statistical difference and show stable performance with high average ammonia nitrogen removal efficiencies of 96.8%, 97.0%, 92.0% and low average solids production rates of 0.19, 0.23, 0.25g TSS/d at NH_4^+-N SALR of 0.8, 1.6 and 1.8 g NH_4^+-N/m^2·d, respectively. The relationship between SALR and surface area removal rate (SARR) indicates a transition from a first-order kinetic relationship at SALR values of 0.8 and 1.6 g NH_4^+-N/m^2·d to a zero order kinetic relationship at loadings of 1.8 g NH_4^+-N/m^2·d. The transition along with the significant decrease in percent ammonia removal efficiency at NH_4^+-N SALR of 1.8 g NH_4^+-N/m^2·d demonstrates that the biofilm transitions from limited ammonia mass transfer rate to limited oxygen mass transfer rate at NH_4^+-N SALR of 1.8 g NH_4^+-N/m^2·d. A loss of system stability and biofilm sloughing, identified as fluctuating ammonia removal rates and solids production rates, were observed at elevated SALRs of 2.1 and 2.4 g -N/m^2·d. Hence, the application of various SALRs demonstrates that BioCord substrata PP + K-45, MK-PP50 and SP-100 may be efficiently operated at SALRs up to 1.8 g NH_4^+-N/m^2·d, providing steady ammonia-nitrogen removal efficiencies greater than 90%. The efficient and steady operational rate at an SALR of 1.6 NH_4^+-N/m^2·d and up to an SALR of 1.8 g NH_4^+-N/m^2·d demonstrates a recommended design rate for the post-carbon treatment, nitrifying BioCord technology above conventional nitrification attached growth design rates of 1 g NH_4^+-N/m^2·d.

At the meso and micro-scales, biofilm porosity was shown to decrease with increasing SALR for all three substrata. The reduction in porosity with increased loadings and decreased ammonia removal efficiency that was observed in this study implies that the excessive growth of biofilm restricts substrate mass transfer through the biofilm under high loading condition, which significantly reduced the nitrifying kinetics and the system stability. The acquired variable pressure scanning electron microscope (VPSEM) images highlight differences in the protozoa, water mite

and rotifer communities across different operational conditions. At high loading conditions, filamentous morphology of biofilms and more advanced microorganisms were observed on all three BioCord substrata. These results demonstrate that the operational conditions directly affect the meso-scale structure of the biofilm and the micro-animal populations. Furthermore, no statistical difference in biofilm coverage and live cell percentage was observed at various SALRs, which indicates the activity of the cells themselves and not cell coverage was linked to the kinetic performance.

The study of the effects of air scouring demonstrated that no statistical improvement was observed in the average SARR or solids production rate. However, sloughing events along with subsequent variance in effluent solids concentrations were shown to be reduced at elevated loading conditions under enhance air scouring conditions. Moreover, biofilm porosity was significantly increased with enhanced air scouring at elevated loading conditions. Hence air scouring was shown as a potential strategy to prevent the clogging of biofilm pores and improve the system stability in terms of solids production rate in the BioCord technology.

7.1.1 Novel contribution and practical implication

This study represents the first performance and optimization study of the BioCord technology for post-carbon removal tertiary nitrification. This study provides novel information at the macro, meso, and micro-scale and develops new fundamental knowledge necessary to design, operate and optimize a post-carbon removal, nitrification BioCod system. This study demonstrates that the BioCord technology is a promising and cost-feasible upgrade solution for rural municipal wastewater treatment systems to achieve consistent ammonia removal. The findings of this study provide empirical evidence for the system to determine the maximum ammonia removal and aeration system optimization. Our industrial partner, Bioshop Water Technologies, has used the

findings of this study, with the first full-scale BioCord post-carbon, nitrifying system being built at Limoges wastewater treatment facility, Ontario.

7.2 Inoculation and carrier modification strategies for the rapid start-up of attached growth anammox technologies.

Chapter 5 and 6 provide the findings on optimizing the start-up of attached growth anammox technologies using a novel seeding strategy and carrier surface modification strategies. Chapter 5, the first part of this study, compares a novel method of using detached anammox biofilm as inoculation to reduce the start-up time of attached growth anammox technologies on non-modified virgin carriers (control) and pre-seeded denitrifying carriers (readily available carriers worldwide due to number of attached growth denitrifying MBBR systems in operation) to the more common use of attached anammox carriers for anammox MBBR inoculation. The results demonstrate significantly higher NH_4^+-N SARRs along with higher biofilm thickness development rates for both virgin and pre-seeded denitrifying carriers with detached biofilm seeding during the first 71 days of start-up than those with attached anammox carriers seeding. Moreover, the anammox bacteria specific attachment-growth rates for virgin carriers inoculated with detached anammox biofilm mass were 38.1% greater for the first 25 days, leading to approximately 30% less time required to achieve complete biofilm coverage than those measured in attached biofilm carrier inoculated systems during the attachment and early biofilm growth stages. The significantly lower anammox bacteria abundance attached to virgin and pre-seeded denitrifying carriers with attached anammox carriers seeding analyzed during the first 39 days of operation along with the significantly lower anammox kinetics indicate that seeding with anammox carriers may be rate limited by the dispersal and detachment rate of anammox cells from the seeding anammox carriers in the anammox attachment process. After approximately 106 days of start-up operation, the

anammox population and ammonia removal rates for both carrier types were peaked and no longer distinguishing between the detached anammox biofilm inoculation and attached anammox carrier inoculation. This lack of distinction after longer operation times are indicative of the carriers of both systems having achieved biofilm maturation before or on day 106. However, the very slow but higher specific attachment-growth rates for both carrier types with attached anammox carrier seeding than those with detached anammox biofilm seeding are an indicative of a delay in the time towards anammox biofilm maturation in the system seeded with attached anammox carriers.

The differences observed in anammox attachment, biofilm growth and maturation rates between the carriers exposed to detached biofilm mass and attached biofilm carriers resulted in improved biofilm development and subsequently enhanced reactor performance. The findings of this study indicate that detached biofilm seeding outperformed the conventional means of using attached biofilm carrier seeding during the early stages of start-up and suggest that the use of a detached anammox biofilm inoculation with pre-seeded denitrifying carriers has the greatest potential to accelerate the anammox biofilm attachment, growth and maturation.

Chapter 6, the second part of the study, is built on the findings from the first part of the study (Chapter 5), which identifies detached anammox biofilm inoculation as a preferred seeding strategy to reduce the start-up time of attached growth anammox systems. As such, Chapter 6 compares multiple carrier modification strategies seeded with detached anammox biofilm during the start-up of an MBBR anammox system. Ammonia removal kinetics, biofilm attachment and growth as well as anammox bacteria enrichment for dextran-functionalized carriers (chemically enhanced), silica-functionalized carriers (roughness enhanced), and pre-seeded denitrifying carriers (biologically enhanced) were compared to non-modified virgin carriers (control). The results demonstrate that silica-functionalized carriers along with pre-seeded denitrifying carriers

induced significantly higher kinetics, faster biofilm growth and greater anammox bacteria enrichment during the first 71 days of operation compared to dextran-functionalized carriers and non-modified virgin carriers. The results hence indicate that the silica-functionalized and pre-seeded denitrifying carriers offer attachment, growth and subsequently kinetic advantages during the early stage of start-up while the dextran-functionalized carriers did not reduce the start-up period for anammox biofilm.

7.2.1 Novel contribution and practical implication

This study contributes new knowledge on the inoculation and carrier modification start-up strategies for reducing the onerous start-up time of an attached growth anammox technology. It provides the first fundamental understanding of anammox bacterial attachment, growth and maturation at the meso (biofilm) and genetic level. The findings of this research are being directly used by the industrial partner as empirical evidence for optimizing seeding strategies to achieve the rapid start-up an attached growth MBBR anammox system. In addition, this study provides a feasible carrier modification strategy of using pre-seeded denitrifying carriers to reduce the start-up of attached growth anammox systems as these carriers are easily accessible in many countries due to the large volume of pre-seeded denitrifying carriers from denitrification facilities around the world.

Appendix A: Supporting Material

The two reactors, R1 and R2, were operated as SBR for a period of 65 days. SBR operation during inoculation of attached growth anammox technologies is common. Between days 1 to 10, the target feed concentration of NH_4^+-N and NO_2^--N were 200 mg/L each to allow the reactors to start-up without substrate limitation. On day 11 the target feed concentrations were modified to 150 mg/L of NH_4^+-N and 50 mg/L NO_2^--N based on the measured performance of the reactors. In this start-up period between day 11 to day 22, both R1 and R2 showed a similar ammonia removal rate and a small production of nitrate. However, the detached biofilm mass seeded reactor (R1) demonstrated a consumption of nitrite between days 11 to 22 (Figure C-5.1a), while nitrite production was observed in the attached biofilm carrier seeded reactor (R2) (Figure C-5.1b). This distinction is indicative of enhanced anammox bacteria activity in R1 between days 11 to 22 compared to R2. From day 23 to day 64 (the latter period of the SBR operation period), both R1 and R2 showed similar performances and the ratio of ammonia consumed, nitrite consumed and nitrate produced supports anammox as a pathway for nitrogen removal in the both the R1 and R2 reactors.

On day 65, the start-up operation of R1 and R2 shifted to continuous feed operation. A shift to continuous feed following SBR operation is common during start-up as the steady operation of these systems operate as continuous flow systems. Between day 65 to day 98, the target influent concentration for both reactors were approximately 150 mg/L of NH_4^+-N and 75 mg/L of NO_2^--N. It took approximately 6 days for the carriers in R1 to acclimate from SBR to continuous feed operation. As the mass transfer rate was limited by complete NO_2^--N consumption in R1, the target feed nitrite concentration was augmented to approximately 150 mg/L of NO_2^--N from day 99 (Figure C-5.1a). While in R2, the transition period was approximately 13 days and

nitrite augmentation started on day 106 to a target nitrite feed concentration of 125 mg/L of NO_2^- N (Figure C-5.1b). Higher ammonia removal and nitrite consumption along with shorter transition time observed in R1 to acclimate to continuous flow operation indicates that the start-up activity of systems seeded with detached biofilm mass has the potential to achieve elevated kinetics at early stages of start-up compared to inoculation using pre-seeded carriers. On day 128, a new batch of centrate was collected for the feed. However, this batch of centrate showed different characteristics to previous centrate batches collected causing system fluctuation. In particular, the C/N ratio was significantly higher for this batch.

Appendix B: Supporting Tables

Table B1. Influent synthetic wastewater characterization at the ammonia SALR of 1.8 g NH_4^+-N/m^2·d.

Constituent	Value
Ammonia (NH_4^+-N)	27.1 ± 0.5 mg/L
Nitrite (NO_2^--N)	0.1 ± 0.0 mg/L
Nitrate (NO_3^--N)	0.2 ± 0.0 mg/L
TN	27.7 ± 0.5 mg/L
sCOD	10.0 ± 0.9 mg/L
TSS	0 mg/L
pH	7.1 ± 0.2
Temperature	23.0 ± 1.0°C

Appendix C: Supporting Figures

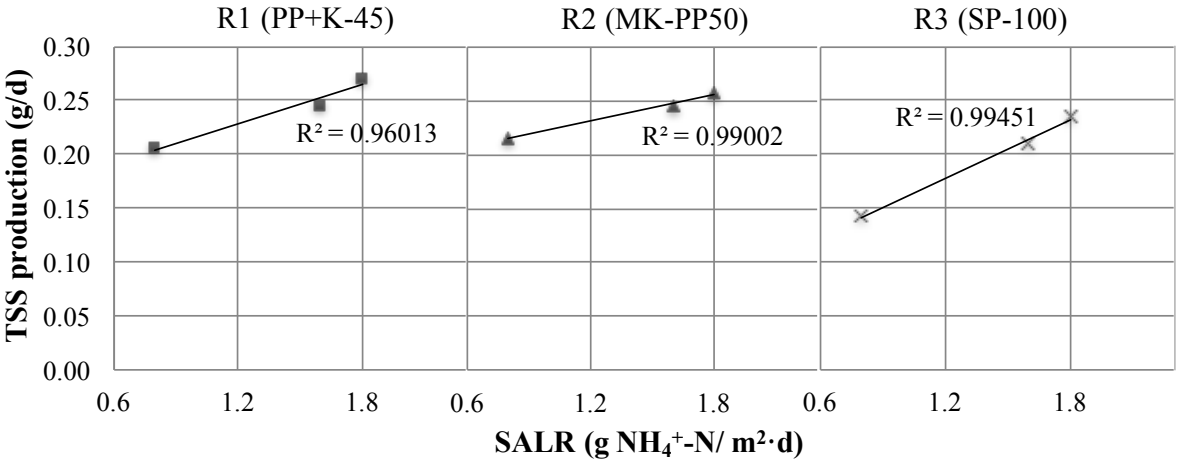

Figure C-3.1 TSS productions versus ammonia nitrogen loading

(a) (b)

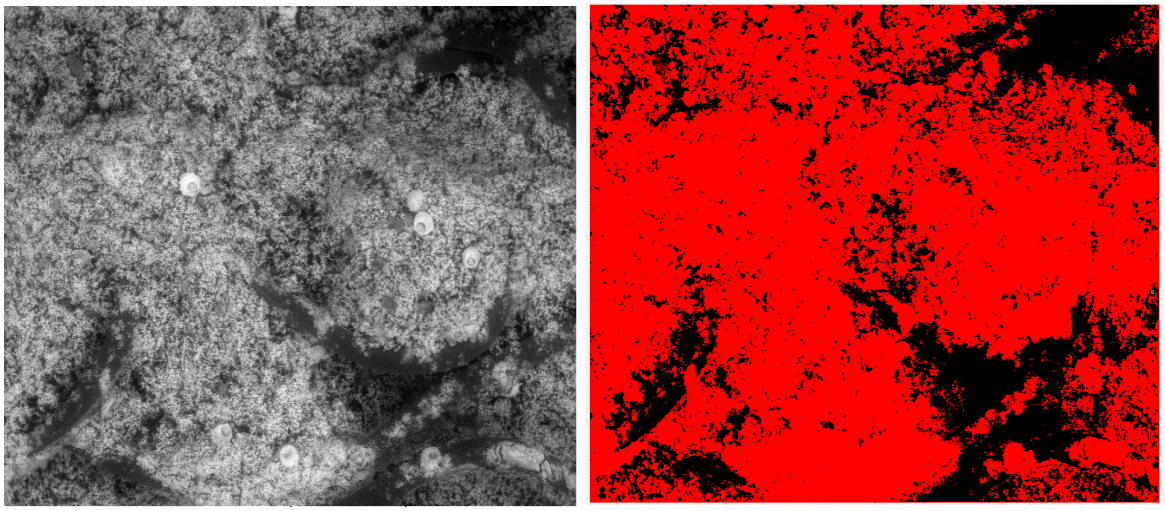

Figure C-4.1 Analysis of VPSEM images at ×60 magnification using NI Vision assistant: (a) Original VPSEM image of bio-cord substratum MK-PP50 (b) Highlighted biofilm area.

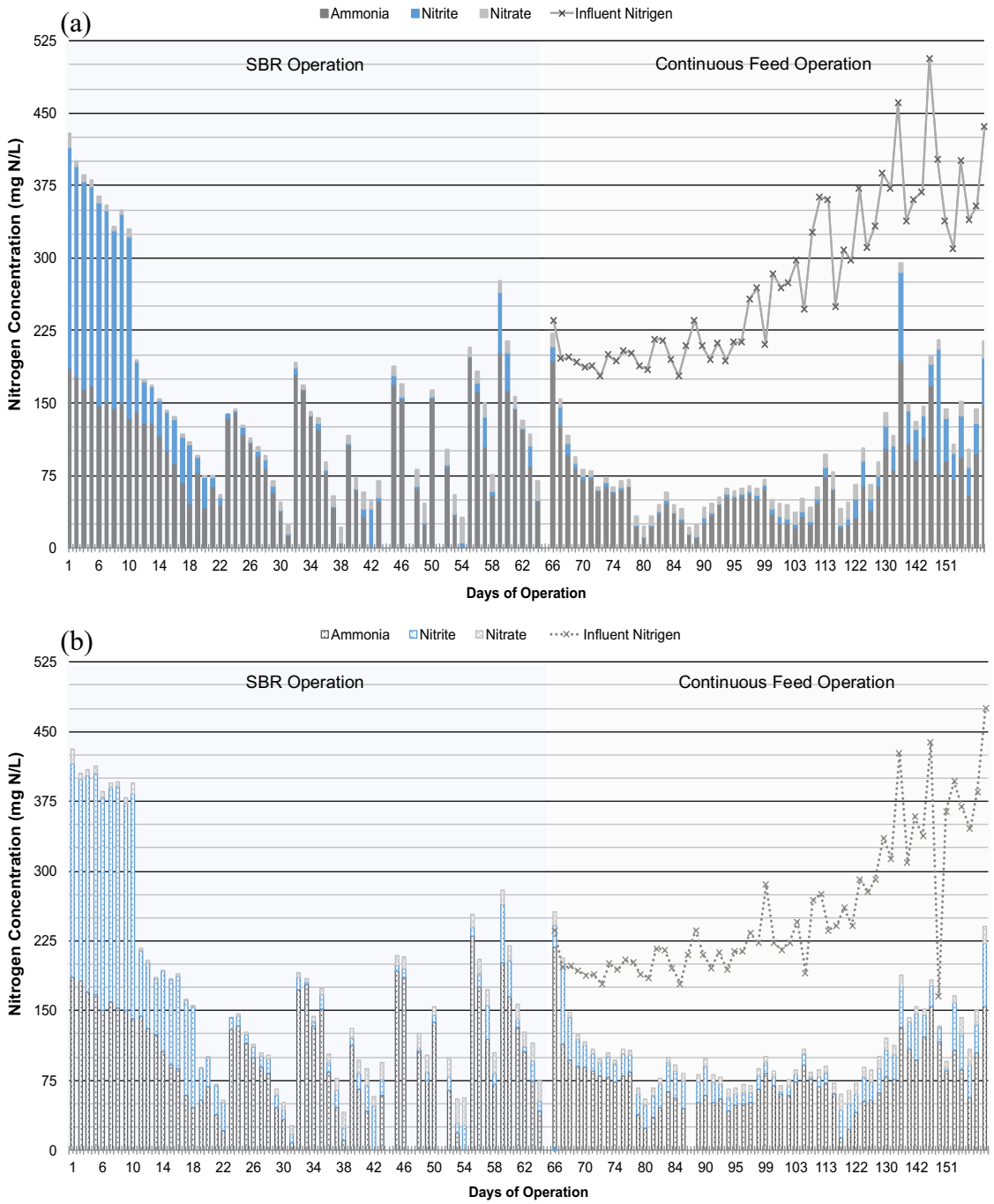

Figure C-5.1 Daily effluent nitrogen concentration and influent nitrogen concentration during SBR and continuous feed operation for (a) R1 – detached biofilm inoculation and (b) R2 – attached carrier inoculation.

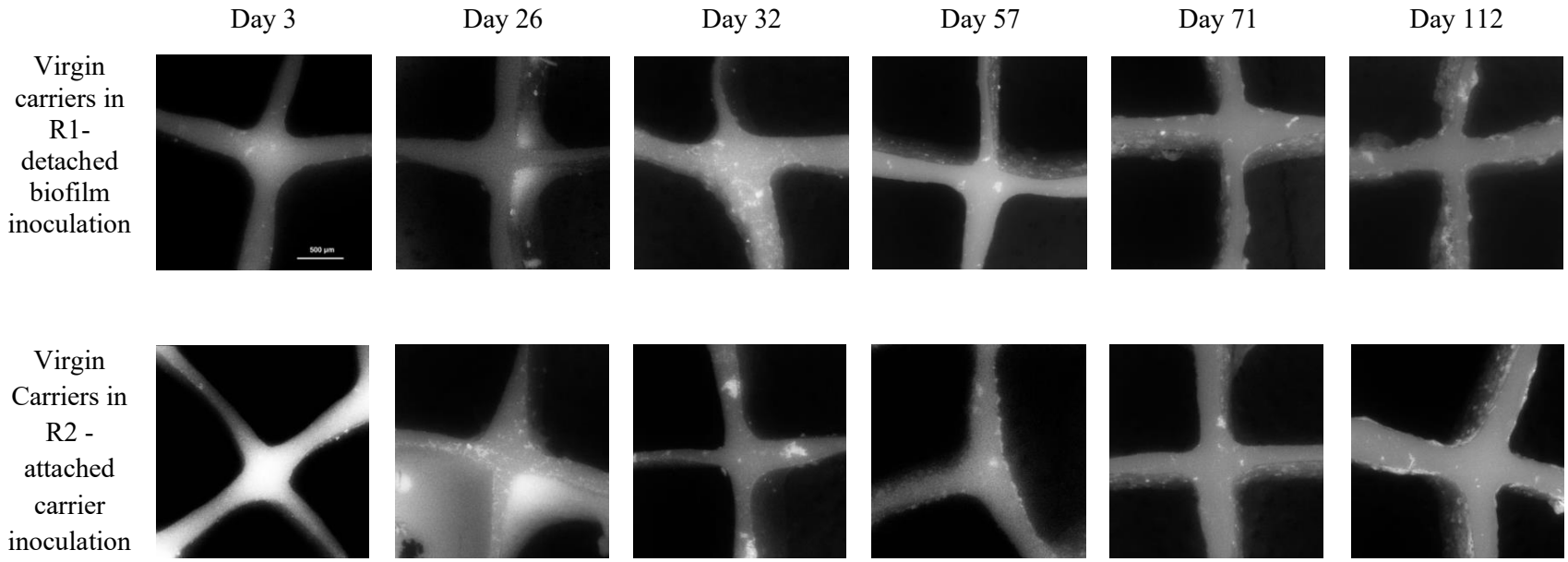

Figure C-5.2 VPSEM images of virgin carriers in R1 and R2 at 60x magnification. Images took on day 26 were overexposure.

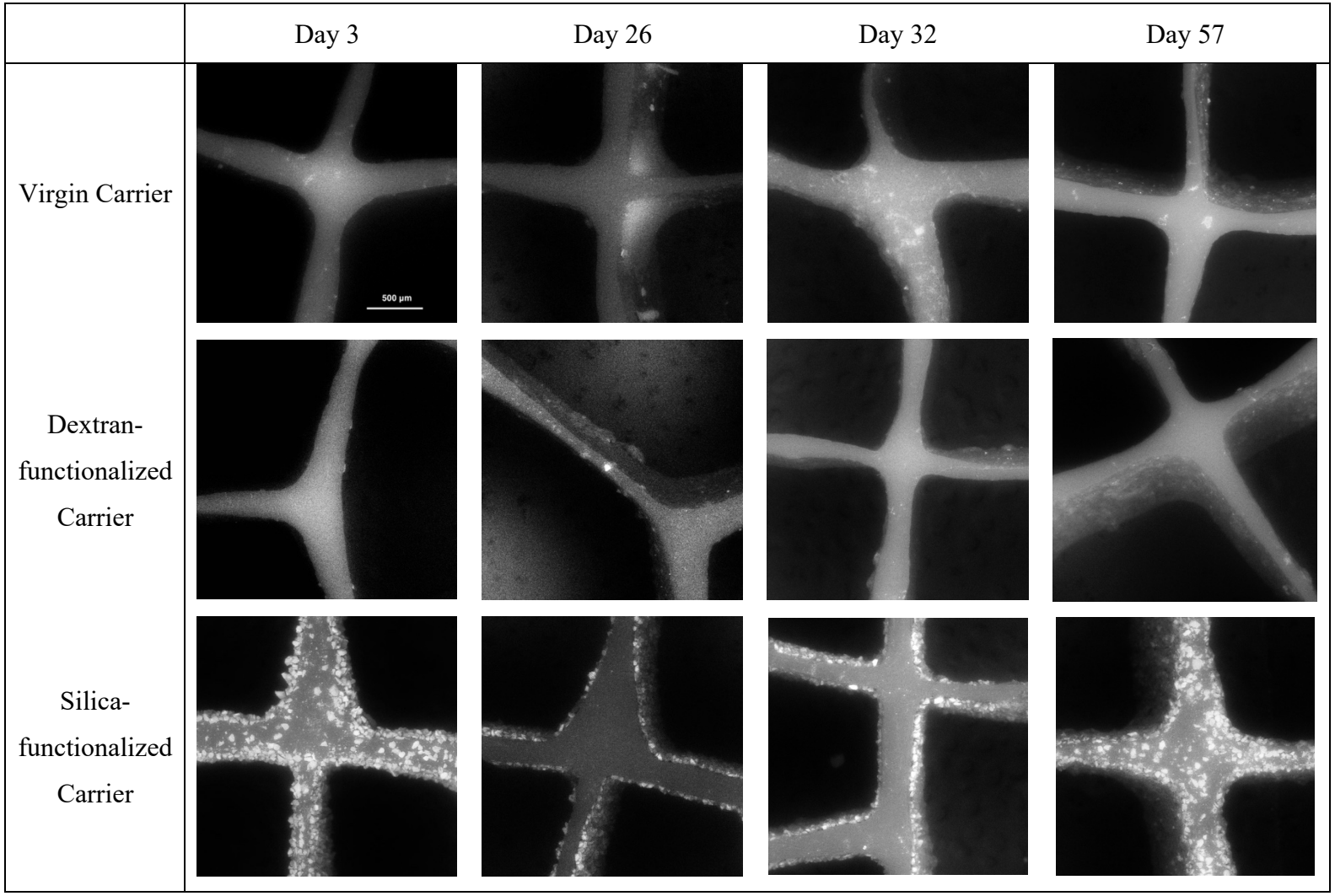

Figure C-6.1 VPSEM images of carriers at 60x magnification.