

Sequencing Batch Moving Bed Biofilm Reactors for Treatment of Cheese Production Wastewater

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Abstract

Discharging cheese production wastewater with high concentrations of organics and nutrients is detrimental to receiving aquatic systems, as the release of these deleterious substances cause oxygen depletion, and eutrophication respectively. On-site treatment of cheese production wastewater requires the removal of high concentrations of organics and nutrients with a small land footprint to meet regulations. There is therefore a critical need for compact, high-rate, cost-effective wastewater technologies such as the moving bed biofilm reactor (MBBR). Although MBBR systems have been well established for carbon and nitrogen removal, to date only a limited number of studies have achieved enhanced biological phosphorous removal in sequencing batch moving bed biofilm reactor (SB-MBBR) systems, and only for municipal-strength wastewater. Operating SB-MBBR systems under sequencing batch mode enables the reactor operation to be well synced to the fluctuating influent concentrations and flow characteristics of cheese production wastewaters. Furthermore, cycling between anaerobic and aerobic conditions can be achieved in a single sequencing batch reactor, which can promote the proliferation of poly-phosphate accumulating organisms. The SB-MBBR is studied in this research for the removal of carbon, nitrogen, and phosphorous from cheese production wastewaters. Specifically, the effects of anaerobic staging time, aeration rate, enhanced aerobic operation, and adding a second reactor in series was studied by analyzing the kinetics, biofilm characteristics, and microbiome. Extending the anaerobic staging time was shown to achieve aerobic soluble chemical oxygen demand removal rates of $92.5 \pm 2.8 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$, by selecting for a thinner biofilm with, with a lower biofilm dry-density and a more rough biofilm surface, and therefore likely a biofilm with an enhanced mass transport. A significant shift in the microbiome was observed with longer anaerobic staging times and lower aeration, whereby possible putative poly-phosphate accumulating organisms

including *Brachymonas*, and *Dechloromonas* were selected for in greater relative abundances compared to anaerobic bacteria. The total phosphorous removal in the possibly resulted from enhanced biological phosphorous removal, supported by the high abundance of putative polyphosphate accumulating organisms ($43.1 \pm 8.4\%$), which dominated the biofilms in the SB-MBBRs with 120 and 168 minute anaerobic staging times. Finally, total ammonia nitrogen oxidation was achieved through partial nitrification with a two reactor in series configuration with a removal rate of $1.07 \pm 0.05 \text{ g-N} \cdot \text{m}^{-2} \text{d}^{-1}$. Two SB-MBBRs operated in series was shown to be the superior strategy for achieving TAN compared to a single SB-MBBR with extended aerobic operation. By operating two SB-MBBRs in series, competition between autotrophic nitrifiers and heterotrophs is averted, and AOB proliferate in the biofilm, achieving TAN oxidation. Since TAN oxidation is likely achieved through partial nitrification, the SB-MBBR technology may be incorporated in a deammonification treatment train. The overall study presents novel information for the SB-MBBR design and operation, along with biofilm and microbiome fundamental findings that will guide future pilot- and full-scale applications of the SB-MBBR to treat cheese production wastewater.

Preface

The dissertation is an original work performed by Alexandra Tsitouras, under the supervision of Dr. Robert Delatolla, and includes four manuscripts. The manuscripts herein have either been published, submitted, or will be submitted for publication in peer reviewed journals.

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Alexandra Tsitouras: Contributed to the experimental design, performed the research, analyzed the data, and wrote the original draft of the paper.

Nour Al-Ghussain: Contributed to data collection and analysis.

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Robert Delatolla (supervisor): Developed the research question, the experimental design, directed the research, and revised 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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LIST OF ACRONYMS

AMO	Ammonia Monooxygenase
ANAMMOX	Anaerobic Ammonia Oxidation
AOB	Ammonia Oxidizing Bacteria
AS	Activated Sludge
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BNR	Biological Nutrient Removal
bsCOD	Biodegradable Soluble Chemical Oxygen Demand
CAS	Conventional Activated Sludge
cBOD	Carbonaceous Biological Oxygen Demand
CLSM	Confocal Laser Scanning Microscopy
COD	Chemical Oxygen Demand
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
EBPR	Enhanced Biological Phosphorous Removal
EPS	Extracellular Polymeric Substance
FISH	Fluorescence <i>in situ</i> Hybridization
GAO	Glycogen Accumulating Organism
HAC	Acetic Acid
HAO	Hydroxyl-Amine Oxidoreductase
HGT	Horizontal Gene Transfer

HRT	Hydraulic Retention Time
MBBR	Moving Bed Biofilm Reactor
NOB	Nitrite Oxidizing Bacteria
OLR	Organic Loading Rate
ORP	Oxidation-Reduction Potential
PAO	Polyphosphate Accumulating Organism
PCR	Polymerase Chain Reaction
PHA	Polyhydroxyalkanoate
PHB	Poly- β -hydroxybutyrate
PN	Partial Nitritation
poly-P	Polyphosphate
PPK1	Polyphosphate Kinase 1
QIIME	Quantitative Insights into Microbial Ecology
RNA	Ribonucleic Acid
rRNA	ribosomal Ribonucleic Acid
SALR	Surface Area Loading Rate
SARR	Surface Area Removal Rate
SB-MBBR	Sequencing Batch-Moving Bed Biofilm Reactor
SEM	Scanning Electron Microscope
sCOD	soluble Chemical Oxygen Demand
SDS	Sodium Dodecyl Sulfate
SND	Simultaneous Nitrification and Denitrification
SWW	Synthetic Wastewater

TAN	Total Ammonia Nitrogen
TCA	Tricarboxylic Acid
TN	Total Nitrogen
TP	Total Phosphorous
TSS	Total suspended solids
uBOD	ultimate Biological Oxygen Demand
VFA	Volatile Fatty Acids
VSS	Volatile Suspended Solids
WRRF	Water Resource Recovery Facilities

1 Introduction

1.1 Background

Cheese is one of the main agricultural commodities produced worldwide, and the production of cheese is a major source of industrial effluent (Demirel et al., 2005). Wastewater is generated at approximately 4 times the volume of the milk utilized in the production of cheese. Cheese production wastewater is comprised of clean in place wastewaters produced by washing equipment and milk containers; laboratory analyses wastewaters; and by-product whey cheese wastewaters (Prazeres et al., 2012; Carvalho et al., 2013; Tatoulis et al., 2015). Thus, cheese production wastewater contains diluted milk products, alkaline and acidic chemicals, resulting in a chemical oxygen demand (COD) that commonly ranges from 0.8–102 g·L⁻¹, while the total nitrogen (TN) and total phosphorous (TP) concentrations commonly range from 0.01-1.7 and 0.006-0.5 g·L⁻¹, respectively (Carvalho et al., 2013).

Discharging cheese production wastewater results in environmental degradation of receiving waters, such as excessive oxygen depletion and eutrophication (WEF, 2005). Eutrophication occurs due to excessive growth of algae and cyanobacteria resulting from the discharge of macronutrients; which leads to the depletion of oxygen, causing hypoxic zones in receiving water bodies, and may ultimately lead to a loss in biodiversity (Hutchinson, 1973; Boesch et al., 2006). Specifically, for many lakes in Eastern Canada, phosphorous is the limiting nutrient, and cyanobacterial blooms occur when phosphorous is in abundance; while in marine environments nitrogen is the limiting nutrient (Howarth and Marino, 2006), highlighting the need for control of both phosphorous and nitrogen discharge (Conley et al., 2009). To prevent environmental degradation, many governing agencies are implementing increasingly stringent

regulations to municipal water resource recovery facilities (WRRFs) (Gazette, 2012; Román-Sánchez et al., 2015; Hendriks & Langeveld, 2017). Since industries may contribute a significant portion of the organic and nutrient load to municipal WRRFs; municipalities are also limiting industrial effluent concentrations discharged to sewer collectors by implementing financial and regulatory industrial discharge bylaws, requiring on-site treatment of cheese production wastewater (Rusten et al., 1996; Irish EPA, 2008; Tatoulis et al., 2015; Van Lier et al., 2015).

Biological removal of nitrogen and phosphorous from municipal and industrial wastewater has been conventionally achieved through nitrification-denitrification of nitrogen, and chemical precipitation of phosphorous. Nitrification is the process whereby total ammonia nitrogen (TAN) is oxidized to nitrite by ammonia oxidizing bacteria (AOB), and nitrite is subsequently oxidized to nitrate by nitrite oxidizing bacteria (NOB). TAN removal may be followed by nitrate reduction to achieve TN removal, where denitrification is the process of reducing nitrate to nitrogen gas by denitrifying organisms (Madigan et al., 2008). Biological removal of phosphorus is achieved through enhanced biological phosphorous removal (EBPR), whereby poly-phosphate (poly-P) accumulating organisms (PAOs) uptake poly-P during aerobic conditions through energy derived from carbon previously stored during anaerobic conditions (Mino et al., 1995).

For cheese production wastewater, organic treatment is conventionally achieved with lagoons, while both carbon and nutrient removal has been achieved through conventional activated sludge (CAS) (Bortone and Piccinini, 1991; Donkin and Russell, 1997), and sequencing batch reactors (SBRs) (Sirianuntapiboon and Tondée, 2000; Li and Zhang, 2002; Sirianuntapiboon et al., 2005; Asadi et al., 2012). However, various disadvantages and challenges have been attributed to each of these technologies for cheese production wastewater treatment. Lagoons require large areas, and provide an effluent of fluctuating quality (Metcalf & Eddy, 2014); and CAS requires

high energy inputs and elevated operational intensity (Sirianuntapiboon and Tondee, 2000). SBR technologies produce excessive sludge with high sludge volume indexes, and require elevated operational intensity (Keller et al., 1997). Therefore, to meet increasing regulatory requirements, within the space limitation necessary for on-site treatment of cheese production wastewater, there is a need for the application of more compact treatment technologies. One such technology is the moving bed biofilm reactor (MBBR), which has been shown to be feasible for handling the elevated organic loading requirements, and space limitations for on-site treatment of carbon from cheese production wastewater (Rusten et al., 1996; Andreottola et al., 2002; Tatoulis et al., 2015). Combined carbon and nitrogen treatment have also been achieved with MBBRs, whereby nitrogen was removed with the MBBR technology following anaerobic treatment of carbon (Andreottola et al., 2002).

MBBR systems operated under sequencing batch operation are referred to as sequencing batch MBBR (SB-MBBR) systems. Sequencing batch operation permits anaerobic and aerobic conditions to be achieved in a single reactor, whereby the anaerobic and aerobic phases are separated by timed stages in an operational cycle. The cycles of the SBR operation also permit operational modification in response to variations in wastewater quality and flow patterns (Wilderer et al., 2001), of which are characteristic of cheese production wastewaters (Andreottola et al., 2002). In addition, SB-MBBR systems allow for treatment with a reduced land footprint permitting on-site treatment of cheese production wastewaters. To date, phosphorous treatment from cheese production wastewater with the MBBR technology has been limited to continuous operation, and with assimilation by heterotrophic growth (Rusten et al., 1996; Andreottola et al., 2002). EBPR with SB-MBBR systems has only been achieved for municipal-strength wastewater, with very specific loading conditions (Pastorelli et al., 1999; Helness and Ødegaard, 2001; Gieseke

et al., 2002; Humbert et al., 2018). EBPR has not been achieved in SB-MBBR systems treating industrial wastewater, nor have the microbial characteristics and the microbiome community of the SB-MBBR treating industrial wastewater been previously studied. Studying the biofilm morphology and microbiome communities can allow for improvements in the design and operation of the SB-MBBR technology, by identifying operational conditions that enhance the survival and performance of key microorganisms in SB-MBBR systems (Mohan et al., 2010; Cydzik-Kwiatkowska and Zielińska, 2016). Fundamental knowledge of the SB-MBBR design and operation can allow for the full-scale implementation of the SB-MBBR technology for carbon, nitrogen, and phosphorous removal from high-strength wastewaters, including cheese production wastewaters.

1.2 Research objectives

The overall objectives of this research are to investigate the potential and to in-turn optimize the design and operation of the SB-MBBR technology for the treatment of carbon, nitrogen, and phosphorous from cheese production wastewater. The research moves beyond design and optimization of the SB-MBBR technology and endeavors to provide new knowledge of this technology at the macro-, meso-, micro-, and molecular-scale. Specifically, this research will (i) study the effects of anaerobic staging on the kinetics of carbon, nitrogen, and phosphorous removal of a single SB-MBBR system; (ii) study the effects of aeration rates on the kinetics of carbon, nitrogen, and phosphorous removal of a single SB-MBBR system; (iii) study the effects of anaerobic staging on the biofilm dry-mass, thickness, morphology, biomass viability, and microbiome of a single SB-MBBR system; (iv) study the effects of aeration rates on biofilm dry-mass, thickness, morphology, biomass viability, and microbiome of a single SB-MBBR system; (v) compare two strategies for achieving TAN oxidation in a high loaded SB-MBBR system; (vi)

compare the biofilm dry-mass, thickness, dry-density, morphology, viability and microbiome of the two strategies for achieving TAN oxidation in a high loaded SB-MBBR system; (vii) study the kinetics of the carbon, nitrogen, and phosphorous of a two SB-MBBR in series system; and (viii) study the biofilm dry-mass, thickness, dry-density, and morphology of the of the two SB-MBBR in-series system.

1.3 Thesis Organization

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

Chapter 2 presents a literature review of the fundamental knowledge for biological organic and nutrient removal for cheese production wastewater, specifically for the MBBR and for carbon, nitrogen, and phosphorous removal.

Chapter 3 is a published manuscript entitled “*Kinetic effects of anaerobic staging and aeration rates on sequencing batch moving bed biofilm reactors treating carbon, nitrogen, and phosphorous from cheese production wastewater*”. The article was published in Chemosphere in 2020, and the article addresses the research objectives i and ii. The kinetics of carbon, nitrogen, and phosphorous removal from cheese production wastewater with a single SB-MBBR system are studied in this manuscript. The effects of anaerobic staging and aeration rates are tested in three parallel single SB-MBBR systems operated at various anaerobic staging times, with an enhanced aeration test being studied in one system.

Chapter 4 encompasses a research manuscript entitled “*Two attached growth sequencing batch reactors in-series - carbon, nitrogen, and phosphorous removal from high organic wastewaters.*” This manuscript was submitted to the Water Process Engineering in 2021 and addresses the research of objectives vii and viii. The manuscript investigates the operation of two

SB-MBBRs in series to achieve TAN removal, with the first reactor being operated with anaerobic and aerobic stages, and the second reactor being operated under solely aerobic conditions to oxidize TAN. The kinetics are quantified in the two reactors across the 16-hour cycle of the two reactors after steady-state TAN oxidation is achieved. In addition, the biofilm dry-mass, thickness, dry-density, and morphology are quantified in the two reactors following steady-state operation.

Chapter 5 entails a research manuscript entitled “*Biofilm morphology and microbiome of sequencing batch moving bed biofilm reactors treating cheese production wastewater*”. This manuscript is currently in progress and will be submitted to the Water Research Journal in 2021. The manuscript addresses research objectives iii, and iv, through microbial analyses of a single SB-MBBR system treating cheese production wastewater, operating with anaerobic and aerobic stages. Specifically, this research studies the effects of anaerobic staging and aeration rates on the biofilm morphology, biofilm thickness, biofilm dry-mass, biofilm dry-density, embedded biomass viability, and microbiome community to develop a fundamental understanding of the mechanisms of wastewater treatment in SB-MBBR systems treating high-strength wastewater.

Chapter 6 is a research manuscript entitled “*Microbiome of two strategies for ammonia removal of the sequencing batch moving bed biofilm reactor treating cheese production wastewater*”. The manuscript herein is currently in progress with plans to be submitted to the Bioresource Technology Journal in 2021. The manuscript addresses the research objectives v, and vi. The research of this manuscript compares two strategies to achieve TAN oxidation in SB-MBBR systems treating cheese production wastewater. The first strategy involves extending the aerobic operation of a single SB-MBBR system, until TAN oxidation is achieved. The second strategy involves operating two SB-MBBRs in-series, whereby the first SB-MBBR operates with anaerobic and aerobic stages, fed into a second SB-MBBR that operates under exclusively aerobic

conditions. The study herein compares the two start-up strategies for TAN oxidation by comparing the viability makeup of the embedded biomass, and the microbiome community following steady state conditions.

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2 Literature review

2.1 The prokaryotic cell

Prokaryotic cells exist in a range of shapes including spherical, rod (cylindrical), and spirillia (rods in spiral shapes) (Figure 2.1); with diameters ranging from 0.2 μm to 700 μm , and the average cell being closer to 1-2 μm . By comparison, the diameter of eukaryotic cells typically range from 10 μm to over 200 μm . Prokaryotic cells comprise a cell wall, cell membrane, nucleoid, cellular inclusions, and cellular appendages (Figure 2.1). For bacteria, the cell walls are either Gram-positive, containing a thick cell wall made-up of layers of peptidoglycan; or Gram-negative, where the cell wall exists between an outer membrane and a periplasmic space, equipped with transport proteins, signaling proteins, and degradative enzymes (Figure 2.1) (Maier et al., 2009). The cytoplasmic membrane is a thin barrier that surrounds the cell separating the cytoplasm from the environment, allowing the cell to concentrate metabolites and secrete waste materials, while preventing passive leakage from the cell. The lipid bilayer is stabilized by magnesium and calcium, which prevent passage of charged molecules. For example, phosphate must become neutralized by bonding with positively charged magnesium and potassium before passing the lipid bi-layer (Pattarkine, 1991). The membrane is composed of phospholipids with a hydrophobic fatty acid and a hydrophilic glycerol-phosphate, of which form a layer of hydrophobic fatty acids aligning inwards, and a hydrophilic glycerol-phosphate layer that are exposed to the environment (Figure 2.1) (Madigan et al., 2008).

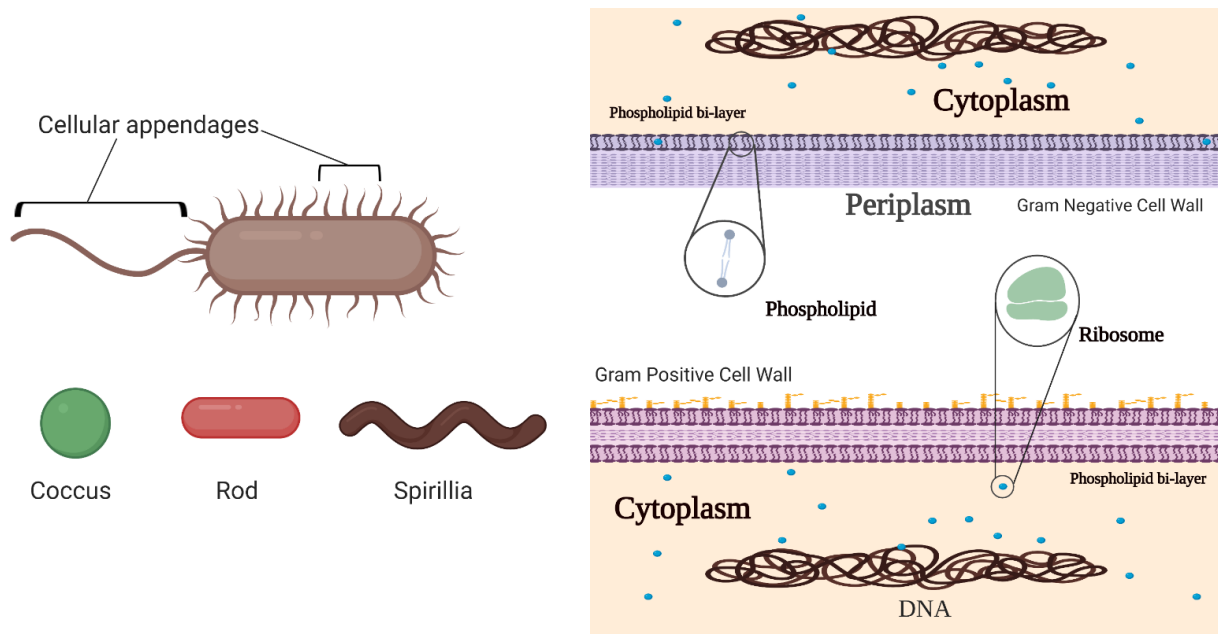


Figure 2.1 Prokaryote cell morphology (left), and inside the bacterial cell (right). Adapted from: (Madigan et al., 2008).

2.2 Taxonomy

Deoxyribonucleic acids (DNA) are used to identify microorganisms within a sample, and Carl Woese pioneered the use of the 16S ribosomal RNA (rRNA) gene to categorize organisms based on similarities of this gene. Woese's work led to the discovery of archaea, and to the development of the categorization of life into 3 domains: eukarya, bacteria, and archaea (Woese and Fox, 1977). These 3 domains of life can be depicted in the form of phylogenetic trees that consist of nodes and branches (Figure 2.3), showing relationships between taxa. Nodes are defined as the point in history where an ancestor diverged into two new entities, and a branch is representative of the time in which the divergences occurred (Woese and Fox, 1977; Madigan et al., 2008).

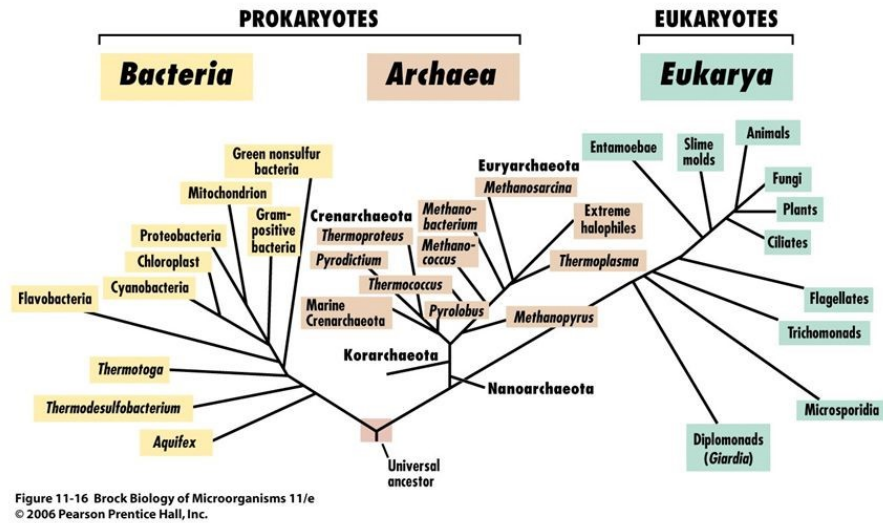


Figure 2.2 Phylogenetic tree of life. From: Brock Biology of Microorganisms (Madigan & Martinko, 2006).

The 16S rRNA gene was selected for categorizing phylogenetic relationships, since at least one gene copy is present in prokaryotic genomes (Acinas et al., 2004), the gene contains hypervariable regions distinct in different species, and the gene is ancient in origin and therefore highly conserved (Vandekerckhove et al., 1999; Klindworth et al., 2013). The 16S gene forms complex and stable secondary structures in the form of loops and stems (Figure 2.4) (Noller et al., 1985). The loop structures contain sequences that are conservative across bacterial species, as the structure of the loops are linked to a particular function. Contrarily, the stem structures in the 16S gene are considerably more variant and can be sub-divided into 9 hypervariable regions (Van de Peer et al., 1996; Jonasson et al., 2002; Wang and Qian, 2009). These hypervariable regions begin with a region that is highly conserved, followed by a downstream region that is variable. The class I hypervariable regions, V4, V5, and V6 region, are thought to be highly sensitive and therefore are recommended for use in phylogenetic studies (Wang and Qian, 2009).

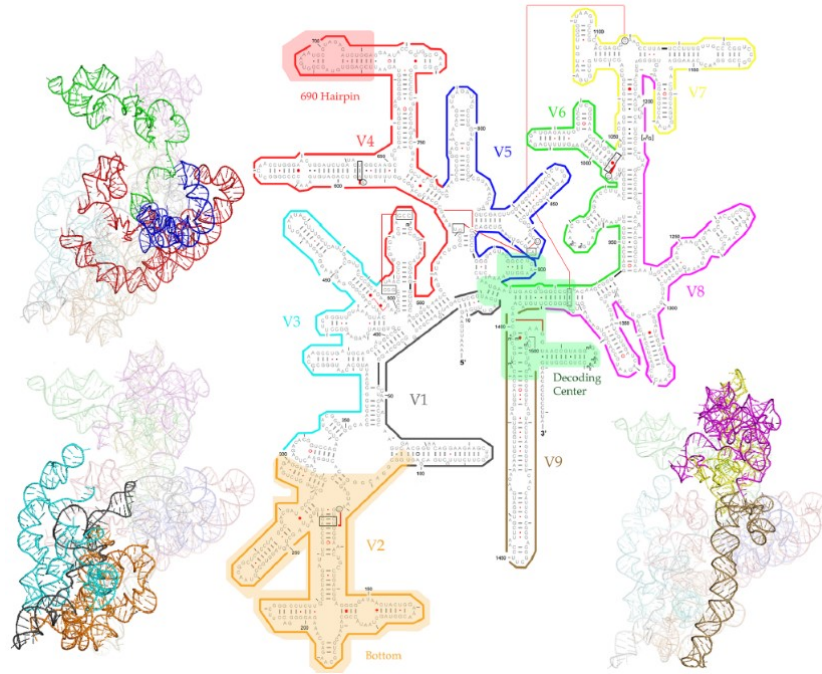


Figure 2.3 Structure of the 16S gene and the hypervariable regions. From: (Wang and Qian, 2009).

2.3 Bacterial metabolism

Bacterial growth requires energy, carbon, nutrients, electron donors and electron acceptors to allow for synthesis reactions that permit the formation new cellular material including nucleic acids, proteins, and membranes. Bacteria derive energy from either light (phototrophs), or through catabolic reactions from chemical sources (chemotrophs), whereby energy is derived through redox reactions. The following discussion focuses on the growth of chemotrophic bacteria.

Chemotrophs capture energy either through respiration or fermentation (Madigan et al., 2008). In respiration, electrons are transferred from an electron donor to an electron acceptor (oxygen, or oxygen containing species nitrite/nitrate) across an electron transport chain, mediated by enzymes, which capture the energy as proton-motive force. Then, proton-motive force fuels the production of ATP by ATPase (Madigan et al., 2008) (Figure 2.6), which holds approximately 32 $\text{KJ}\cdot\text{mol}^{-1}$ of energy. Fermentative growth, on the other, hand occurs through the transfer of

electrons from an electron donor directly to an electron acceptor, other than oxygen, where the transfer of electrons occur within the cell. Growth through fermentation produces significantly less energy compared to respiration, since only 2 ATP molecules are formed per glucose molecule in fermentation, while 32 molecules of ATP are formed per molecule of glucose in respiration (Madigan et al., 2008).

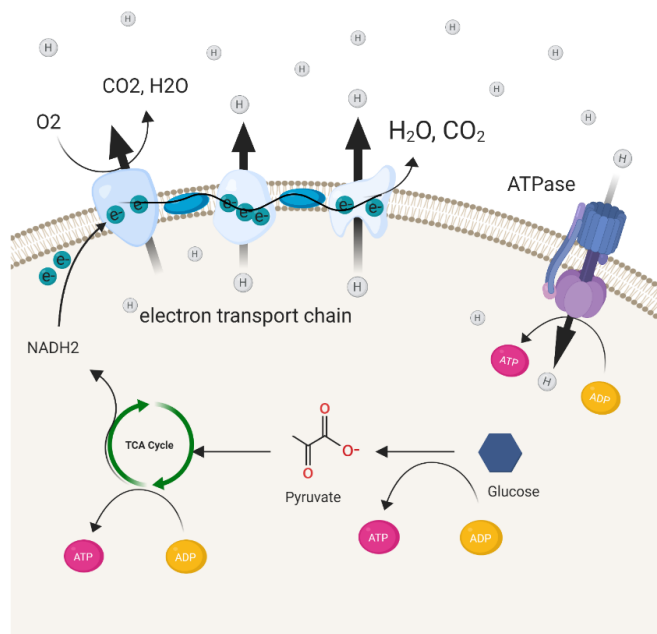


Figure 2.4 Cellular respiration. Adapted from: (Madigan et al., 2008).

Bacteria are categorized based on the electron donor utilized in catabolic reactions, and the carbon source utilized for cellular synthesis. First, aerobic organisms use oxygen as an electron acceptor, while anoxic organisms use nitrate/nitrite as electron acceptors. Anaerobes exhibit fermentative growth, utilizing iron (III), sulfate, or organic carbons as electron acceptors. Obligate anaerobes lyse in the presence of oxygen, while facultative anaerobes can shift their metabolism from fermentative growth to aerobic respiration depending on the absence or presence of oxygen, or oxygen-containing molecular species. Finally aerotolerant organisms perform strictly fermentative growth, however are insensitive to oxygen (Madigan et al., 2008; Metcalf & Eddy,

2014). Bacteria can be further categorized based on their carbon source, being either organic or inorganic, whereby bacteria that uptake organic carbon are termed heterotrophs, and organisms that uptake inorganic carbon are termed autotrophs. Since inorganic carbon must be converted to organic carbon to be utilized in synthesis reactions, autotrophic growth requires a net input of energy. For example the autotrophic bacteria *Nitrosomonas sp.*, requires 30 g of ammonia (electron donor) to form 1 g of cell dry mass (Gallert and Winter, 2005).

Cell growth can be quantified based on cellular yield values, which vary depending on the energy and carbon source used by the microbe. Biomass yields can be determined by directly measuring the biomass produced, often as volatile suspended solids (VSS), divided by the measurement of substrate consumed.

$$Y = \frac{g\text{-biomass produced}}{g\text{-substrate consumed}} \quad \text{Equation 2.1}$$

VSS is a measurement used to estimate the active biomass within a reactor, however the VSS also includes other factors such as cell debris, consisting of non-biodegradable portions such as the cell wall, from endogenous decay, and non-biodegradable material from the influent. (Metcalf & Eddy, 2014).

Biomass yield values are also estimated based on the bioenergetics of the redox reactions and synthesis reactions that occur during cellular growth. During these chemical reactions, there is a change in Gibbs free energy, which is determined through standard thermodynamic half reactions, defined as the transfer of one electron (McCarty, 1965; McCarty, 1971). The change in Gibbs free energy is noted as “ ΔG° ”, whereby “ $^\circ$ ” indicates that free energy is measured at standard conditions, i.e. with a pH of 7, and at 25°C. The biothermodynamics are calculated based on the energy released from the oxidation reduction reaction between the electron donor and acceptor, and the energy requirement for the synthesis reactions, together forming a net reaction. Synthesis

yields, estimated through biothermodynamics, will differ depending on the electron acceptor and electron donor that is utilized by the organisms (Table 2.1) (Metcalf & Eddy, 2014).

Table 2.1 Categorization of metabolic activity in biological wastewater treatment (Metcalf & Eddy, 2014).

Treatment type	Organism	Carbon Source	Electron donor	Electron Acceptor	Biomass yield
Biological oxygen demand (BOD) removal	Aerobic Chemoorganoheterotroph	Organic Carbon	Organic carbon	O ₂	0.45 g-VSS·g-chemical oxygen demand (COD) ⁻¹
Denitrification	Anoxic Chemoorganoheterotroph	Organic Carbon	Organic Carbon	NO ₃ /NO ₂	0.30 g-VSS·g-COD ⁻¹
Nitrification	Aerobic Chemolithoautotroph	Inorganic Carbon	NH ₄	Oxygen	0.12 g-VSS·g-NH ₄ -N ⁻¹
Fermentation	Facultative Chemoorganoheterotrophs	Organic Carbon	Organic Carbon	Organic Carbon	0.06 g-VSS·g-COD ⁻¹
Acetogenesis	Anaerobic Chemolithoautotroph	CO ₂	H ₂ , CO, formate	CO ₂	0.05 g-VSS·g-COD ⁻¹
Methanogenesis	Anaerobic Chemolithoautotroph	CO ₂	H ₂	CO ₂	0.03 g-VSS·g-COD ⁻¹

2.4 Biofilm

Biofilms were first observed using primitive microscopic investigations of tooth surfaces by van Leeuwenhoek (1684), when the presence of aggregates of “animalcules” was described. In 1933, Henrici examined marine populations using direct microscopy, and noted that bacteria were present in significantly greater numbers when attached to a surface compared to the surrounding environment (Henrici, 1933; Bjarnsholt, 2013). Biofilms were later observed with scanning electron microscopes (SEMs), which permit higher resolution and higher magnification, enabling a detailed examination of biofilms. Using the SEM technology, Jones et al. (1969), examined a trickling filter and observed a diverse morphology, and community of organisms in the biofilms (Donlan, 2002). Today, biofilms are defined as: “a structured community of bacterial cells

enclosed in a self-produced polymeric matrix and adherent to an inert or living surface” (Costerton, 1999).

Microbes that exist in biofilms acquire advantages compared to microbes that exist in suspension. Firstly, biofilms provide a barrier that protects against grazing protozoa, while the reduced diffusion of compounds through the biofilm matrix provides protection from antimicrobial agents and toxic compounds (Jefferson, 2004). Furthermore, the biofilms naturally adsorb nutrients, and the housed microbes in biofilms exhibit reduced growth rates; together protecting the microbes against starvation. 97% of the biofilm consists of water, which protects the inhabiting microbes from desiccation (Burmølle et al., 2014). Finally, microbes exist in close proximity within the biofilm, permitting the exchange of genetic material, termed horizontal gene transfer (HGT), and multi-species biofilms provide an opportunity for new genetic combinations to form through HGT across different species (Burmølle et al., 2014). Communication also occurs between bacteria in the biofilm through quorum sensing, whereby a single cell can sense when a threshold number of cells has nearly been reached. Quorum sensing is so vital to the formation of biofilms, that in the absence of essential quorum sensing genes biofilms do not form (Maier et al., 2009; Burmølle et al., 2014).

The biofilm life cycle includes attachment, irreversible attraction, biofilm maturation, and dispersal/detachment (Figure 2.5). First, reversible attachment is initiated through surface coating of polymers that modifies the surface electric charge, and increases the nutrient level compared to the surrounding environment, together promoting the attachment of microbes (Garg et al., 2009). Next, loose attachment is formed through fimbriae, mainly serving to align the microbe with the surface, thereby enabling irreversible attraction. Then extracellular polymeric substance (EPS) is produced forming the irreversible attraction (Costerton, 1999). EPS is a fundamental attribute of

the biofilm structure, accounting for 50% to 90% of the biofilm's organic carbon (Flemming et al., 2000). The production of EPS promotes the attachment of other microbes, and microbial colonies begin to form. As the biofilm continues to mature, the biofilm will grow into a 3-dimensional structure with enlarged microcolonies, which continue to secrete EPS and intracellular adhesion polymers that secure the bonds between cells and the biofilm architecture. Water channels will form permitting the transport of nutrients, electron acceptors and donors; permitting the formation various internal structures. Eventually, shapes will develop such as: cone, mushroom or column (Maier et al., 2009). Once the biofilm is matured, it reaches a state of dynamic equilibrium, and cells will begin to fragment from the biofilm, and potentially colonize new surfaces (WEF, 2010).

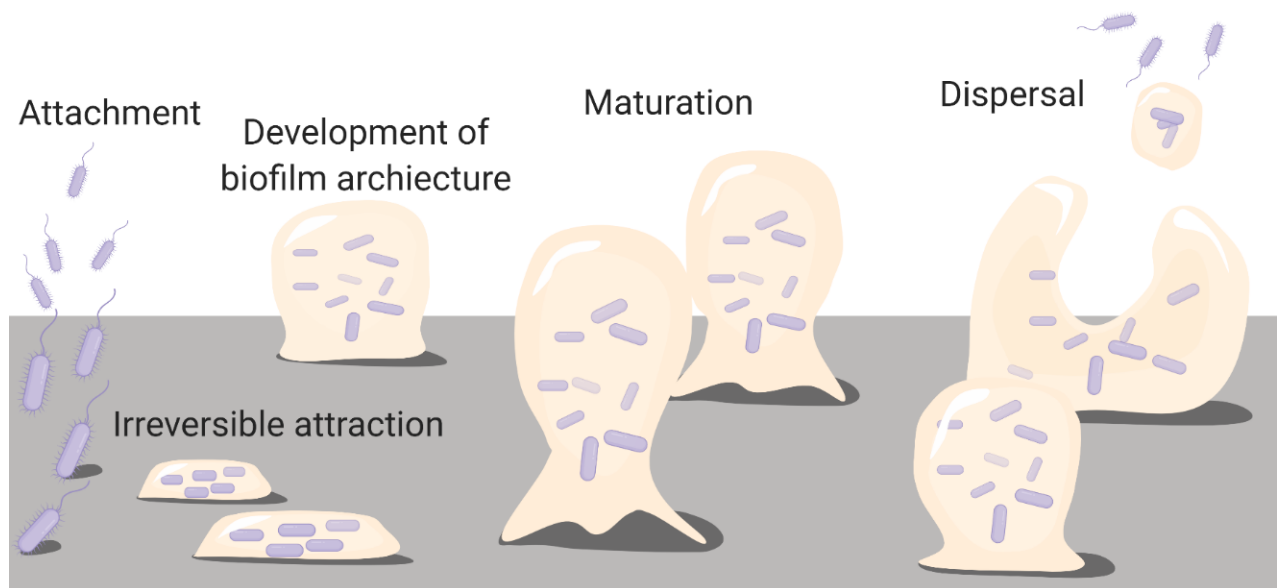


Figure 2.5 Biofilm life cycle. Adapted from: (Hall-Stoodley et al., 2004; Cunningham and Ross, 2006).

Biofilm detachment is categorized as abrasion, erosion, or sloughing. First, abrasion is the detachment due to collision of particles from the bulk liquid, erosion is the continuous removal of small portions of the biofilm, and sloughing is the massive removal of biofilm. Erosion is known

to increase with an increase in biofilm thickness, as well as an increase in fluid shear at the biofilm-bulk-liquid interface. Finally, sloughing is a more random process and is thought to occur as a result of nutrient or oxygen depletion in the biofilm (Hall-Stoodley et al., 2004; Cunningham and Ross, 2006).

2.5 Biological Wastewater Treatment

Biological treatment of wastewater exploits the natural metabolic growth of microorganisms to oxidize biodegradable compounds into acceptable end products, capture non-settleable and colloidal solids, and to transform or capture nutrients including nitrogen and phosphorous. Oxidation and transformation of organic compounds first requires the degradation from larger complex structures; including proteins, lipids, and sugars into simple monomers that are small enough to be taken up by the microbial cell. The action of degradation is achieved through hydrolysis by extracellular enzymes from facultative aerobic and obligate anaerobic bacteria. Biological treatment is feasible for both the treatment of domestic and industrial wastewater, and is administered through either suspended growth treatment or attached growth treatment (Metcalf & Eddy, 2014).

2.5.1 Carbon removal

The oxidization of organic carbon is essential to prevent excessive oxygen depletion in receiving waterbodies; thus oxidation of organic carbon has been the aim of biological wastewater treatment since the 1900s. Carbon removal from wastewater is achieved either through aerobic or anaerobic operation (Metcalf & Eddy, 2014).

Aerobic carbon removal involves heterotrophic organisms that use organic pollutants as electron donors; and supplied oxygen, through aeration, as electron acceptors. The heterotrophs convert the organic wastes to biomass and carbon dioxide (Chan et al., 2009). Aerobic treatment

is advantageous over anaerobic treatment since aerobic treatment requires a startup period ranging from 2-4 weeks, compared to 2-4 months for anaerobic operation; is less sensitive to temperature; and produces superior overall effluent quality (Aziz et al., 2019). Aerobic treatment is considered ideal for wastewaters with concentrations $< 1000 \text{ mg-COD}\cdot\text{L}^{-1}$, and more specifically, a crossover point of 300-700 $\text{mg}\cdot\text{L}^{-1}$ of ultimate BOD (uBOD) is considered, where above these concentrations anaerobic treatment becomes more advantageous (Cakir and Stenstrom, 2005).

Anaerobic wastewater treatment of carbon involves a series of processes that degrade complex organic molecules into methane and hydrogen (Figure 2.7) (Wolin, 1976; Bryant, 1979). First, hydrolysis occurs whereby particulate matter is degraded to organic monomers that can be taken up by bacteria. Next, the organic monomers are fermented to intermediate volatile fatty acids (VFAs), and then to acetic acid and hydrogen. The acetic acid is then metabolized to methane, water, and carbon dioxide by methanogens (Gallert and Winter, 2005; Metcalf & Eddy, 2014). Anaerobic treatment reduces the overall cost to treatment plants, compared to aerobic treatment, due to the lower sludge production and energy inputs required due to the low growth yields, of $0.06 \text{ g VSS}\cdot\text{g-COD}^{-1}$ and $0.03 \text{ g-VSS}\cdot\text{g-COD}^{-1}$ for fermentation and methanogenesis respectively (Table 2.1) (Metcalf & Eddy, 2014). Furthermore, methane produced through anaerobic treatment can be used as methane-rich biogas (Batstone et al., 2006).

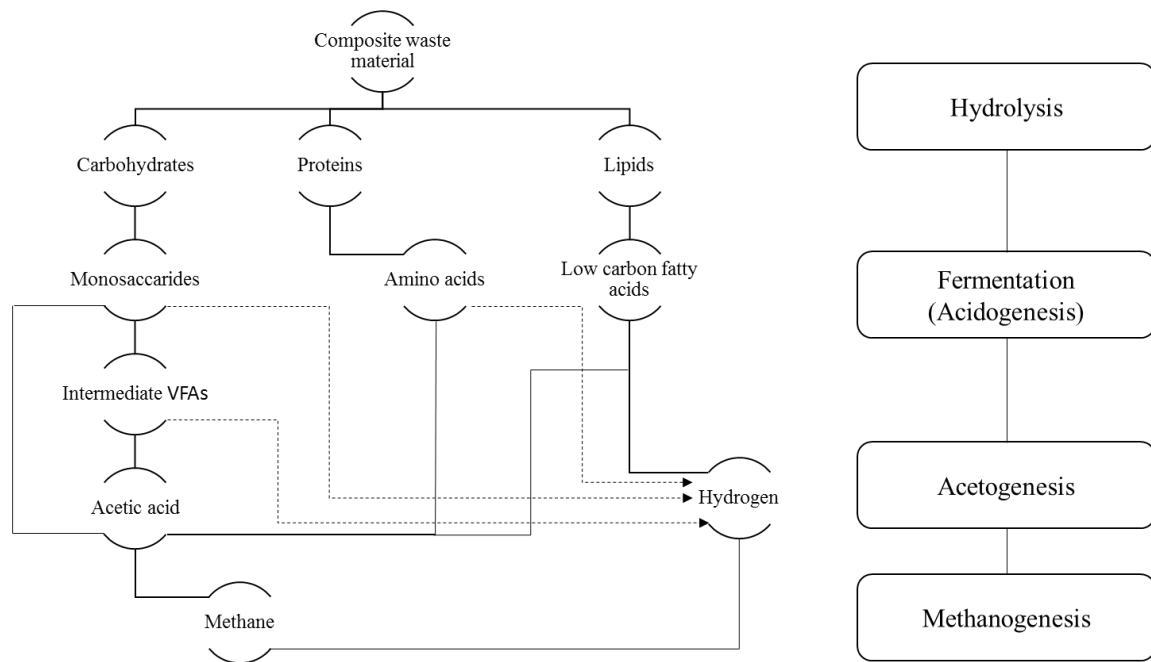


Figure 2.6 Anaerobic degradation of the biodegradable components of wastewater. Adapted from: (Batstone et al., 2006; Metcalf & Eddy, 2014)

Limitations to the widespread implementation of anaerobic treatment prevail, since anaerobic reactors can potentially release unpleasant odors, and do not respond well to fluctuations in load variations. With load fluctuations the equilibrium between acidogenic bacteria, acetogens, and methanogens may shift resulting from a drop in pH and alkalinity (Leitão et al., 2006). Since anaerobic digestion requires the concurrent selection of acetogenic populations and methanogenic populations (Nieuwenhof, 1984), without the development of methanogenic populations, a build-up of VFAs occurs, COD removal ceases, and further treatment is required (Fu et al., 2011). A potential solution is to separate anaerobic reactors into acidogens and acetogens from methanogens, or to combine anaerobic treatment with aerobic treatment to ensure complete carbon removal is accomplished (Chan et al., 2009).

2.5.2 Nitrogen removal

Nitrogen exists in wastewater as total ammonia nitrogen (TAN), or bound in urea and other organic molecules such as the amino groups of proteins (Seviour and Nielsen, 2010). As bacteria grow, nitrogen is taken up through assimilation, and for example heterotrophs will take up nitrogen with carbon at a C:N ratio of 100:12, assuming acetate is the carbon source, is consumed at a ratio of $1.08 \text{ g-COD} \cdot \text{g-acetate}^{-1}$, and with an A value of 0.69 (McCarty, 1965; McCarty, 1971). Organic forms of nitrogen are also degraded to TAN through hydrolysis and ammonification via facultative organisms. Inorganic nitrogen is removed conventionally through nitrification/denitrification processes, whereby TAN is converted to nitrate via nitrification, and then to nitrogen gas via denitrification (Robertson and Kuenen, 1984; Paredes et al., 2007; WEF, 2011).

Nitrification

Complete nitrification proceeds via the endergonic reaction, in which TAN is oxidized to nitrate in a step-wise manner, providing the energy required for nitrifier growth (Madigan et al., 2008). First, ammonia oxidizing bacteria (AOB) oxidize TAN to nitrite (Equation 2.2), then nitrite oxidizing bacteria (NOB) oxidize nitrite to nitrate (Equation 2.4) (WEF, 2011). Various species have been identified as AOBs and NOBs (Table 2.2). A total of $4.57 \text{ g-O}_2 \cdot \text{g-NH}_4\text{-N}^{-1}$ is required for complete nitrification, whereby $3.43 \text{ g-O}_2 \cdot \text{g-NH}_4\text{-N}^{-1}$ is required for TAN oxidation, and $1.14 \text{ g-O}_2 \cdot \text{g-NO}_2\text{-N}^{-1}$ for nitrite oxidation (Metcalf & Eddy, 2014).



Table 2.2 Taxonomy of nitrification (Schmidt et al., 2003).

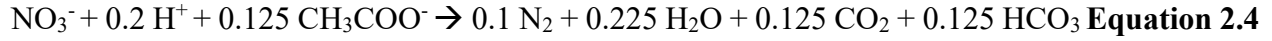
Type	Phyla	Class	Order	Family	Genus
AOB	<i>Proteobacteria</i>	<i>Betaproteobacteria</i>	<i>Nitrosomonadales</i>	<i>Nitrosomonadales</i>	<i>Nitrosomonas</i>
AOB	<i>Proteobacteria</i>	<i>Gammaproteobacteria</i>	<i>Chromatiales</i>	<i>Chromatiaceae</i>	<i>Nitrosococcus</i>
NOB	<i>Nitrospirae</i>	<i>Nitrospira</i>	<i>Nitrospirales</i>	<i>Nitrospiraceae</i>	<i>Nitrospira</i>
NOB	<i>Proteobacteria</i>	<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	<i>Bradyrhizobiaceae</i>	<i>Nitrobacter</i>
NOB	<i>Nitrospinae</i>	<i>Nitrospina</i>	<i>Nitrospinales</i>	<i>Nitrospinaceae</i>	<i>Nitrospina</i>
NOB	<i>Proteobacteria</i>	<i>Gammaproteobacteria</i>	<i>Chromatiales</i>	<i>Chromatiaceae</i>	<i>Nitrococcus</i>

Nitrification wastewater treatment systems require the control of the following parameters: dissolved oxygen (DO), pH, and alkalinity. Firstly, nitrifiers are obligate aerobes, with a low KO_2 value (0.5) (Wett et al., 2011), therefore sufficient oxygen must be supplied to the system for the proliferation of nitrifiers. Wide variations of pH are known to be detrimental to the performance of nitrification, therefore maintaining the pH between 6.5-8.0 is necessary for nitrification to proceed. Finally, nitrification consumes 7.07 g- $CaCO_3$ of alkalinity per gram of TAN-N oxidized, and 7.23 g alkalinity as $CaCO_3$ per NO_2^- -N formed, accounting for ammonia assimilation and biomass growth (WEF, 2011).

Denitrification

Denitrification is the biological reduction of nitrate or nitrite to nitrogen gas, which is performed by various chemoorganotrophic, lithoautotrophic, and phototrophic bacteria; and certain fungi (WEF, 2005). Denitrification is achieved through anoxic respiration; whereby electrons are transferred from organic matter, reduced sulfur compounds, or molecular hydrogen to oxidized nitrogen compounds. Complete denitrification occurs first through the reduction of nitrate to nitrite, catalyzed by a membrane-bound enzyme nitrate reductase A. Then nitrite is reduced to

gaseous nitrogen compounds by nitrite reductase, then to nitrous oxide by nitrous oxide reduces, and finally to nitrogen gas (Seviour and Nielsen, 2010).



Denitrification typically requires anoxic environments, where oxygen is absent or in low concentrations and nitrate or nitrite is present. Denitrifying bacteria represent diverse taxonomic groups of microorganisms including: *Pseudomonas*, *Bacillus*, *Paracoccus*, and *Thiobacillus denitrificans* (Pajares and Bohannon, 2016). Several examples of denitrifiers are presented (Table 2.3).

Table 2.3 Taxonomy of denitrification (Carlson, 1983).

Phylum	Class	Order	Family	Genus
<i>Proteobacteria</i>	<i>Gammaproteobacteria</i>	<i>Pseudomonadales</i>	<i>Pseudomonadaceae</i>	<i>Pseudomonas</i>
<i>Proteobacteria</i>	<i>Gammaproteobacteria</i>	<i>Pseudomonadales</i>	<i>Pseudomonadaceae</i>	<i>Pseudomonas</i>
<i>Proteobacteria</i>	<i>Gammaproteobacteria</i>	<i>Pseudomonadales</i>	<i>Moraxellaceae</i>	<i>Acinetobacter</i>
<i>Firmicutes</i>	<i>Bacilli</i>	<i>Bacillales</i>	<i>Bacillaceae</i>	<i>Bacillus</i>
<i>Firmicutes</i>	<i>Bacilli</i>	<i>Bacillales</i>	<i>Bacillaceae</i>	<i>Bacillus</i>

2.5.3 Enhanced Biological Phosphorous Removal (EBPR)

EBPR utilizes the dynamic metabolism of poly-P accumulating organisms (PAOs) to remove phosphorous from wastewater by storing poly-P within cellular granules in excessive quantities. PAOs store energy in the form of polyhydroxyalkanoate (PHA) (Figure 2.2a), and poly-phosphate (poly-P) (Figure 2.2b). PHAs are lipids derived from β -hydroxybutyric acid, and exist in the form of structures that differ by a substitution of hydrocarbon chains on the β -carbon (Satoh et al., 1992). Poly-P is a linear polymer stored in granules within cells (Figure 2.2b), and range from 3-1000 monomer-phosphate units linked by adenosine triphosphate (ATP)-like bonds (Rao et al., 2009).

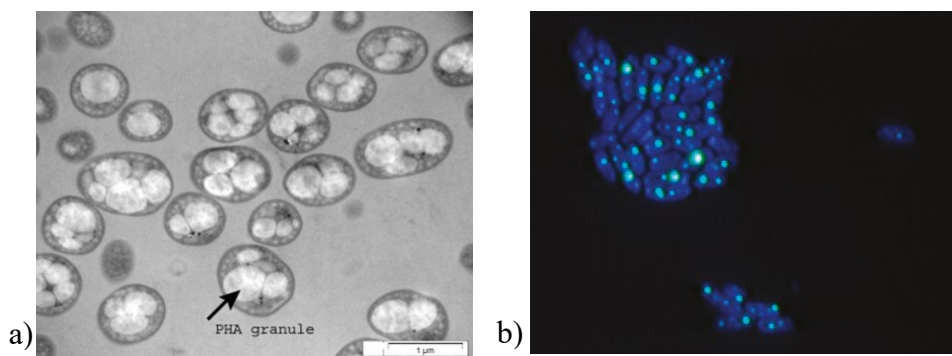


Figure 2.8 Transmission electron micrograph image of the accumulation of PHA in *Pseudomonas putida* cells. From: (Ward, De Roo, & O'Connor, 2005) (a), and ',6-diamidino-2-phenylindole (DAPI)-stained cells of *P. putida* (b). Intracellular poly-P granules appear as yellow/green inclusions within cells. From: (Kulakova et al., 2011).

EBPR was initially observed in the 1970s by James Barnard in South Africa (Barnard, 1976). According to the classical metabolic model of PAOs, under anaerobic conditions, VFAs are taken up and converted to PHA molecules through energy derived from the degradation of stored glycogen, the tricarboxylic acid (TCA) cycle, and the degradation of stored poly-P that releases orthophosphate to the environment (Liu et al., 1994). Under aerobic conditions the PHAs are degraded fueling the uptake of orthophosphate, to form storage poly-P, and cell growth; resulting in a net removal of phosphorous in the surrounding environment (Figure 2.8). More recently, evidence of *actinobacterial*-related genera that ferment sugars and amino acids, but also store poly-P under aerobic conditions has been observed. Therefore, PAOs have recently been more broadly defined as organisms that use aerobically stored poly-P to fuel anaerobic carbon uptake (Seviour and Nielsen, 2010). The storage of poly-P allows PAOs to manage their energy, giving them a competitive edge over other heterotrophs (WEF, 2010b). PAO activity is detected in wastewater treatment plant based on various ratios with carbon, phosphorous, and VSS observed in the wastewater treatment systems (Table 2.4).

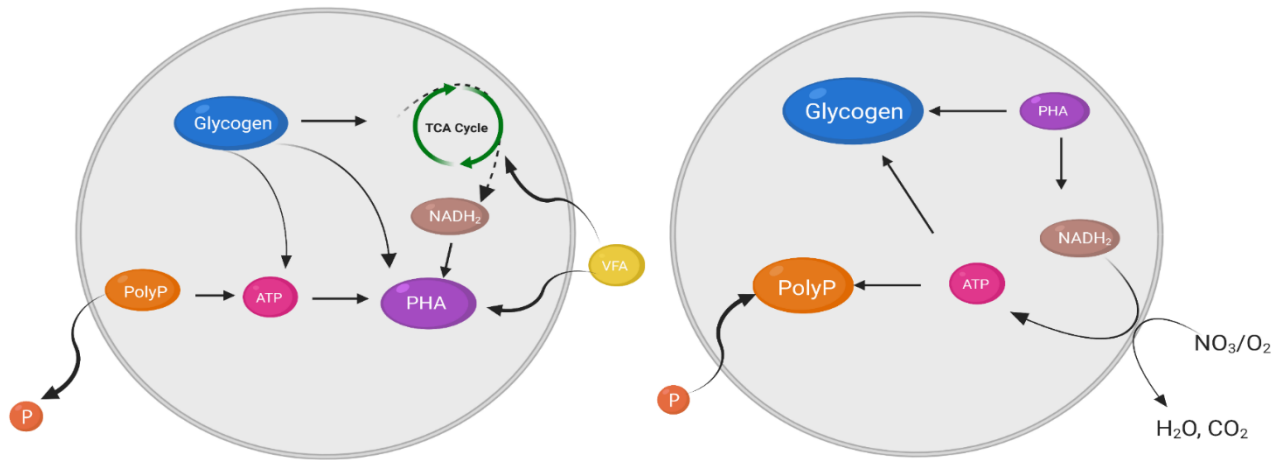


Figure 2.8 Metabolism of PAOs. Adapted from: (Tarayre et al., 2016).

Table 2.4 EBPR ratios in wastewater treatment (Metcalf & Eddy, 2014).

Parameter	Ratio
VSS/Total suspended solids (TSS) ratio in EBPR enriched system	60-65%
Biomass produced per acetate uptake by PAOs	0.45 g-VSS·g-acetate ⁻¹
Cell P content in PAOs	0.20-0.30 g-P·g-VSS ⁻¹
P/C uptake ratio observed in EBPR system	8-20 g-P·g-rbCOD ⁻¹

Taxonomy of EBPR

According to early investigations of EBPR microbial communities, based on culture-dependent techniques, the main PAO taxa was thought to be the genus *Acinetobacter* (Fuhs and Chen, 1975). More recently, culture-independent studies identified a species in the *Rhodocyclus* genus that dominates EBPR communities, through the construction of 16S rRNA clone libraries (Bond et al., 1995), and has since been given the name *Candidatus Accumulibacter phosphatis* (Hesseltmann et al., 1999). In full-scale EBPR plants, *Accumulibacter* has been observed at abundances of 40-70%

of the total PAO population, and studies investigating the *ppk1* gene found that *Accumulibacter* varied in different full-scale EBPR plants (McMahon et al., 2002). *Accumulibacter* subpopulations have also been observed in freshwater and estuarine sediments. From these studies 12 clades of *Accumulibacter* have been identified, and these fall into two groups: clade type I and clade type II (Peterson et al., 2008). Although *Accumulibacter* has not been isolated, the full genome sequence of *Accumulibacter* has been sequenced by Martin et al. (2006) (*Accumulibacter* UW-1) (Nielsen et al., 2012), and later by Flowers et al. (2013) (*Accumulibacter* UW-2) through metagenomic sequencing.

Accumulibacter is the most frequently studied PAO, however other taxa have also been shown to accumulate poly-P, and as such are considered putative PAOs, including: *Microthrix phosphovorans* (Nakamura et al., 1995), *Tetrasphaera* (Kong et al., 2005; Nguyen et al., 2011), *Pseudomonas* (Günther et al., 2009), *Halomonas* (Nguyen et al., 2012), *Comamonadaceae* (Ge et al., 2015), and the species *Candidatus Dechloromonas phosphatis* and *Candidatus Dechloromonas phosphovorans* (Petriglieri et al., 2020). These organisms were shown to store high quantities of poly-P through DAPI staining (Nakamura et al., 1991; Günther et al., 2009), and were observed in high abundances in EBPR sludge through studying 16S rRNA gene clone libraries (Christensson et al., 1998; Günther et al., 2009), pyrosequencing the 16S rRNA gene (Ge et al., 2015), and fluorescence *in situ* hybridization (FISH) analysis (Bond et al., 1999). *Tetrasphaera*-related PAOs do not uptake of VFAs like the *Accumulibacter*, but instead have been known to take up glucose or amino acids, and store glycogen (Kong et al., 2005; Nguyen et al., 2011; Marques et al., 2017). *Tetrasphaera*-related PAOs uptake glucose through energy derived from fermentation as well as poly-P degradation (Marques et al., 2017), whereby glycogen is the storage compound, opposed to PHA (Kong et al., 2005; Nguyen et al., 2011; Marques et al., 2017). These

fermenting PAOs occupy a different niche from *Accumulibacter*, and are therefore thought to contribute to the stability of the EBPR process (Mielczarek et al., 2013).

Operational conditions for EBPR

The anaerobic staging, carbon to phosphorous ratio, pH, and DO concentration must be operated to control the PAO metabolism (Table 2.5). To ensure sufficient EBPR activity is achieved, in the anaerobic stage the DO concentration must remain $< 0.2 \text{ mg}\cdot\text{L}^{-1}$, and an ORP value of -300 mV or less must be maintained, otherwise PAOs will exhibit aerobic metabolism, and less PHAs will be stored. Since particular PAOs use nitrate as an electron acceptor, the influent should also not contain nitrate (WEF, 2011). Finally, mixing is required in the anaerobic stage, however if mixing is too rigorous then oxygen will be introduced to the system resulting in aerobic metabolism in the anaerobic stage resulting in EBPR failure (WEF, 2011).

Table 2.5 Conditions required for EBPR in conventional activated sludge (CAS) systems.

Parameter	Condition	Reference
Anaerobic oxidation reduction potential (ORP)	-300 mV	(WEF, 2011)
Anaerobic DO	$< 0.2 \text{ mg}\cdot\text{DO}\cdot\text{L}^{-1}$	(WEF, 2011)
COD	$< 211.7 \text{ mg}\cdot\text{L}^{-1}$	(WEF, 2011)
Carbon: phosphorous ratio (Complex carbon)	41-48	(Carrera et al., 2001)
Temperature	$< 30^{\circ}\text{C}$	(Whang and Park, 2006; López-Vázquez et al., 2008)
Cation requirement (P:Mg:K:Ca)	1.0:0.28:0.26:0.09	(Metcalf & Eddy, 2014)

The C:P ratio must be controlled in EBPR systems, since wastewaters that are organic-substrate limiting will have insufficient substrate available to fuel the uptake of available phosphorous. On the other hand, in a phosphorous-limiting system non-PAO organisms will be selected for, and therefore may lead to EBPR failure in CAS (Liu, 1997). For effective EBPR treatment, a ratio of carbonaceous (cBOD) to total phosphorous (TP) ratios of at least 20:1 is

recommended (WEF, 2011). When instead complex carbon is used, the optimum COD:P ratio to be 41-48:1 for CAS (Carrera et al., 2001).

EBPR failures have been observed in CAS even under optimum operational parameters for PAO metabolism under laboratory-scale (Nielsen et al., 1999; Oehmen et al., 2006), and full-scale operations (Saunders et al., 2003; Kong et al., 2006). These failures were thought to be linked to competition with GAOs; of which cycle PHA when exposed to cycling anaerobic and aerobic conditions, but do not uptake poly-P (Wong et al., 2004). However, GAO competition is suggested to not be as important as previously stated, since evidence of GAO-related failures of full-scale EBPR plants has not been identified. Furthermore, evidence of GAOs found in high abundances have been observed in conjunction with stable EBPR treatment plants (Saunders et al., 2003; Lanham et al., 2013; Tu and Schuler, 2013; Stockholm-Bjerregaard et al., 2017; Nielsen et al., 2019).

2.6 Treatment of Cheese Production Wastewater

Cheese production wastewater is treated through chemical and physical means, including coagulation/flocculation and chemical precipitation (Tirado et al., 2018; Prazeres et al., 2020); or biological means, with biological processes being the more economical treatment method for organic and nutrient removal (Vidal et al., 2000; Sathasivan, 2009). Biological treatment of organic carbon from cheese production wastewater is achieved through anaerobic technologies, aerobic technologies, or a combination of the two. Examples of anaerobic treatment technologies for cheese production wastewater include: anaerobic digestion (Plan et al., 1995), and membrane reactor systems (Li and Corrado, 1985; Paçal et al., 2019). Anaerobic degradation of cheese production wastewater is limited, since complete organic degradation is often not achieved, and nutrient removal is not possible with anaerobic treatment. Aerobic treatment of organic carbon

from cheese production wastewater has been achieved with technologies including: CAS (Donkin and Russell, 1997; Lateef et al., 2013), SBRs (Torrijos et al., 2001; Li and Zhang, 2002; Asadi et al., 2012), and membrane bioreactors (Fraga et al., 2017). However, conventional suspended growth treatment of industrial wastewaters produce excessive sludge and exhibit fluctuating effluent qualities (Sirianuntapiboon and Tondée, 2000).

Aerobic conditions are necessary for both nitrogen and phosphorous removal. As such aerobic treatment technologies have been implemented in the treatment of nitrogen through nitrification and denitrification from cheese production wastewater, including with CAS (Bortone and Piccinini, 1991; Donkin and Russell, 1997), membrane bioreactors (Fraga et al., 2017), and wetlands (Kasapgil et al., 1994). EBPR has been studied with limited success for dairy production wastewater with CAS in continuous anoxic/anaerobic reactors (Kolarski and Nyhuis, 1995), and SBRs (Comeau et al., 1996; Papagiannis, 1996). Successful EBPR was achieved treating cheese production wastewater in a SBR following an equalisation tank (Comeau et al., 1996), and more recently with intermittently aerated SBRs (Gil-Pulido et al., 2018).

2.7 Attached-growth biological wastewater treatment

Attached-growth biological wastewater treatment, with biofilms attached to an inert media, permits a higher concentration of biomass compared to CAS, and thus permits higher removal rates with a more compact system. By maintaining the biofilm on a surface, the need for sludge recycling is avoided, permitting a simpler system, and the proliferation of more specialized microorganisms in the biofilm compared to CAS (Ødegaard, 1999; WEF, 2010a). Downfalls of attached growth technologies include the potential for clogging, excessive growth, and inadequate mixing or short-circuiting that can result in inefficient use of the media. Disadvantages are generally linked to the specific technology in attached growth wastewater treatment (WEF, 2011),

and examples include the trickling filter, rotating biological contactor, biologically aerated filter, and the moving bed biofilm reactor (MBBR) (Table 2.6).

Table 2.6 Common attached growth treatment systems (WEF, 2010b).

Technology	Definition
Trickling filter	Open media supporting biomass growth with wastewater distributed over the top that trickles down outer the media.
Rotating biological contactor	Circular plastic discs mounted on a horizontal shaft, usually submerged 40% in wastewater. Rotates 1 to 2 rpm, alternating exposure to water and air cannot be used for heavy organic loading or for roughing
Biologically aerated filter	Combines aerobic biological treatment with filtration, eliminates the need for separate solids removal. Configured as up flow or downflow. Air is sparged into the bottom to provide an aerobic environment. Requires backwashing to removed excess solids
MBBR	Biomass growths on small polyethylene carriers in either aerobic, anoxic, or anaerobic environments. Mixed through coarse-bubbled diffused aeration (aerobic), or mixing with submerged mixers (anoxic)

2.7.1 MBBR

The MBBR is an attached-growth wastewater treatment system, in which biofilms are maintained on high density polyethylene carriers, such as the K5 AnoxKaldnes carrier (Figure 2.9) (Ødegaard et al., 1994). The MBBR was developed in Norway in the 1980s; and as of 2009, 500 MBBR plants exist in over 50 countries (McQuarrie and Boltz, 2011). The polyethylene carriers have a specific gravity similar to water, allowing the carriers to remain buoyant and move freely through the reactor (Ødegaard, 2006). These carriers are kept in constant motion permitting efficient mass transfer of substrate and DO, while eliminating clogging within the media, thereby eliminating the need for backwashing (Rusten et al., 2006). Carriers are kept in motion through aeration in aerobic reactors and mechanical mixing in anaerobic reactors (Morgan-Sagastume, 2018). The specific

design of the carriers allow for a high surface area, and a structure that protects the biofilms from abrasion (Johnson et al., 2000; Ødegaard et al., 2000). In addition, MBBRs can be easily used to upgrade existing CAS treatment plants, by simply adding carriers to existing CAS sludge tanks. MBBR plants can therefore be upgraded by increasing the fill percentage, which range from 25-70% of the reactor volume (Rusten et al., 2006). To date, the MBBR has been well established for the treatment of BOD, TAN, and total nitrogen (TN) (WEF, 2010a; Zinatizadeh and Ghaytooli, 2015).

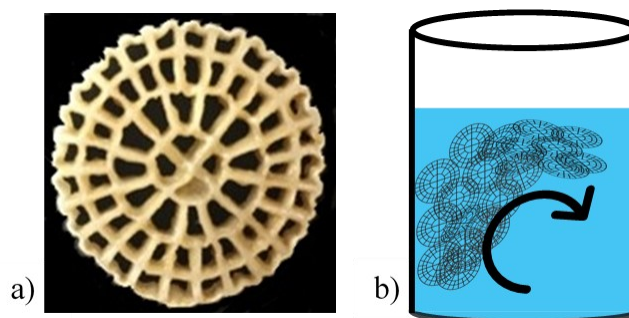


Figure 2.9 K5 carrier (a), and MBBR operation (b).

2.7.2 EBPR with MBBRs treating municipal wastewater

By alternating between anaerobic and aerobic conditions, EBPR can be achieved in MBBRs in addition to carbon and nitrogen removal. With a continuous operation, Kermani et al. (2008) achieved EBPR and nitrogen removal in a series of 4 continuous flow reactors, which consisted of an anaerobic reactor, followed by two anoxic reactors, and finally an aerobic reactor. The reactors achieved 99.7% TN removal through nitrification and denitrification, and a TP removal efficiency of 87.9% achieved through EBPR in the MBBR system (Kermani et al., 2008).

Sequencing-batch operation permits EBPR activity to occur in a single reactor, for a more compact operation, and MBBRs operated under sequencing batch operation are termed sequencing batch moving bed biofilm reactors (SB-MBBRs). EBPR has been achieved in SB-MBBRs with

simultaneous nitrogen and phosphorous removal (Pastorelli et al., 1999; Helness and Ødegaard, 2001; Gieseke et al., 2002; Humbert et al., 2018), and with removal of micropollutants (Torresi et al., 2019). Since SB-MBBR biofilm communities are more susceptible to competition from heterotrophic bacteria that can inhibit PAOs compared to CAS (Helness and Ødegaard, 2001), SB-MBBR studies have only achieved EBPR in a single reactor under specific loading rates and staging times (Table 2.7). To limit the non-phosphorous accumulating heterotrophic community, it is recommended that the carbon be completely treated in the anaerobic stage (Helness and Ødegaard, 2001). For example, both Pastorelli et al. (1999), and Gieseke et al. (2002) achieved successful EBPR with complete COD removal in the anaerobic stage, while Helness and Ødegaard (2001) only achieved EBPR when the anaerobic COD removal efficiency was at least 88%. Furthermore, EBPR depends on the energy obtained from PHAs formed from COD taken up in the anaerobic stage, therefore the greater quantity of COD that is taken up in the anaerobic stage, the more energy will be available for phosphorous uptake.

Table 2.7 Loading Rates for 95% P-removal. Adapted from: (Helness and Ødegaard, 2001).

Constituent	Requirement
COD:N:P	~ 100:10:2
COD-loading rate	< 4 g-COD·m ⁻² d ⁻¹
Anaerobic biodegradable soluble COD (bsCOD)-loading rate	<5 g-COD·m ⁻² d ⁻¹
bsCOD / PO₄³⁻-P	20

2.7.3 Industrial wastewater treatment with the MBBR technology

MBBRs are well suited to treat industrial wastewater, as MBBRs are resilient to fluctuations in water quality, and the high concentration of biomass allows for the treatment of high organic loads with a compact land footprint, ideal for on-site treatment. Specifically, MBBRs have been shown

to be feasible for the treatment of industrial wastewaters including: chemical processing (Rusten et al., 1999), landfill (Chen et al., 2008), and the food and beverage industries (Rusten et al., 1996; Andreottola et al., 2002; Chai et al., 2014; Tatoulis et al., 2015; di Biase et al., 2018). Anaerobic treatment alone is limited for industrial wastewater treatment with MBBRs due to excessive hydraulic retention times (HRTs) that are required for complete treatment (Chai et al., 2014; di Biase et al., 2018), and nutrient removal is not possible. On the other hand, aerobic treatment alone with MBBRs can lead to excessive production of solids, and nitrification is not achieved in a single reactor due to the carbon load of cheese production wastewater (Andreottola et al., 2002). Aerobic MBBRs have therefore been combined with anaerobic treatment as a polishing step, and separate anaerobic and aerobic MBBRs in series to permit nitrification (Chen et al., 2008). Nitrification was also achieved in dairy parlor wastewater treatment following anaerobic treatment in continuous MBBR units (Luostarinen et al., 2006). Nitrogen and phosphorous treatment from cheese production wastewater with the MBBR technology has been achieved through assimilation pathways (Andreottola et al., 2002), and with precipitation (Rusten et al., 1996), respectively.

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3 Article 1 - Kinetic effects of anaerobic staging and aeration rates on sequencing batch moving bed biofilm reactors: carbon, nitrogen, and phosphorous treatment of cheese production wastewater

3.1 Abstract

The food and beverage industry produces wastewaters containing high concentrations of organic carbon and nutrients, which when discharged leads to eutrophication and algal blooms. Given recent stringencies in effluent regulations, industries are required to treat their wastewater on-site. There is a critical need for compact, high-rate, cost-effective wastewater technologies to treat industrial wastewaters, such as the sequencing batch moving bed biofilm reactor (SB-MBBR). The aim of this study is to investigate the potential and evaluate the performance of the SB-MBBR cycling between anaerobic and aerobic stages to treat high-strength food and beverage wastewaters. Specifically, this study focuses on the effects of anaerobic staging times and enhanced aeration on the removal of carbon, nitrogen, and phosphorous from cheese production wastewaters. Increasing anaerobic staging times was found to improve the removal rates of carbon beyond previously reported moving bed biofilm reactor (MBBR) results. Increasing the anaerobic stage however decreased the total nitrogen removal, with organic nitrogen undergoing ammonification during the anaerobic stage. This study demonstrates an optimum anaerobic staging time of 138 minutes; with a carbon removal rate of $31.1 \text{ g-sCOD} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ and a nitrogen removal rate of $1.3 \text{ g-N} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$. Enhanced aeration was found to be detrimental to phosphorous removal, where a moderate aeration rate demonstrated a net total phosphorous removal of approximately $22 \text{ mg-P} \cdot \text{l}^{-1}$ with the phosphorous-content of the suspended solids being approximately 4%. Finally,

the sequencing batch moving bed biofilm reactor shows potential for on-site treatment of carbon, nitrogen, and phosphorous from cheese production wastewater.

3.2 Introduction

Cheese production generates wastewater containing by-products of cheese whey and/or second cheese whey (from cottage cheese production), combined with wastewaters generated by on-site laboratory analyses and washing equipment; thereby producing wastewaters with high concentrations of organic content and nutrients (Prazeres et al., 2012; Carvalho et al., 2013; Tatoulis et al., 2015). Discharging cheese production wastewater is deleterious to receiving aquatic systems, since organic content and nutrients cause excessive oxygen depletion and eutrophication (Hutchinson, 1973; Boesch et al., 2006). To mitigate these effects, many countries are implementing increasingly rigorous regulations for municipal wastewater treatment plants (Gazette, 2012; Román-Sánchez et al., 2015; Hendriks and Langeveld, 2017). The food and beverage industry, along with cheese production facilities, often contribute significantly to the organic and nutrient load of municipal wastewater treatment plants and in smaller communities can overload wastewater treatment lagoons (Delatolla and Babarutsi, 2005). As such, municipalities limit industrial effluent concentrations discharged to sewers through financial and regulatory pressures, requiring industries to treat their wastewaters on-site (Rusten et al., 1996; Tatoulis et al., 2015; Van Lier et al., 2015). To meet the required elevated loading conditions and space limitations for on-site treatment of industrial wastewater, there is a need for advanced treatment technologies.

Attached-growth technologies provide advantages compared to conventional suspended growth treatment systems by utilizing biofilms that house a high concentration of microbial consortium attached to a surface. Biosolids produced, requiring separation and disposal, is reduced

compared to conventional suspended growth systems, and the attached biomass also becomes increasingly specialized in biofilms with an ability to maintain higher concentrations of key organisms. Together these features allow for a more compact system without the need of sludge recycling (Ødegaard, 1999). One such attached-growth system is the MBBR, in which biofilms are maintained on engineered carriers (Ødegaard et al., 1994; Rusten et al., 2006). The carriers are kept in constant motion in MBBR basins with the design of these carriers is often based on maximizing the active biofilm surface area, permitting a minimal land footprint of the treatment system (Johnson et al., 2000; Ødegaard et al., 2000). The MBBR has demonstrated the successful treatment of organic matter and nitrogen from high-strength wastewaters from chemical processing (Rusten et al., 1999), landfill (Chen et al., 2008), and the food and beverage industries (Chai et al., 2014; di Biase et al., 2018) including dairy industries (Rusten et al., 1996; Andreottola et al., 2002; Tatoulis et al., 2015). In these studies MBBR systems were operated as a single anaerobic reactor when treating carbon alone (Chai et al., 2014; di Biase et al., 2018); and as a 2-reactor system when treating carbon and nitrogen, where the MBBRs comprised the polishing step following anaerobic treatment (Andreottola et al., 2002), or were operated as separate anaerobic and aerobic MBBRs in series (Chen et al., 2008). Specifically for dairy wastewater, studies have demonstrated that the MBBR technology is capable of achieving an efficiency of 80% at loads of up to $52.7 \text{ g-chemical oxygen demand (COD)·m}^{-2}\text{d}^{-1}$ (Andreottola et al., 2002) while operated as continuous flow and a removal rate of $26.3 \text{ g-dissolved COD·L}^{-1}\text{d}^{-1}$ while operated as a sequencing batch reactor (Tatoulis et al., 2015). The MBBR technology was also successfully demonstrated as an upgrade to an existing trickling filter system for cheese production wastewater treatment to meet increased regulation standards (Rusten et al., 1996).

Enhanced biological phosphorous removal (EBPR) is a more economical means of phosphorous removal compared to chemical treatment (Sathasivan, 2009). Early EBPR studies for the treatment of dairy wastewater showed limited success with CAS in continuous anoxic/anaerobic reactors (Kolarski and Nyhuis, 1995), and sequencing batch reactors (SBRs) (Papagiannis, 1996). Successful EBPR was first observed with a SBR following an equalisation tank (Comeau et al., 1996), and more recently with intermittently aerated SBRs (Gil-Pulido et al., 2018). EBPR has also been achieved using the SB-MBBR technology by cycling between anaerobic and aerobic stages at the laboratory scale (Helness and Ødegaard, 1999), at the pilot scale (Pastorelli et al., 1999) with simultaneous nitrogen and phosphorous removal (Pastorelli et al., 1999; Helness and Ødegaard, 2001; Gieseke et al., 2002; Humbert et al., 2018), and with removal of micropollutants (Torresi et al., 2019). The duration of these anaerobic and aerobic stages is an important factor in selecting for polyphosphate accumulating organisms (PAOs) in the biofilm (Helness and Ødegaard, 1999, 2001), while the control of aeration is considered necessary for overcoming oxygen-diffusion limitations that may impair EBPR in biofilms (Morgenroth and Wilderer, 1999; Chiou and Yang, 2008). EBPR has only been achieved to date in MBBRs under specific organic and nutrient loading conditions, where most of the limited number of studies that currently exist have been performed on municipal-strength wastewater (Helness and Ødegaard, 1999, 2001). Hence, a gap in the knowledge exists with respect to EBPR in MBBRs with high-strength wastewater, and furthermore no studies have investigated the treatment of food and beverage wastewater in a single SB-MBBR with anaerobic and aerobic stages. Identifying the potential for the treatment of organic carbon and nutrients with SB-MBBRs with alternating anaerobic and aerobic stages can provide industry a cost-effective and compact technology capable of on-site treatment of carbon, nitrogen, and phosphorous. New information on the design and

operation will allow for improved performance of the SB-MBBR and will guide future pilot- and full-scale studies. Therefore, the main objectives of this research are to provide new fundamental knowledge with respect to the operation and design of the SB-MBBR treating wastewater with high concentrations of organic-content and nutrients. Specifically, this study aims to determine the effect of the anaerobic staging times and increased aeration on carbon, nitrogen, and phosphorous removal of the SB-MBBR system treating cheese production wastewaters.

3.3 Materials and methods

3.3.1 Experimental start-up, design, and operation

The experiments were conducted using three identical laboratory-scale SB-MBBR reactor systems. Each reactor was operated at room, with an average temperature of 22°C, and were designed with a total operating volume of 2.3 L and each reactor housed K5 AnoxKaldnes carriers (Veolia, Sweden) at a fill percent of 47%. Seeded carriers harvested from the Hawkesbury integrated fixed film activated sludge (IFAS) wastewater treatment plant in Ontario, Canada were used to fill the three reactors and initiate the start-up phase of the systems. The seeded carriers contained a low percent abundance of nitrifying bacteria, as described by Young et al. (2017). The three reactors were operated at stable conditions for a period of 5 months prior to the profile testing of the study, where steady operation was observed, and the influent and effluent concentrations were measured 6 consecutive times throughout each operation to determine steady state. Profile studies were subsequently conducted over the entire cycle, which were repeated a minimum of 3 times for each condition studied. Steady state operation was defined in this study by $\pm 10\%$ variance in the reactors' performance. Following the 5-month period, the enhanced aeration was tested in reactor 2. The reactors were each operated for an additional 7 months, and profile studies were conducted again for each reactor following the 7-month period.

All three reactor systems were operated for 8 hours cycle times with the following stages: fill, anaerobic, aerobic, anoxic, decanting, idle (Table 3.1). The duration of the anaerobic and subsequent aerobic stage were varied throughout the study (Table 3.1). The start of the cycle for all three reactors was initiated by peristaltic pumps filling 2 L of the same feed into the three reactors over a 5 minute period, which was then followed by an anaerobic and aerobic stage. Succeeding the aerobic stage, the air pumps were turned off for 14 minutes during the anoxic stage prior to decanting. The anoxic stage was included in the cycle to allow for denitrification in the case of nitrate formation. The cycle ended after the entire volume was decanted for a duration 5 minutes, equating to a total cycle HRT of 8 hours for each reactor (Figure 3.1). Mixing was applied by mechanical mixers during the anaerobic stage, and by aeration at a rate of $2.4 \text{ L} \cdot \text{min}^{-1}$, and the dissolved oxygen (DO) ranged between 0.06 and $7.2 \text{ mg} \cdot \text{L}^{-1}$ during the AE stage.

For this study, the effects of anaerobic staging times and enhanced aeration rate during aerobic staging were tested. The 3 reactors were operated with anaerobic staging times of: 72 minutes (reactor 1), 120 minutes (reactor 2), and 168 minutes (reactor 3); followed by an aerobic staging time of 384 minutes (reactor 1), 336 minutes (reactor 2), and 288 minutes (reactor 3). Note that the increase in operation times of the anaerobic stage was compensated by decreased aerobic staging times in each reactor, hence maintaining the total cycle time of 8 hours (Table 3.1). Since the DO dropped to concentrations below $2.0 \text{ mg} \cdot \text{L}^{-1}$ during the first 2 hours of aeration in the reactors, an enhanced aeration test in reactor 2 (120 minute anaerobic stage) was performed in this study by doubling the aeration rate to $4.8 \text{ L} \cdot \text{min}^{-1}$, elevating the DO concentration to $3.2\text{-}7.4 \text{ mg} \cdot \text{L}^{-1}$. The aeration was maintained at $4.8 \text{ L} \cdot \text{min}^{-1}$ in the system during the first 2 hours of the aerobic stage, maintaining the DO concentration above $2 \text{ mg} \cdot \text{L}^{-1}$ throughout the entire aerobic phase of

operation, then after 2 hours, the aeration returned to $2.4 \text{ L} \cdot \text{min}^{-1}$. This enhanced aeration operation of reactor 2 was maintained for 7 months prior to data collection.

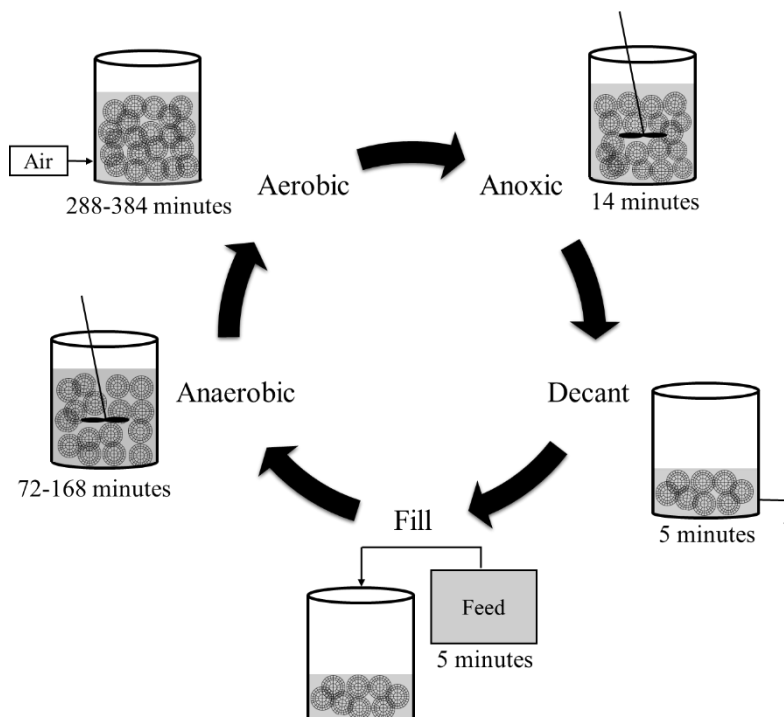


Figure 3.1 Operation of the SB-MBBRs showing the stages and range of staging times.

Table 3.1 Reactor staging times across the three reactors.

Stage	Reactor 1 (min)	Reactor 2 (min)	Reactor 3 (min)
Fill	5	5	5
Anaerobic	72	120	168
Aerobic	379	336	283
Anoxic	14	14	14
Decant	5	5	5
Idle	5	5	5

3.3.2 Wastewater source

The synthetic wastewater used for this study simulated the carbon, nitrogen, and phosphorous composition of the cheese production wastewater from the St. Albert Cheese Factory, located in Ontario, Canada. The cheese production wastewater at this plant was stored in an onsite lagoon

designed to dampen load fluctuations entering a proposed on-site treatment system (Delatolla and Babarutsi, 2005). The composition of the synthetic wastewater is as follows: NH_4Cl : $89 \text{ mg}\cdot\text{L}^{-1}$, KH_2PO_4 : $69 \text{ mg}\cdot\text{L}^{-1}$, NaHCO_3 : $304 \text{ mg}\cdot\text{L}^{-1}$, $\text{MgCl}_2\cdot\text{H}_2\text{O}$: $58 \text{ mg}\cdot\text{L}^{-1}$, $\text{CaCl}_2\cdot\text{H}_2\text{O}$: $29 \text{ mg}\cdot\text{L}^{-1}$, and FeCl : $5 \text{ mg}\cdot\text{L}^{-1}$ (Delatolla et al., 2012) carbon sources were comprised of: dextrose: $510 \text{ mg}\cdot\text{L}^{-1}$, sodium acetate: $340 \text{ mg}\cdot\text{L}^{-1}$, and peptone: $510 \text{ mg}\cdot\text{L}^{-1}$. The carbon sources were selected to mimic the readily and non-readily degradable carbon characteristic of cheese production wastewater and to replicate the biological oxygen demand (BOD) to COD ratio of 0.7 with approximately $86 \text{ mg}\cdot\text{L}^{-1}$ of the soluble COD (sCOD) being slowly biodegradable COD. The carbon, nitrogen and phosphorous concentrations of the synthetic wastewater were as follows: sCOD: $2500 \text{ mg}\cdot\text{L}^{-1}$, total ammonia nitrogen (TAN): $100 \text{ mg}\cdot\text{L}^{-1}$, total nitrogen (TN): $250 \text{ mg}\cdot\text{L}^{-1}$, and total, and total phosphorous (TP): $40 \text{ mg}\cdot\text{L}^{-1}$. The synthetic wastewater of this study was designed with the same BOD:COD, sCOD concentration, TAN concentration, TN concentration and TP concentration of the St. Albert cheese production wastewater. For each profile and each condition, the confidence interval between the feed concentrations was below 10%, therefore the differences in influent concentrations was not significant in this study.

3.3.3 Constituent analysis

HACH methods 8000-sCOD, 10205-TAN, 10072-TN, and 1012-TP (Delatolla et al., 2012) were used to test bulk liquid samples of sCOD, TAN, TN, and TP respectively, and used to calculate the surface area loading rates (SALRs), and surface area removal rates (SARRs) of the SB-MBBR. Bulk-liquid samples were collected 9 times throughout the 8-hour cycle and were then filtered through a $0.45 \mu\text{m}$ -pore sized filter before testing. Triplicate samples were performed on the influent and effluent samples following the anaerobic and aerobic staging times of each reactor. Standard methods 2450 D-total suspended solids (TSS dried at $103\text{-}105^\circ\text{C}$) and E-volatile

suspended solids (VSS ignited at 550°C) were used to analyze the suspended solids in the bulk liquid, acquired from the influent and effluent at the end of the anaerobic and aerobic stages. Triplicate samples were performed for each solids sample analyzed. Finally, DO and pH measurements were acquired at 5 minute intervals throughout the 8-hour cycle using a HACH multi-meter with a DO and pH probe (HACH, USA, Colorado).

3.3.4 Statistical methods

The statistical significance of the sCOD surface area removal rate (SARR), TN SARR; the sCOD, TAN, TN and TP concentrations; and the TN and TP content were calculated using the t-test using a *p*-value <0.05 to identify significance. Pearson's R was used to determine second order polynomial correlation between both the sCOD SARR and anaerobic staging time; and between oxygen uptake rate (OUR) and anaerobic staging time.

3.4 Results and discussion

3.4.1 Anaerobic sCOD removal

The sCOD concentrations were monitored across the anaerobic stage (Figure 3.2). The SARR of the anaerobic stage for all systems were calculated after 72 minutes of operation in the anaerobic stage ($SARR_{AN-72min}$) (Table 3.2). For all reactors, the sCOD concentration of the systems decreased linearly, then plateaued following 72 minutes of the anaerobic stage. $SARR_{AN-72min}$ values were as follows: $32.5 \pm 0.6 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$, $43.3 \pm 0.9 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$, $38.3 \pm 1.2 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ for anaerobic staging times of 72, 120, and 168 minutes, respectively. sCOD concentrations reached values of $2040 \pm 38 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$, $1740 \pm 70 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$, and $1720 \pm 52 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ at anaerobic staging times of 72, 120, and 168 minutes. Following 72 minutes of operation, the removal was limited to $80 \text{ mg} \cdot \text{L}^{-1}$ of sCOD between 72 and 120 minutes and was limited to $60 \text{ mg} \cdot \text{L}^{-1}$ of sCOD being removed between 120 and 168 minutes. Increasing the aeration rate from $2.4 \text{ L} \cdot \text{min}^{-1}$ to $4.8 \text{ L} \cdot \text{min}^{-1}$ in the aerobic

stage did not affect the $SARR_{AN-72min}$ ($p = 0.5$). The pH was also found to decrease linearly in the anaerobic stage, and then plateau at 72 minutes where the final pH of 6.0 ± 0.1 was reached for each system that operated with varying anaerobic staging times. The pH trend was similar to the sCOD concentration removal, suggesting acidogenesis (Fu et al., 2011; Chai et al., 2014; di Biase et al., 2018) occurred. It is suspected that the acidogens active in the biofilm are aerotolerant, and certain strains of fermenting bacteria are known to be aerotolerant (Kataoka and Tokiwa, 1998). Furthermore, no biofilm sloughing or excess production of biomass in the suspended phase was observed that would suggest lysis of anaerobic bacteria. Finally, complete acidogenesis was shown to likely be reached after approximately 72 minutes where the sCOD removal in the anaerobic phase is the more efficient.

3.4.2 Aerobic sCOD removal

The sCOD was again monitored over the aerobic phase (Figure 3.2). In the aerobic stage the sCOD decreased reaching a final concentration of $47.5 \pm 1.8 \text{ mg} \cdot \text{L}^{-1}$ for all reactors, likely consisting of the recalcitrant sCOD of the feed. As such, the sCOD removal kinetics were calculated in this stage ($SARR_{AE-47mg/L}$) up to the time where the sCOD reached a concentration of $47.5 \pm 1.8 \text{ mg} \cdot \text{L}^{-1}$ (Table 3.2). The final sCOD concentration was reached following 323 (Figure 3.2.a), 180 (Figure 2b), and 72 (Figure 3.2d) minutes of the aerobic stage for the reactors operating with 72, 120, and 168 minute anaerobic staging, respectively. This corresponds to total removal times of 395, 300, 240 minutes for the reactors operating with 72, 120, and 168 minute anaerobic stages, respectively. The $SARR_{AE-47mg/L}$ increased from $29.5 \pm 0.6 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ to $92.5 \pm 2.8 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ ($p < 0.0001$) when increasing the anaerobic staging time from 72 to 168 minutes, while the total SARR of the anaerobic and aerobic stages up to a final sCOD concentration of $47.5 \pm 1.8 \text{ mg} \cdot \text{L}^{-1}$ ($SARR_{AN\&AE-47mg/L}$) increased from $24.8 \pm 0.5 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ to $41.6 \pm 1.3 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ ($p < 0.0001$) when again increasing

the anaerobic staging time from 72 to 168 minutes (Table 3.2). Therefore, although the extended anaerobic staging times (to 120 minutes and 168 minutes) did not increase sCOD removal during the anaerobic stage, the extended anaerobic staging times enhanced the aerobic sCOD removal rates along with the total sCOD removal rates of the combined anaerobic and aerobic stages.

Conversely, the aeration rate had no effect on the $SARR_{AE-47mg/L}$ ($p = 0.5$) (Figures 3.2b and 2c). The SB-MBBRs showed improved removal efficiency for cheese production wastewaters (Table 3.2) when compared to aerobic ponds, where 49-82% reduction of COD was achieved in 5 days (Britz et al., 2006). Also, a previous study that used aerobic MBBRs to treat anaerobically pre-treated wastewater required an HRT of 4 days to reach similar removal (Chen et al., 2008). Other studies using anaerobic MBBRs required an HRT of 1.5 days to treat winery wastewater (Chai et al., 2014), and 2 days to treat brewery wastewater (di Biase et al., 2018) even while operating at 35°C, while 24 hours to achieve a similar removal from synthetic wastewater with an influent concentration of 295 mg·L⁻¹ while operating at room temperature, as in this study (Rezaee et al., 2012). Therefore, the SB-MBBR technology (which exposes the same biofilm to an anaerobic stage followed by an aerobic stage) demonstrates a potential improved effect on the required HRT and hence the sizing and footprint of the system required to treat high concentrated wastewaters with MBBRs.

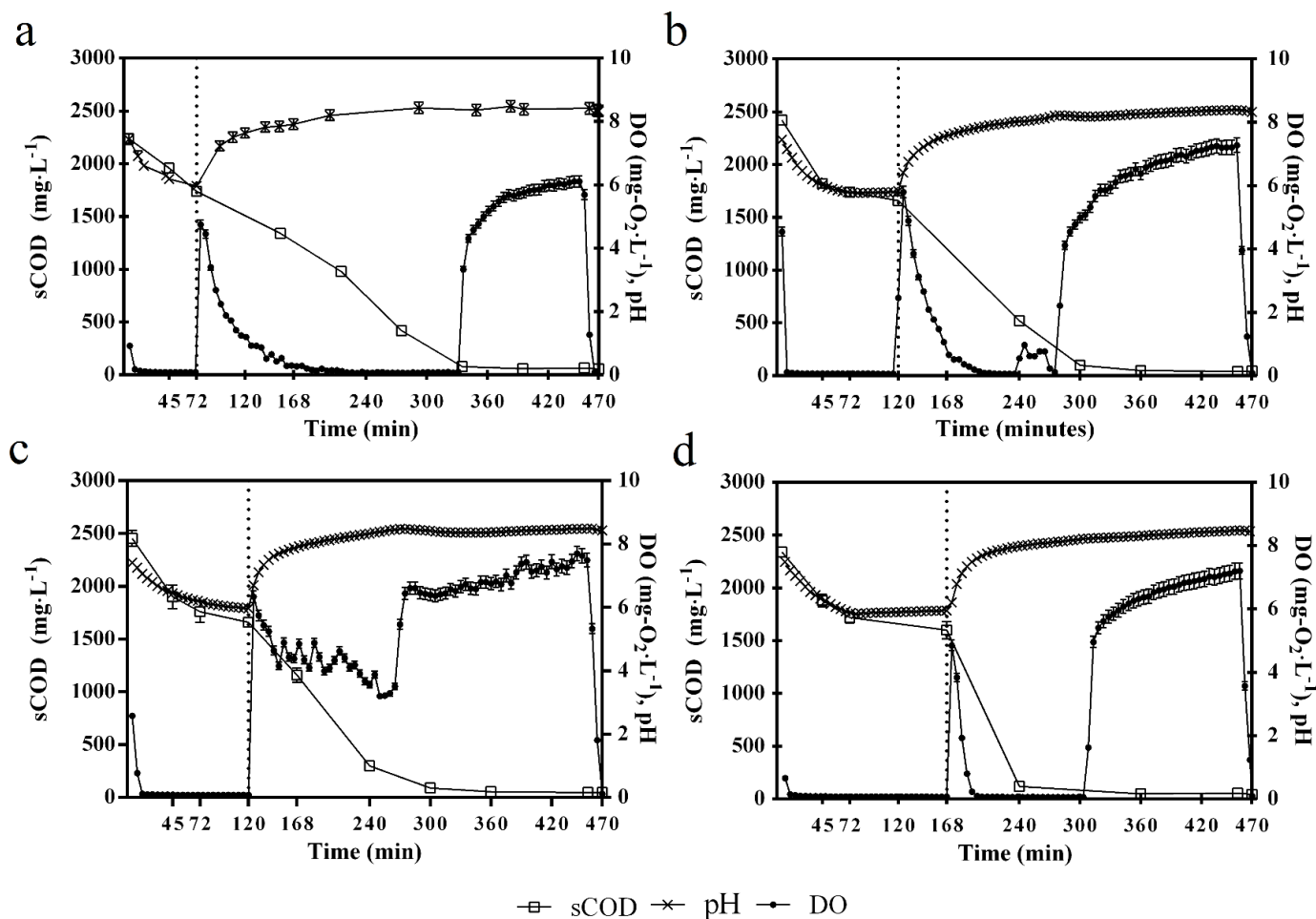


Figure 3.2 Average and 95% confidence interval of sCOD, pH, and DO of one representative profile study following steady-state conditions. (a) Reactor 1, with an anaerobic staging time of 72 minutes and an aeration rate of $2.4 \text{ L} \cdot \text{min}^{-1}$. (b) Reactor 2 operated with an anaerobic staging time of 120 minutes and an aeration rate of $2.4 \text{ L} \cdot \text{min}^{-1}$. (c) Reactor 2 operated with an anaerobic staging time of 120 minutes and an aeration rate of $4.8 \text{ L} \cdot \text{min}^{-1}$. (d) Reactor 3, with an anaerobic staging time of 168 minutes and an aeration rate of $2.4 \text{ L} \cdot \text{min}^{-1}$. The vertical dotted lines indicate the end of the anaerobic phase and the start of the aerobic phase for each reactor and each operational condition.

Table 3.2. Anaerobic, aerobic and total sCOD SALR, SARR, removal efficiency across the removal time of one representative profile study following steady-state conditions.

Anaerobic ²					Aerobic ³				Total ⁴			
Staging time (min)	Removal Time (min)	SALR _{AN} 72min (g·m ⁻² d ⁻¹)	SARR _{AN} 72min (g·m ⁻² d ⁻¹)	Removal efficiency (%)	Removal Time (min)	SALR _{AE} 47mg/L (g·m ⁻² d ⁻¹)	SARR _{AE} 47mg/L (g·m ⁻² d ⁻¹)	Removal efficiency (%)	Removal Time (min)	SALR _{AN} &AE-47mg/L (g·m ⁻² d ⁻¹)	SARR _{AN} &AE-47mg/L (g·m ⁻² d ⁻¹)	Removal Efficiency (%)
72	72	140.0±2.7	32.5±0.6	22.3±0.4	323	30.4±0.6	29.5±0.6	97.2±1.8	395	25.5±0.5	24.8±0.5	97.3±1.8
120	72	90.8±1.8	42.6±0.9	31.4±0.6	180	41.5±0.6	41.1±0.8	96.8±1.9	300	36.3±0.7	29.6±0.6	97.9±2.0
120 ¹	72	92.0±5.5	43.3±2.6	32.3±1.9	180	41.5±2.5	39.3±2.4	94.6±5.7	300	36.8±2.2	35.5±2.1	96.3±5.8
168	72	62.7±1.9	38.8±1.2	31.6±0.9	72	95.4±2.9	92.5±2.8	97.0±2.9	240	42.4±1.3	41.6±1.3	98.1±2.9

¹Operated at an aeration rate of 4.8 L·min⁻¹, all other conditions operated at 2.4 L·min⁻¹.

²Removal time used to calculate SALR, SARR and removal efficiency is set to 72 minutes of operation during the AN stage.

³Removal time used to calculate SALR, SARR and removal efficiency set to time when sCOD concentration decreases to the recalcitrant sCOD concentration of 47 mg/L.

⁴Removal time used to calculate SALR, SARR and removal efficiency set to time when sCOD concentration decreases to the recalcitrant sCOD concentration of 47 mg/L.

Due to the consumption of the organic-content of the synthetic wastewater, the DO was found to decrease rapidly in the system to concentrations as low as 0.06 mg·L⁻¹. Therefore, the OUR was calculated from the difference between the DO at the onset of the aerobic phase to when the DO reached below 0.06 mg·L⁻¹, and this value was used to assess the microbial activity in the SB-MBBR. OUR has been previously used to determine optimum conditions (Ganesh et al., 2006), assess microbial activity and determine when the microbial activity is occurring in sequencing batch reactor (SBR) systems treating industrial wastewater (Pijuan et al., 2009). In the current study, the OUR increased from 2.6±0.1 mg·L⁻¹h⁻¹ to 11.4±0.4 mg·L⁻¹h⁻¹ ($p = 0.0001$) following a second order polynomial ($R = 1.0$), with increasing anaerobic staging time (Figure 3.3). The sCOD SARR_{AE-47mg/L} also increased in the aerobic stage with increasing anaerobic staging time following a second order polynomial ($R = 1.0$), and this finding was similar to previous studies where OUR profiles were reported to closely follow COD removal in SBR treatment of industrial wastewater

(Ganesh et al., 2006). Extending the anaerobic stage likely converted more complex sCOD to more readily degradable sCOD that was more quickly oxidized in the aerobic stage. Therefore, the increase in $SARR_{AE-47mg/L}$ is likely due to the degradation of peptone in the anaerobic stage (Fu et al., 2011), resulting in a greater fraction of readily degradable carbon available in the aerobic stage, optimizing the aeration used in the system. With a 168 minute anaerobic stage, the overall efficiency of the system increased, which reduces the sizing and footprint of the system, while reducing aeration costs. Reducing footprint, operating costs and energy consumption fits in-line with the design and operational initiatives of modern wastewater treatment facilities (Ødegaard, 2016; Henriques and Catarino, 2017).

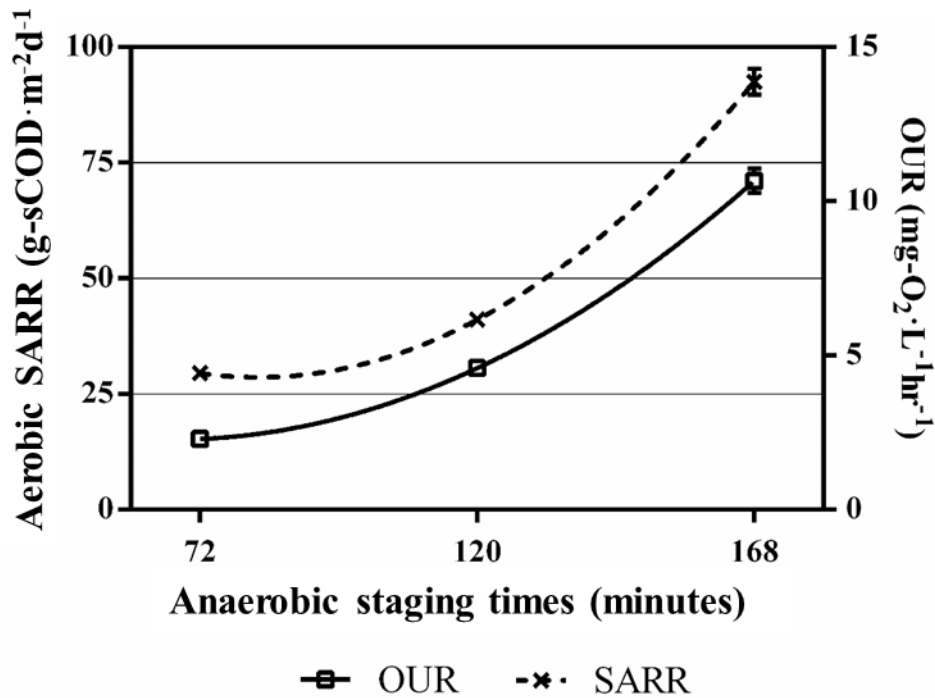


Figure 3.3 Average and 95% confidence interval of OUR, and aerobic SARR across anaerobic staging time of one representative profile study following steady-state conditions.

3.4.3 Anaerobic nitrogen removal

TN and TAN were monitored across the operational cycle of the reactors as well. TAN concentrations were found to increase significantly during the anaerobic stage (Figure 3.4), as is

expected with the acidogenesis of peptone (Ellis et al., 2014). The net production of TAN measured at the end of the anaerobic stages of the reactors is statistically greater following the 168 minute anaerobic staging time compared to 72 and 120 minutes of anaerobic staging times ($p < 0.0001$), suggesting that increasing anaerobic staging allowed more time for the degradation of peptone and the subsequent release of more readily degradable sCOD, ammonification and hence production of TAN (Maier et al., 2009). These findings support the observed increase in sCOD removal rates in the aerobic stages with longer anaerobic staging times (Figure 3.3)

3.4.4 Aerobic nitrogen removal

In the subsequent aerobic stage likely, limited autotrophic nitrification occurred since nitrate concentrations remained below $0.4 \text{ mg}\cdot\text{L}^{-1}$ throughout the cycle for each operational condition. Given the C:N ratios in the feed (2.0:1.0) and at the onset of the aerobic phase (1.2:1.0) being non-favourable for nitrification. The high carbon loading likely resulted in the autotrophs being out competed by heterotrophs. Both high COD loads (Figuerola and Silverstein, 1992; Bassin et al., 2015) and C:N ratios (Tijhuis et al., 1994; Van Benthum et al., 1997; Bassin et al., 2012) favour heterotrophic growth over autotrophic nitrification due to competition for oxygen, space and nutrients. Conventionally for attached growth systems, nitrification requires BOD to be limited to $20 \text{ mg}\cdot\text{L}^{-1}$ of carbonaceous BOD (WEF, 2005). For example, in MBBRs, BOD loads $> 5 \text{ g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$ resulted in no nitrification (Hem et al., 1994), and soluble BOD₅ load of $1.5 \text{ g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$ reduced the bulk liquid DO concentrations available for nitrification by approximately $2.5 \text{ g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$ (Rusten et al., 2006; McQuarrie and Boltz, 2011). Furthermore, the promotion of heterotrophic growth may have resulted in a thick biofilm, limiting the diffusion of oxygen into deeper layers where the nitrifiers may be located (Bassin et al., 2012). Finally, given the low K_{O_2} of nitrifiers (0.5) (Wett et al., 2011), the anaerobic stage may have prevented the growth of nitrifiers without sufficient

aerobic conditions to compensate for the adverse effects of the anaerobic stage times. However, following the depletion of sCOD, TAN concentration decreased by 16.0 ± 0.4 , 11.0 ± 0.2 , and 20.5 ± 0.6 $\text{mg-N} \cdot \text{L}^{-1}$ for the reactors operating with 72, 120, and 168 minute anaerobic staging times; and 9.2 ± 0.2 $\text{mg-N} \cdot \text{L}^{-1}$ for the reactor operating with 120 minute anaerobic staging time and enhanced aeration. Therefore, it is possible small populations of nitrifiers were active following carbon depletion, along with endogenous denitrification, however populations were not established or active enough to allow for complete nitrification. Therefore, to achieve complete nitrification, it is suggested that a second reactor should be utilized to separate the nitrifying population from the heterotrophic population.

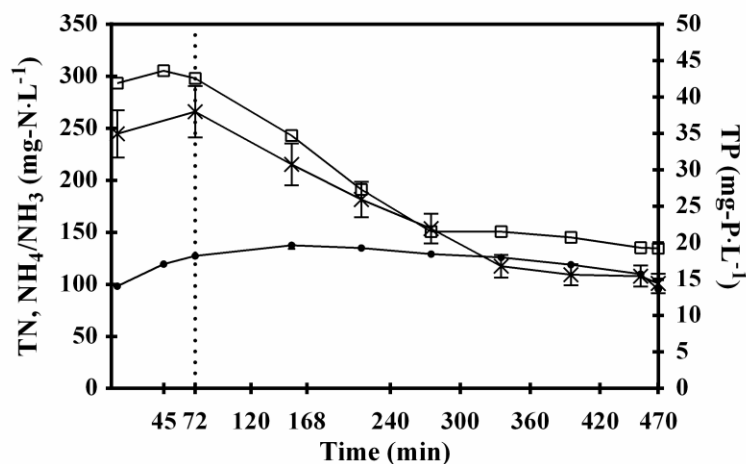
3.4.5 Anaerobic/aerobic phosphorous removal

As EBPR occurs across the anaerobic and aerobic stages, the TP removal results of this study are herein discussed across the combined anaerobic and aerobic stages (Figure 3.4). No phosphorous release is observed in the anaerobic stage, which was expected of PAO metabolism (Gujer et al., 1995). This may be due to the assimilation of phosphorous caused by anaerobic growth, which could be masking the phosphorous release. It should be noted that further microbial and/or molecular analyses are required to confirm PAOs were present in the reactors, both embedded in the biofilm and in the suspended phase.

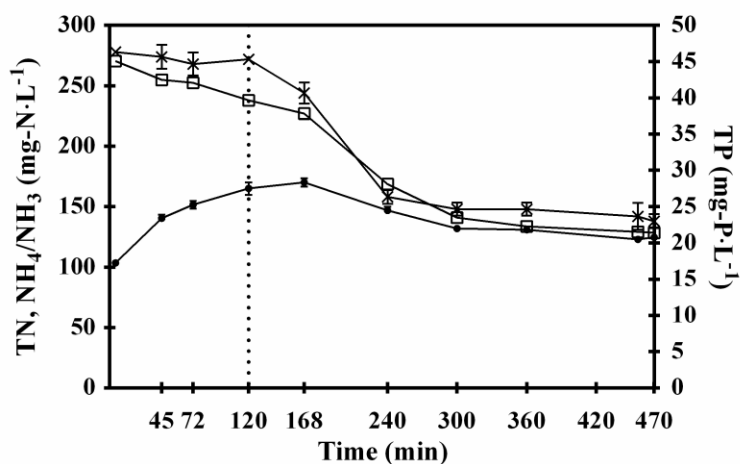
In the subsequent aerobic stage, the SARR was found to be 0.36 ± 0.01 $\text{g-P} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$, 0.40 ± 0.01 $\text{g-P} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$, and 0.54 ± 0.02 $\text{g-P} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ for the reactors operating with anaerobic staging times 72 minutes, 120 minutes, and 168 minutes each with $2.4 \text{ L} \cdot \text{min}^{-1}$ aeration rate; and 0.33 ± 0.01 $\text{g-P} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ for the reactor operating with 120 minutes and $4.8 \text{ L} \cdot \text{min}^{-1}$ aeration rate. These SARR in the current study are lower than previous reports of MBBRs treating municipal-strength wastewater ($0.85 \text{ g-P} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$) (Helness and Ødegaard, 2001), which suggests that the SARR are

lower for industrial wastewaters. On the other hand, the TP removal of the system was found to be $23.4 \pm 1.3 \text{ mg-P} \cdot \text{L}^{-1}$, $20.8 \pm 0.4 \text{ mg-P} \cdot \text{L}^{-1}$, and $21.8 \pm 0.7 \text{ mg-P} \cdot \text{L}^{-1}$ for the reactors operating with anaerobic staging times of 72 minutes, 120 minutes, and 168 minutes (Table 3.3), and $18.3 \pm 0.2 \text{ mg-P} \cdot \text{L}^{-1}$ with 120 minute anaerobic staging and enhanced aeration. These phosphorous removal rates are more than triple compared to previous studies treating municipal-strength wastewater ($4.5 \text{ mg-P} \cdot \text{L}^{-1}$) (Pastorelli et al., 1999), and more than double rates observed with an aerobic MBBRs treating dairy wastewater with a similar carbon loading ($6.3 \text{ mg-P} \cdot \text{L}^{-1}$) while operating at an average of 14°C , which is thought to be a more ideal temperature to select for PAOs (López-Vázquez et al., 2008), compared to room temperatures as used in this study (Andreottola et al., 2002). As such, these enhanced removal rates are indicative of EBPR occurring in the SB-MBBR system treating cheese production wastewater.

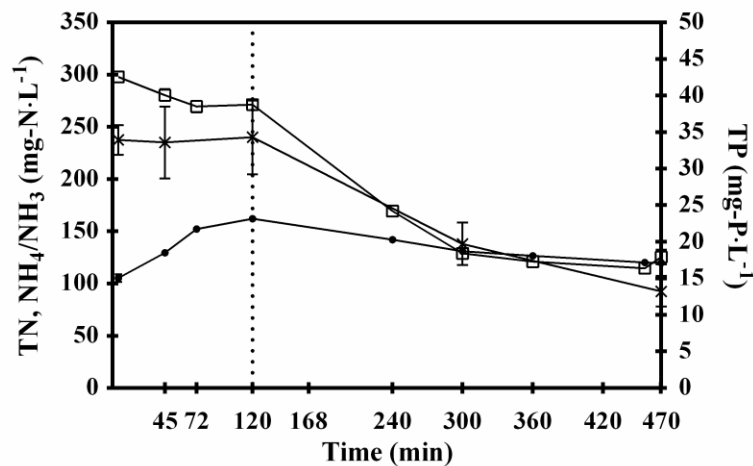
a



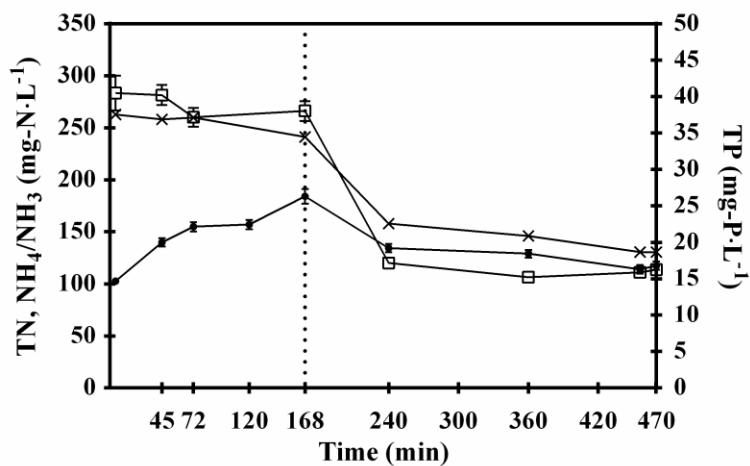
b



c



d



□ TP × TN ● TAN

Figure 3.4 Average and 95% confidence interval of TN, $\text{NH}_3/\text{NH}_4^+$, and TP of one representative profile study following steady-state conditions. (a) Reactor 1, with an anaerobic staging time of 72 minutes and an aeration rate of $2.4 \text{ L} \cdot \text{min}^{-1}$. (b) Reactor 2 operated with an anaerobic staging time of 120 minutes and an aeration rate of $2.4 \text{ L} \cdot \text{min}^{-1}$. (c) Reactor 2 operated with an anaerobic staging time of 120 minutes and an aeration rate of $4.8 \text{ L} \cdot \text{min}^{-1}$. (d) Reactor 3, with an anaerobic staging time of 168 minutes and an aeration rate of $2.4 \text{ L} \cdot \text{min}^{-1}$. The vertical dotted lines indicate the end of the anaerobic phase and the start of the aerobic phase for each reactor and each operational condition.

3.4.6 Aerobic nutrient removal, yields, VSS content and ratios

The aerobic yields were calculated as the production of VSS normalized per sCOD removed in the aerobic phase. When operating with an aeration rate of $4.8 \text{ L} \cdot \text{min}^{-1}$ the aerobic yield was found to be $0.432 \pm 0.007 \text{ g-VSS} \cdot \text{g-sCOD}^{-1}$ (Table 3.3), which is similar to the expected yield for heterotrophic organisms ($0.42 \text{ g-VSS} \cdot \text{g-sCOD}^{-1}$) (McCarty, 1965; McCarty, 1971). However, with the lower aeration rate, the aerobic yields were 0.326 ± 0.021 , 0.339 ± 0.038 , and $0.328 \pm 0.015 \text{ g-VSS} \cdot \text{g-sCOD}^{-1}$ for systems with upfront anaerobic staging times of 72, 120, and 168 minutes, respectively. Although these yields are in range with ratios previously reported ($0.12\text{-}0.56 \text{ kg-TSS} \cdot \text{kg-total COD}^{-1}$) for influent COD concentrations ranging from $500\text{-}8000 \text{ mg} \cdot \text{L}^{-1}$ (Andreottola et al., 2003; Aygun et al., 2008), and with organic loading rates of 2 and 35 $\text{total COD} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ (Orantes and González-Martínez, 2004), these yields are approximately 30% lower than expected for an aerobic heterotrophic populations. Produced and detached biological solids (released cells and EPS, etc.) may hydrolyse in the systems and may have contributed to increased sCOD consumption during the process, which is supported by the fact that the measured VSS yields were slightly lower than conventional values. Hence the consumption of hydrolysed biological solids may have been a pathway for N and P removal (Karizmeh et al., 2014; Forrest et al., 2016). Another possibility is that, given the low DO in the bulk liquid (< 0.2) throughout a portion of the aerobic stage (Figure 3.2), deeper biofilm layers may remain anaerobic during the aerobic stage due to limitations in oxygen diffusion (Bishop et al., 1995), and the sCOD may be consumed by anaerobic bacteria that have lower cell yields (McCarty, 1965). Finally anoxic bacteria may be metabolizing the carbon in the aerobic stage as well, since studies have found anoxic yield values to be approximately $0.36 \text{ g-VSS} \cdot \text{g-sCOD}^{-1}$ (Orhon et al., 1996; Muller et al., 2003), which is similar to the measured yields observed in this study at the lower aeration rate.

The C:N:P ratio removed/consumed was calculated based on the sCOD removed in the aerobic phase (Table 3.3). The C:N:P ratio removed/consumed was found to be 100:53.4:7.9, 100:49.3:7.0, 100:38.4:7.6, and 100:44.8:6.1 for systems that operated with upfront anaerobic staging times of 72, 120, and 168 (with an aeration rate of $2.4 \text{ L} \cdot \text{min}^{-1}$), and 120 minutes (with an aeration rate of $4.8 \text{ L} \cdot \text{min}^{-1}$) respectively. Each condition achieved a greater ratio of N and P removal than would be expected for aerobic heterotrophs, which would be approximately 100:12:0.4; based on the assumption that the carbon source consumed in the aerobic stage was acetate and an assumed ratio of $1.08 \text{ g-COD} \cdot \text{g-acetate}^{-1}$ and an A value of 0.69 (McCarty, 1965; McCarty, 1971). Furthermore, the nitrogen to carbon removal in this study ranged from $0.384 \pm 0.003 \text{ g-N} \cdot \text{g-C}^{-1}$ to $0.533 \pm 0.048 \text{ g-N} \cdot \text{g-C}^{-1}$ (Table 3.3), which was greater compared to what has been previously reported in a single aerobic MBBR (Andreottola et al., 2002), $0.223 \text{ g-N} \cdot \text{g-C}^{-1}$, which was assumed to be due to cellular synthesis of TN alone with a similar organic load. Acetate is assumed to be present in the aerobic stage as the glucose and peptone were likely degraded to acetate in the anaerobic stage. The elevated N and P removal relative to C removal observed in this study may have resulted from N and P constituents being adsorbed to the EPS of the biofilm and hence increasing the overall TN and TP removal (Yang et al., 2017). The C:N ratios and cell-yields are similar to anoxic heterotrophs ($100:53; 0.36 \text{ g-VSS} \cdot \text{g-sCOD}^{-1}$) (McCarty, 1965), therefore the TN may have been removed through anoxic heterotrophs that consume nitrate (a by-product of nitrification) in the place of oxygen. Since we suspect autotrophic nitrification was not likely, heterotrophic nitrification and nitrifier denitrification (Verstraete and Focht, 1977; Castignetti and Hollocher, 1984b, a; Robertson and Kuenen, 1990; Stein, 2011) may be responsible for the enhanced TN removal. Heterotrophic nitrification and nitrifier denitrification have both been observed in wastewater treatment (Schmidt, 1960; Robertson and Kuenen, 1990), and

specifically in MBBRs (Mannina et al., 2017), and have been shown to withstand high organic loading rates, and perform well at high C:N ratios of up to 8 (Li et al., 2015). Furthermore, it is known that heterotrophic nitrifiers simultaneously perform denitrification under aerobic conditions (Stein, 2011), which would explain why intermediates of nitrate and nitrite were not observed. Heterotrophic nitrifiers have also been observed to use both nitrate and oxygen when both were available (Poth, 1986), and when oxygen was limiting the cell yields were reduced while growth rates were maintained (Robertson et al., 1988; Robertson and Kuenen, 1990), which may explain why the cell yields were reduced with low aeration rates (Table 3.3).

Finally, the TN and TP content were estimated as a percentage of the TN and TP removed during the aerobic stage relative to the VSS produced during the aerobic stage (Table 3.3). The TN and TP content were also higher than expected for assimilation through aerobic heterotrophs, which supports the hypothesis that other metabolic processes are involved in the TN removal (i.e. heterotrophic nitrification and denitrification), and TP removal (i.e. EBPR). The TP content of the cells produced in the reactors operated at an aeration rate of $2.4 \text{ L} \cdot \text{min}^{-1}$ was greater than the reactor operated at an aeration rate of $4.8 \text{ L} \cdot \text{min}^{-1}$ ($p = 0.0001$); where the TP content of cells at the elevated aeration rate was similar to theoretical TP content of aerobic heterotrophs (Gujer et al., 1995; Fagerbakke et al., 1996). Since at an aeration rate of $2.4 \text{ L} \cdot \text{min}^{-1}$ a greater TP content was observed (ranging from $4.1 \pm 0.2\%$ to $4.5 \pm 0.2\%$), it is likely that PAO activity was observed. Previous studies on EBPR in conventional activated sludge systems demonstrate cellular phosphorous contents, measured as a ratio of g-TP to g-VSS, that range from 13.5-17.5% (Wentzel et al., 1989; Crocetti et al., 2000), with some studies demonstrating values of approximately 7.6% (Comeau et al., 1996; Broughton et al., 2008). It was speculated that the phosphorous content in Comeau et. al's study was lower compared to other work due to the use of real wastewater, a higher proportion of non-

PAO being present and hence a lowering of the overall phosphorous content of the total cells (Broughton et al., 2008). Similarly, in this study, where the biofilm includes an acidogenesis community and peripheral biofilm communities that contribute to the structure of the biofilm microbiome, the presence of these bacterial community would result in an overall lower TP content of the collected cells. The findings in this study suggest that lower aeration, where the DO reached minimum concentrations of $0.06 \text{ mg}\cdot\text{L}^{-1}$, may be required for EBPR in SB-MBBRs treating cheese production wastewater. The lower DO appears to better select for PAOs over heterotrophic community and/or glycogen accumulating organisms (GAOs) (Oehmen et al., 2007), which may explain the presence of PAOs at the lowers aeration rate. Further work on the microbiome and metabolic processes of the SB-MBBR system is as such required.

Table 3.3 TN and TP removal in the aerobic stage, with respect to VSS production and carbon removal of one representative profile study following steady-state conditions.

Anaerobic (min)	Aerobic Yield (g-VSS·g-sCOD ⁻¹)	Δ TN (mg-N·L ⁻¹)	TN content (%)	Δ TP (mg-P·L ⁻¹)	TP content (%)	C:N:P
72	0.326±0.021	165±15	30.3±2.8	23.4±1.30	4.5±0.2	100: 53.4: 7.9
120	0.339±0.038	148±9	27.0±1.6	20.8±0.4	4.1±0.2	100: 49.3: 7.0
120 ¹	0.432±0.007	134±5.4	19.2±0.7	18.3±0.2	2.3±0.1	100: 44.8: 6.1
168	0.328±0.015	111±1	21.7±0.2	21.8±0.7	4.3±0.2	100: 38.4: 7.6

¹Operated at an aeration rate of $4.8 \text{ L}\cdot\text{min}^{-1}$, all other conditions operated at $2.4 \text{ L}\cdot\text{min}^{-1}$.

3.4.7 Suspended solids production

The bulk-liquid suspended solids measured as VSS were used to determine the suspended solids production during the aerobic stage. The suspended solids production rate during the aerobic stage was found to be $2.48\pm0.30 \text{ g-VSS}\cdot\text{d}^{-1}$, $2.76\pm0.29 \text{ g-VSS}\cdot\text{d}^{-1}$, and $1.95\pm0.05 \text{ g-VSS}\cdot\text{d}^{-1}$ for anaerobic staging times of 72, 120, 168 minutes respectively and $2.4 \text{ L}\cdot\text{min}^{-1}$ aeration rate; and $3.11\pm0.02 \text{ g-VSS}\cdot\text{d}^{-1}$ at a 120 minute anaerobic staging time and an enhanced aeration rate of $4.8 \text{ L}\cdot\text{min}^{-1}$. This value falls in range with previous studies of high-rate MBBRs ($0.35\text{-}12.5 \text{ g-TSS}\cdot\text{d}^{-1}$), where the organic loading rates range from $6\text{-}96 \text{ g-COD}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$ (Aygun et al., 2008). The

suspended solids concentrations at the end of the cycle was $1013 \pm 139 \text{ mg-VSS} \cdot \text{L}^{-1}$, $871 \pm 23 \text{ mg-VSS} \cdot \text{L}^{-1}$, 876 ± 105 for anaerobic staging times of 72, 120, 168 minutes respectively and $2.4 \text{ L} \cdot \text{min}^{-1}$ aeration rate; and $1003 \pm 7 \text{ mg-VSS} \cdot \text{L}^{-1}$ at a 120 minute anaerobic staging time and an enhanced aeration rate of $4.8 \text{ L} \cdot \text{min}^{-1}$. Suspended solids production in MBBRs is thought to be a result of biofilm detachment, and this detachment rate has been shown to increase with organic loading rate (Aygün et al., 2008; Tian and Delatolla, 2019). Furthermore, increasing the SALR from 9 to $64 \text{ g-COD} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ was shown to increase effluent VSS concentrations from 20 ± 2 to $200 \pm 11 \text{ mg} \cdot \text{L}^{-1}$ (Karizmeh et al., 2014). It should finally be noted that considering the VSS in the reactor, it is possible that the PAOs may be residing in the bulk-liquid suspended solids phase, as appose to the biofilm. Further investigation into the biofilm microbiome is required to confirm any PAOs are present in the biofilm, and not in the bulk liquid suspended solids.

3.4.8 Suggested operational conditions

Increasing the anaerobic staging time demonstrated an increase in the $\text{sCOD SARR}_{\text{AN\&AE-47mg/L}}$ following a second order polynomial ($R = 1.0$), while the total TN SARR however decreased with increasing anaerobic staging times between 120 minutes to 168; following a second order polynomial ($R = 1.0$). Clearly there is a trade-off between sCOD SARR and TN SARR with increasing anaerobic staging time, and therefore the optimum operation of the system was calculated by identifying the point where the polynomial curves for both sCOD and TN SARR values across anaerobic staging time converge; identifying the maximum sCOD SARR that can be achieved without significantly decreasing the TN SARR. The optimal anaerobic staging time with respect to both the sCOD and TN SALR of the system is identified as 130 minutes (Figure 3.5). In this case, the $\text{sCOD SARR}_{\text{AN\&AE-47mg/L}}$ is $31.1 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ and the $\text{TN SARR}_{\text{AN\&AE-47mg/L}}$ is $1.3 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$. The aeration rate of $2.4 \text{ L} \cdot \text{min}^{-1}$ demonstrated a greater TP content in the VSS produced

suspended solids and a greater TP removal compared to an aeration rate of $4.8 \text{ L} \cdot \text{min}^{-1}$, with the aeration rate not having a significant effect on the sCOD or TN SARR ($p < 0.1$).

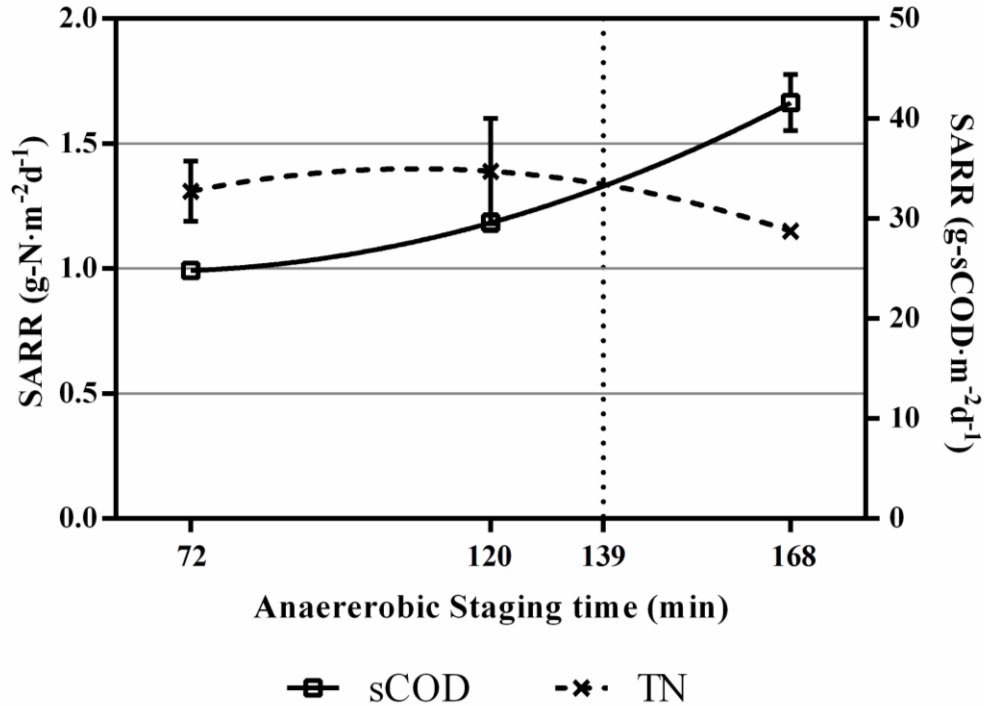


Figure 3.5 Average and 95% confidence interval of TN SARR_{AN&AE}, and sCOD SARR_{AN&AE} of one representative profile study following steady-state conditions.

3.5 Conclusions

This study investigated the potential and performance of the SB-MBBR technology to treat cheese production wastewaters. This research focused on the effects of anaerobic and aerobic staging times and increased aeration rates on carbon, nitrogen, and phosphorous removal. It was concluded that increasing the anaerobic staging time improved the aerobic SARR, to rates greater than previously reported with MBBRs, and TP removal was also found to increase with anaerobic staging times. However, increasing the anaerobic stage decreased the TN removal, identifying a trade-off between carbon removal that must be considered for future application of this technology. An anaerobic stage of 138 minutes is

suggested for the treatment of the wastewater in this study, which results in a sCOD $SARR_{AN\&AE}$ of $31.1 \text{ g}\cdot\text{m}^{-2}\text{d}^{-1}$ and a TN of $SARR_{AN\&AE-47\text{mg/L}}$ of $1.3 \text{ g}\cdot\text{m}^{-2}\text{d}^{-1}$. Contrary to previous reports on attached growth technologies, both lower aeration and longer anaerobic staging time showed improved TP removal, demonstrating that low DO may be required to design future EBPR SB-MBBRs treating high-strength wastewater. Together increasing the anaerobic staging time and decreasing the aeration rate improved the efficiency of carbon, and phosphorous removal, while simultaneously decreasing costly aeration, thereby resulting in a more cost-effective and energy-efficient system. The findings of this study demonstrate the potential of the SB-MBBR for on-site treatment of organic-content and nutrients from cheese production wastewaters and provide guidance for the design and operation of future pilot- and full-scale applications.

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4 Article 2 - Two moving bed biofilm reactors in-series - carbon, nitrogen, and phosphorous removal from high organic wastewaters

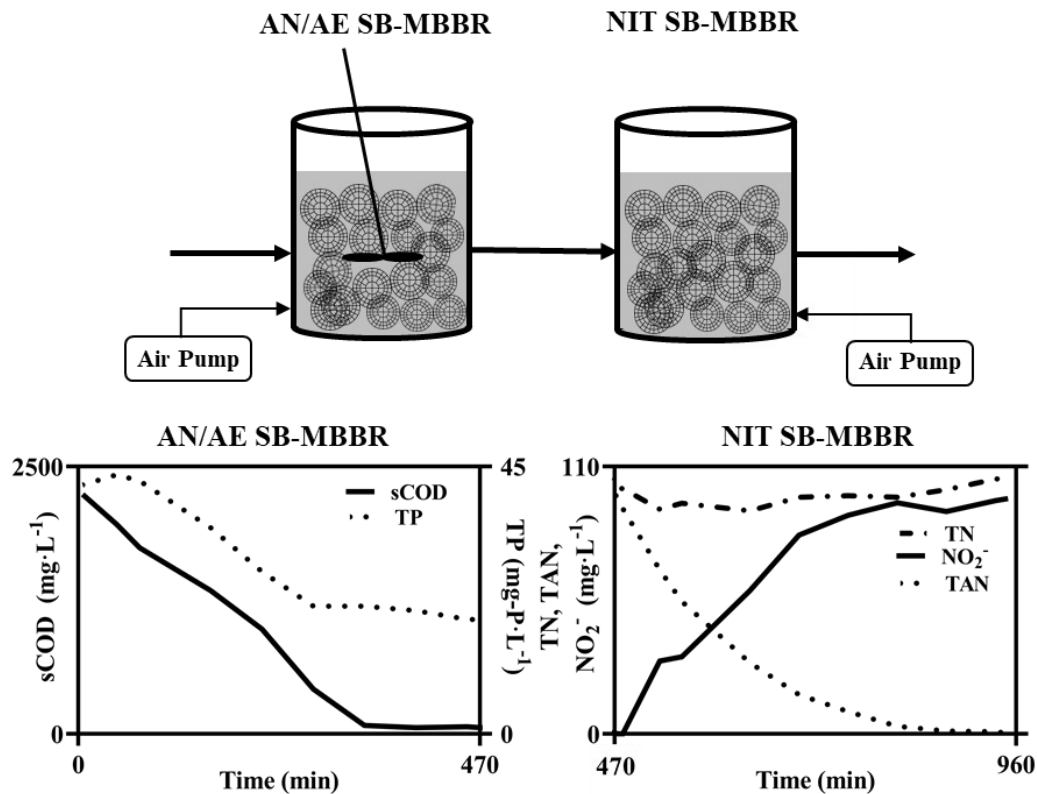


Figure 4.1 Graphical abstract

4.1 Abstract

Government regulations require on-site treatment of industrial wastewater prior to sewage discharge to prevent environmental degradation of receiving waterbodies. The sequencing batch moving bed biofilm reactor (SB-MBBR) can permit on-site treatment of high organic and nutrient content of cheese production wastewater. However, the critical challenge exists of maintaining both an active heterotrophic and a autotrophic population in a single biofilm system when treating carbon and ammonia to low discharge levels from cheese production wastewater. This study

operates two SB-MBBRs in series, thereby separating the heterotrophic and autotrophic communities, circumventing the competition between the heterotrophic and autotrophic populations in a single biofilm system treating wastewater with high carbon to nitrogen ratios. The first reactor (AN/AE reactor) was operated by cycling between anaerobic and aerobic stages to select for polyphosphate accumulating organisms, and the AN/AE reactor degraded carbon and phosphorous. The second reactor (NIT reactor) was operated with aerobic conditions to promote nitrifying bacteria and treat total ammonia nitrogen. The performance of this system for carbonaceous constituents and ammonia was evaluated by quantifying the kinetics, biofilm morphology, thickness, dry-mass, and dry-density of the two SB-MBBR in series technology. The AN/AE reactor achieved $97.5 \pm 1.7\%$, $58.8 \pm 5\%$, and $49 \pm 1\%$ soluble chemical oxygen demand, total nitrogen, and total phosphorous removal efficiencies respectively. The NIT reactor achieved $98.7 \pm 2.4\%$ total ammonia nitrogen removal, though partial nitrification at rates of $1.09 \pm 0.03 \text{ g-N} \cdot \text{m}^2 \cdot \text{d}^{-1}$, indicating this system can be incorporated in an anammox system. The findings of this study demonstrate that enhanced biological phosphorous removal, and potentially anaerobic removal of ammonia can be achieved for on-site treatment of cheese production wastewater.

4.2 Introduction

Dairy processing is considered the largest source of food and beverage wastewater, and is growing annually at a rate of approximately 2.8% (Wang et al., 2005). Cheese production from raw milk produces wastewater volumes up to 4 times that of the milk production product (Prazeres et al., 2012; Carvalho et al., 2013; Tatoulis et al., 2015); with the wastewater being derived from cleaning, disinfection, heating and cooling operations. Organic carbon is present in cheese production wastewaters as easily degradable carbohydrates: lactose, proteins and lipids (Fang and Yu, 2000), resulting in chemical oxygen demand (COD) concentrations typically ranging from

0.8–102 g·L⁻¹ (Carvalho et al., 2013). Nitrogen is derived from milk proteins, amino acids and urea, and exists in cheese production wastewaters in ionic forms such as total ammonia nitrogen (TAN), NO₂⁻ and NO₃⁻, with the total nitrogen (TN) concentrations typically ranging from 0.01-1.7 g·L⁻¹ (Carvalho et al., 2013). Finally, total phosphorous (TP) is derived from detergents and cleaners, and exists as ortho-phosphorous, poly-phosphate, and organic phosphorous; the TP concentrations typically range from 0.006-0.5 g·L⁻¹ (Carvalho et al., 2013).

Discharging untreated cheese production wastewater to natural receiving waters begets excessive oxygen depletion and eutrophication due to the high organic and nutrient content of the wastewater respectively (Hutchinson, 1973; Boesch et al., 2006). Therefore, governing agencies are implementing increasingly stringent regulations for discharge from municipal water resource recovery facilities (WRRFs) (Gazette, 2012; Román-Sánchez et al., 2015; Hendriks and Langeveld, 2017). Furthermore, the cheese production industry contributes a significant portion of the organic and nutrient load to municipal WRRFs. Thus, municipalities are also limiting industrial effluents discharged to sewer collectors through financial and regulatory sewer discharge bylaws, requiring the on-site treatment of cheese production wastewater (Rusten et al., 1996; Tatoulis et al., 2015; Van Lier et al., 2015). Biological treatment is more economical than the chemical treatment of organics and nutrients in wastewaters (Sathasivan, 2009), whereby nitrogen is treated traditionally through nitrification and denitrification, and phosphorous through enhanced biological phosphorous removal (EBPR) (Oehmen et al., 2007). However, conventional suspended growth treatment of industrial wastewaters produce excessive sludge and exhibit fluctuating effluent qualities (Sirianuntapiboon et al., 2005). Thus, there is an urgent need for novel attached growth, cost effective, and small footprint treatment technologies to meet effluent targets of on-site industrial wastewater systems.

The moving bed biofilm reactor (MBBR) is an attached-growth wastewater treatment system, whereby biofilms are maintained on bio-carriers. These carriers are kept in constant motion, permitting efficient mass transfer of both substrate and dissolved oxygen (DO), minimizing the risk of clogging within the media, thereby eliminating the need for backwashing. The specific design of the many carriers permits a high surface area and structure while protecting the biofilm from abrasion. Thus, the MBBR maintains significant biofilm, characterized by a high-concentrated microbial consortia within the system (Ødegaard, 1999; Rusten et al., 2006). The MBBR technology has been successfully applied to treat high-strength carbonaceous wastewaters with a limited land footprint, and may be appropriate for the on-site treatment of cheese production wastewaters (Rusten et al., 1996; Andreottola et al., 2002; Tatoulis et al., 2015). The MBBR has been operated under anaerobic conditions for the treatment of wastewater from the food and beverage industries (Chai et al., 2014; di Biase et al., 2018). However, anaerobic MBBR treatment alone does not remove nutrients, and often complete organic carbon removal is also not achieved with anaerobic MBBR treatment. Thus, aerobic MBBRs have been applied following anaerobic treatment (Luostarinen et al., 2006), or with anaerobic and aerobic MBBRs operated in series to treat nitrogen, and residual carbon (Chen et al., 2008) (Table 4.1). Nitrogen removal from cheese production wastewater has been achieved with aerobic operation of the MBBR technology through assimilation pathways (Andreottola et al., 2002). Phosphorous removal in continuous flow MBBR systems has been limited to removal via chemical addition and subsequent precipitation (Rusten et al., 1996). MBBR nitrification has been achieved treating dairy parlor wastewater and cheese production wastewater following anaerobic treatment in continuous MBBR units (Luostarinen et al., 2006; Zkeri et al., 2020). Successful EBPR has been mainly studied in MBBRs treating municipal-strength wastewater (Helness and Ødegaard, 1999; Pastorelli et al., 1999; Helness and

Ødegaard, 2001; Humbert et al., 2018; Rudi et al., 2019; Torresi et al., 2019; Nair et al., 2020; Zhang et al., 2020), and has only recently been studied and achieved in MBBRs treating dairy parlour wastewater (Luostarinen et al., 2006) and cheese production wastewater (Tsitouras et al., 2020).

Table 4.1 Review of MBBR treating carbon, nitrogen, and phosphorous from industrial wastewater.

Reactor Type	Wastewater Type	Loading rate	Retention time	Carbon removal	Nitrogen removal	Phosphorous removal	Reference
Anaerobic MBBR	Winery	29.59 g-COD·L ⁻¹ d ⁻¹	1.55 d	80% (COD)	-	-	(Chai et al., 2014)
Anaerobic MBBR	Brewery	4.0 kg-sCOD·m ⁻³ d ⁻¹	2 d	88%	-	-	(di Biase et al., 2018)
Aerobic MBBR (post treatment)	Dairy parlor	0.023-0.093 kg-COD·m ³ d ⁻¹	2.2 d	40-70%	50-60% (TN)	74% (TP)	(Luostarinen et al., 2006)
Aerobic MBBR	Cheese production	5 kg-COD·m ³ d ⁻¹	11-30 hours	90% (COD)	13.2-96% (TN)	6.3 mg-P·L ⁻¹	(Andreottola et al., 2002)
Aerobic MBBR	Chemical processing	53 g-BOD·m ² d ⁻¹	1.9-3.8 h	60-80% (rbBOD)	-	-	(Rusten et al., 1999)
Aerobic MBBR	Cheese production	1.8-3.6 g-COD·L ⁻¹ d ⁻¹	8 h	80-100%	-	-	(Santos et al., 2020)
Aerobic MBBR	Synthetic	0.5-9 kg-COD·m ³ d ⁻¹	1 d	81-96%	-	-	(Rezaee et al., 2012)
Anaerobic & Aerobic MBBRs in series	Cheese production	2.45 kg-COD ³ ·d ⁻¹	24 h (Anaerobic) 28.8 h (Aerobic)	93% (COD)	97% (NH ₄ -N)	49% (TP)	(Zkeri et al., 2020)
Anaerobic & Aerobic MBBRs in series	Landfill	4.08-15.70 kg-COD·m ³ d ⁻¹	1.25 d	92-94% (COD)	97% (NH ₄ -N)	-	(Chen et al., 2008)

MBBRs may be operated as sequencing batch reactors, termed the sequencing batch MBBR (SB-MBBR) technology. The SB-MBBR is an attractive technology for cheese production wastewaters as the cycles of the system can be modified in real-time response to commonly observed variations in the cheese production wastewater characteristics and flow rates (Andreottola et al., 2002). Furthermore, anaerobic and aerobic conditions can be achieved in a

single SB-MBBR basin, permitting nutrient removal (Kolev Slavov, 2017). Still, challenges in achieving nitrification and EBPR in MBBR units for cheese production wastewater treatment are expected due to competition between heterotrophs, nitrifiers and poly-phosphate accumulating organisms (PAOs) for oxygen and space within the biofilm. The elevated organic carbon loading (FdZ-Polanco et al., 2000), and the high C:N ratios of cheese production wastewaters (Carvalho et al., 2013) may limit the proliferation and maintenance of nitrifiers and PAOs in SB-MBBR cheese production wastewater treatment systems. Nitrification has not been studied with the SB-MBBR technology treating cheese production wastewater specifically, and EBPR has only recently been studied and achieved for cheese production wastewater with the SB-MBBR (Tsitouras et al., 2020). Furthermore, the biofilm morphology has yet to be characterized and assessed for the SB-MBBR technology. In addition, the biofilm morphology of MBBR systems treating TAN from cheese production wastewater has also not been assessed. Knowledge of the system's biofilm morphology can give insight to the performance of the SB-MBBR system due to its effect on the mass transfer rate to the embedded cells and hence the global kinetics of the system. This knowledge can therefore potentially advance the understanding of the SB-MBBR technology for future pilot- and full-scale SB-MBBR installations.

The aim of this study is to provide cheese production industry with a compact biological treatment system to achieve on-site treatment of carbon, nitrogen, and phosphorous. Furthermore, this study aims to present a SB-MBBR treatment system that circumvents heterotrophic and autotrophic competition to achieve TAN removal along with carbon and phosphorous removal from cheese production wastewater. Therefore, this study presents the kinetics of a two SB-MBBR in series system treating high carbonaceous cheese production wastewater, whereby the first reactor cycles between anaerobic and aerobic conditions, and the second reactor is solely aerobic,

separating the heterotrophic aerobes and autotrophic nitrifiers. The study also characterizes the biofilm thickness, dry-mass, dry-density, and morphology of the two SB-MBBR in series system providing new and fundamental information of the biofilm in the SB-MBBR system when treating cheese production wastewater.

4.3 Materials Methods

4.3.1 Experimental set-up

Two SB-MBBR units in series were operated throughout the study, each with a 2.3 L operating volume, and filled to 47% for the AN/AE reactor and 33% for the NIT reactor with K5 AnoxKTM carriers (AnoxKaldnes, Lund, Sweden). The carriers are made of polyethylene plastic, each carrier has a total volume of 3.84 cm³, and a surface area of 800 m²·m⁻³. The system was operated over a 16-hour cycle, with each of the SB-MBBR units operating with an 8-hour hydraulic retention time (Figure 4.1). The first reactor, the anaerobic/aerobic (AN/AE) reactor, was operated to achieve both carbon and phosphorous removal. The AN/AE reactor cycled between anaerobic and aerobic conditions to achieve EBPR, and the second reactor, the nitrifying (NIT) reactor, was operated under aerobic conditions to achieve TAN oxidation. The cycle was initiated with a peristaltic pump filling 2 L of synthetic wastewater from a feed reservoir into the AN/AE reactor; followed by a 72 minute anaerobic stage and a 379 minute aerobic stage. The highest SARR of sCOD was observed with a 168-minute anaerobic staging time (Tsitouras et al., 2020), however for this study a 72 minute anaerobic staging time was chosen to limit the effects of the anaerobic conditions on the nitrifiers, considering the KO₂ of nitrifiers (0.5) (Wett et al., 2011). Following the aerobic stage, the air pumps were turned off, and mechanical mixers were turned on for 14 minutes during the anoxic stage prior to decanting, then the mechanical mixers were shut off. The anoxic stage operated as did the anaerobic stage, where this stage was included in the cycle to achieve

denitrification, if anoxic conditions would have developed in the reactors, in the case of nitrate formation. The entire volume of the AN/AE reactor was then decanted, filling the NIT reactor, which was then aerated until the NIT reactor was itself decanted. The NIT reactor was decanted with a peristaltic pump, signifying the end of the system's cycle (Table 4.2).

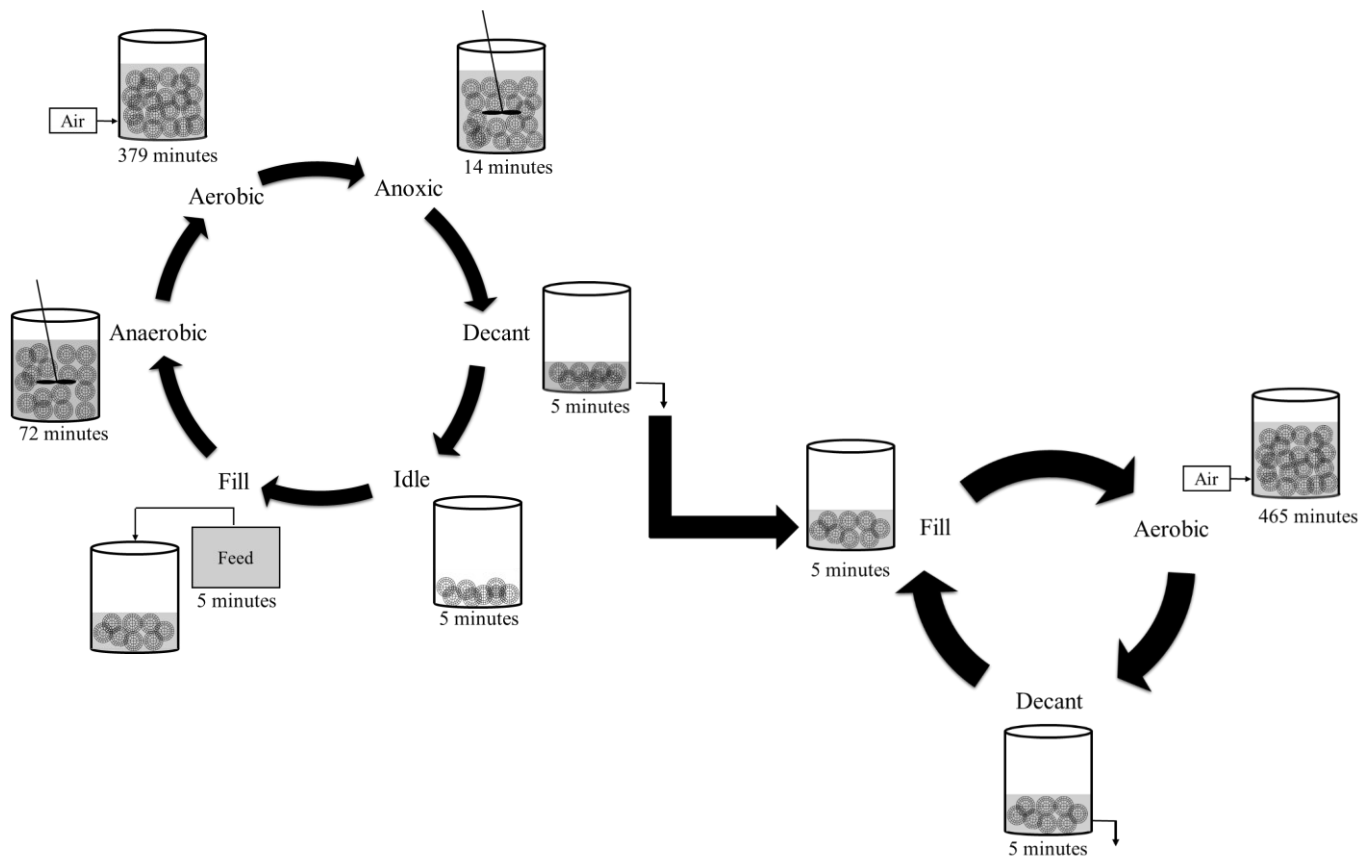


Figure 4.2 Stages of the AN/AE and NIT SB-MBBR reactors.

Table 4.2 Reactor staging times of the AN/AE and NIT reactors.

Stage	AN/AE Reactor (min)	NIT Reactor (min)
Fill	5	5
Anaerobic	72	-
Aerobic	379	465
Anoxic	14	-
Decant	5	5
Idle	5	5

Mixing in the AN/AE reactor was achieved with mechanical mixers during the anaerobic stage. Mixing and DO transfer to the bulk liquid was achieved via aeration at $2.4 \text{ L} \cdot \text{min}^{-1}$ through diffusers positioned at the bottom of the reactors during the aerobic stage of the AN/AE reactor and the entire cycle of the NIT reactor. The aeration of this study was maintained at $2.4 \text{ L} \cdot \text{min}^{-1}$ as previous reports demonstrated that increasing the aeration to $4.8 \text{ L} \cdot \text{min}^{-1}$, maintaining the DO concentration above $2 \text{ mg} \cdot \text{L}^{-1}$, did not improve the carbon removal, and impaired the phosphorous removal (Tsitouras et al., 2020). The two reactors were operated at room temperature, with an average temperature of $22.2 \pm 0.2^\circ\text{C}$.

4.3.2 Reactor inoculation

The carriers for each of the two SB-MBBR reactors were harvested from the operational Hawkesbury integrated fixed film activated sludge Water Resource Recovery Facilities in Ontario, Canada, where secondary treatment is achieved with limited nitrification. The integrated fixed-film activated sludge (IFAS) system operates with a COD and TN loading of $3200 \text{ g-COD} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$ and $220 \text{ g-N} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$ respectively. The average influent concentrations of sCOD, TAN of the IFAS treatment system are $230 \pm 2 \text{ mg-COD} \cdot \text{L}^{-1}$, and $23.9 \pm 0.6 \text{ mg-N} \cdot \text{L}^{-1}$ respectively, and the average influent temperature of the system was $9.0 \pm 0.1^\circ\text{C}$ when the carriers were harvested. 16S rRNA gene sequencing of these carriers demonstrated a low percent abundance of nitrifying bacteria, and demonstrated that the microbiome is dominated by aerobic heterotrophs (Young et al., 2017a).

4.3.3 System start-up

The AN/AE reactor was fed with the synthetic wastewater simulating St. Albert Cheese production wastewater for the start-up and the entirety of the experiment. The AN/AE reactor was operated for 12 months before the NIT reactor was added in series, at which point the AN/AE reactor was operating under steady-state conditions and hence was feeding the NIT reactor with an

effluent/feed of steady concentrations. Both reactors were operated for an additional month before the NIT reactor reached steady-state conditions. During this time, the influent and effluent soluble COD (sCOD), TAN, NO_3^- , and NO_2^- were analyzed across six consecutive cycles across a period of 14 days for each reactor to determine steady state. Once steady state was determined in both reactors, the two reactors in series were operated for an additional two weeks prior to the profile studies, which were subsequently conducted over the entire 16-hour cycle, and repeated two times. For this study, steady state was defined as a variance in sCOD, TAN, NO_3^- , and NO_2^- removal rates within $\pm 10\%$.

4.3.4 Wastewater source

The composition of the synthetic wastewater fed to the AN/AE reactor was as follows: NH_4Cl : $89 \text{ mg}\cdot\text{L}^{-1}$, KH_2PO_4 : $69 \text{ mg}\cdot\text{L}^{-1}$, NaHCO_3 : $304 \text{ mg}\cdot\text{L}^{-1}$, $\text{MgCl}_2\cdot\text{H}_2\text{O}$: $58 \text{ mg}\cdot\text{L}^{-1}$, $\text{CaCl}_2\cdot\text{H}_2\text{O}$: $29 \text{ mg}\cdot\text{L}^{-1}$, and FeCl_3 : $5 \text{ mg}\cdot\text{L}^{-1}$ (Young et al., 2017a); dextrose: $510 \text{ mg}\cdot\text{L}^{-1}$, sodium acetate: $340 \text{ mg}\cdot\text{L}^{-1}$, and peptone: $510 \text{ mg}\cdot\text{L}^{-1}$. The synthetic wastewater simulated the organic and nutrient contents of the wastewater from the St. Albert cheese production Cheese Factory, located in Ontario, Canada (Table 4.3). The carbon sources of the synthetic wastewater were selected to simulate the readily and non-readily degradable carbon characteristic of the St. Albert cheese production wastewater; where the St. Albert cheese production wastewater was comprised of an average sCOD concentration of $2850 \text{ mg}\cdot\text{L}^{-1}$, TN concentration of $125 \text{ mg}\cdot\text{L}^{-1}$ and TP concentration of $41 \text{ mg}\cdot\text{L}^{-1}$. The sCOD concentration of the synthetic wastewater was $2500 \text{ mg}\cdot\text{L}^{-1}$, the biological oxygen demand to COD ratio of the synthetic wastewater was 0.7, with approximately $86 \text{ mg}\cdot\text{L}^{-1}$ of the sCOD being slowly-biodegradable or recalcitrant. The TN, TAN, and soluble TP concentrations were $250 \text{ mg}\cdot\text{N}\cdot\text{L}^{-1}$, $100 \text{ mg}\cdot\text{N}\cdot\text{L}^{-1}$, and $40 \text{ mg}\cdot\text{P}\cdot\text{L}^{-1}$, respectively. Note that the TN of the synthetic wastewater was augmented compared to the St. Albert cheese production wastewater to represent

higher concentrations of nitrogen that are observed in other cheese production wastewaters and in turn to verify that the SB-MBBR technology is capable of treating these higher TN concentrations. The TAN concentration is also augmented compared to the St. Albert cheese production wastewater, as the TAN concentrations also represent the NO_3^- and NO_2^- concentrations.

4.3.5 Constituent analyses

Wastewater-constituent-profile testing was performed on the two SB-MBBR units in series (AN/AE and NIT) for the following parameters: sCOD, TN, TAN, NO_3^- , and NO_2^- , and soluble TP. Bulk-liquid samples were collected 19 times throughout the 16-hour cycle, including samples taken from the influent and effluent of the two reactors (AN/AE and NIT), and following the anaerobic, and aerobic stages in the AN/AE reactor. Following collections, the samples were filtered to test the sCOD, TAN, N- NO_2^- , N- NO_3^- , TN, and soluble TP. Triplicate bulk-liquid samples were collected three times throughout the cycle, and the sCOD, TAN, N- NO_2^- , N- NO_3^- concentrations were measured for each replicate bulk-liquid sample. The errors from the triplicate samples were averaged and applied to the other measurements to indicate sampling, analytical and human error. Triplicate solids samples were also collected at the end of the anaerobic, aerobic stages, and from the effluent of the AN/AE and NIT reactors to measure the total suspended solids (TSS) and volatile suspended solids (VSS) concentrations. Finally, DO and pH samples were acquired at 5-minute intervals throughout the 16-hour cycle.

HACH methods 8000-sCOD, 10072-TN, 10205-TAN, 835- NO_3^- , and 1012-TP were used for sCOD, TN, TAN, NO_3^- , and soluble TP concentrations in the bulk liquid, respectively. Standard method 4500B- NO_2^- was used to test NO_2^- -N concentrations in the bulk liquid [30]. For HACH method 835- NO_3^- , 50 mg of sulfamic acid was added to the 10 ml sample before testing, to prevent inaccuracies due to nitrite concentrations in the bulk liquid. Standard methods 2450 D-total

suspended solids (TSS dried at 103-105°C) and E-volatile suspended solids (VSS ignited at 550°C) were used to test the solids. DO and pH measurements were acquired with an attached DO probe and pH probe (HACH, USA, Colorado).

4.3.6 Biofilm dry-mass

To measure the biofilm dry-mass, the protocol adopted from Delatolla et al. (Delatolla et al., 2009) was employed. In brief, five carriers were harvested from the AN/AE SB-MBBR and NIT SB-MBBR. The carriers were incubated overnight at 105°C, then weighed. Next, the carriers were cleaned with warm water and a stiff-bristled brush to remove the biofilm. The carriers were dried overnight and weighed again. The difference between the two weights was defined as the biofilm dry-mass. For each condition, 5 carriers were harvested and tested for dry-mass for each reactor for a total of 5 biofilm dry-mass measurements per reactor.

4.3.7 Biofilm morphology and thickness

Images were acquired using a stereoscope (Zeiss Stemi 305) to analyze the biofilm morphology and thickness. Four carriers were harvested from the AN/AE and NIT reactors; five images were taken at random locations, at 2× magnification from the 4 replicate samples from each reactor. The images were analyzed using the software Digimizer V.4.6.1 (Ostend, Belgium), where measurements were recorded from the edge of the carrier to the edge of the biofilm. 200 measurements were acquired per image, from 5 images per carrier, and total of 4 carriers per reactor; thus 1000 thickness measurements were acquired per reactor (APHA, 2005). Images presented were converted from RGB to a grey-scale, and the brightness and contrast were adjusted using the FIJI software V.2.0.0 (Wisconsin, USA) (Figure 4.4).

4.3.8 Statistical methods

The statistical significance of differences in kinetics analysis, biofilm dry-mass, thickness, and dry-density data sets was calculated using the t-test, whereby p -value < 0.05 was considered significant.

4.4 Results and discussion

4.4.1 Carbon and phosphorous profiles

The sCOD was monitored in the two reactors in series (AN/AE and NIT) across the 16-hour cycle (Figure 4.2). The kinetics of the AN/AE reactor are presented as surface area loading rate (SALR) and surface area removal rate (SARR), as is conventional for MBBR technologies (Table 4.3). The surface area of each carrier is $800 \text{ m}^2 \cdot \text{m}^{-3}$, the percent fill was 47% (meaning 47% of the operational volume of the reactors were filled with carriers) in the AN/AE reactor and 33% in the NIT reactor, and thus the total surface area was 640 m^2 in the AN/AE reactor and 528 m^2 in the NIT reactor. The loading rates were $8160 \text{ g-sCOD} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$, $748 \text{ g-N} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$, and $179 \text{ g-P} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$ for the total system. The sCOD loading of the AN/AE reactor is applied at an SALR of $25.5 \pm 0.5 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ with the sCOD being removed at an SARR of $24.8 \pm 0.5 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$, corresponding to a removal efficiency of $97.3 \pm 1.8\%$ (Table 4.3). A final sCOD concentration of $57.0 \pm 2.3 \text{ mg} \cdot \text{L}^{-1}$ is reached following 355 minutes of the cycle in the AN/AE reactor, with the remaining sCOD in the reactor likely being recalcitrant. The sCOD increases in the NIT reactor to a final concentration of $147 \pm 6 \text{ mg} \cdot \text{L}^{-1}$ (Figure 4.2), likely due to lysed cells from the detached biofilm from the AN/AE that is fed to the NIT reactor. The system herein showed favourable removal of carbon compared to anaerobic MBBRs treating food and beverage wastewater with lower organic loading rates, where retention times of over 24 hours were required to achieve 80% removal (Chai et al., 2014; di Biase et al., 2018). The removal of carbon also compared favourably to aerobic MBBRs where retention

times of 1-2.2 days was required to treat dairy wastewaters with organic loading rates ranging from 2.45-15.7 kg-COD·m⁻²·d⁻¹ (Luostarinen et al., 2006; Rezaee et al., 2012; Santos et al., 2020) (Table 4.1). Thus the anaerobic and aerobic cycling in a single SB-MBBR appears to improve carbon removal rates compared to solely anaerobic or aerobic operations of the MBBR.

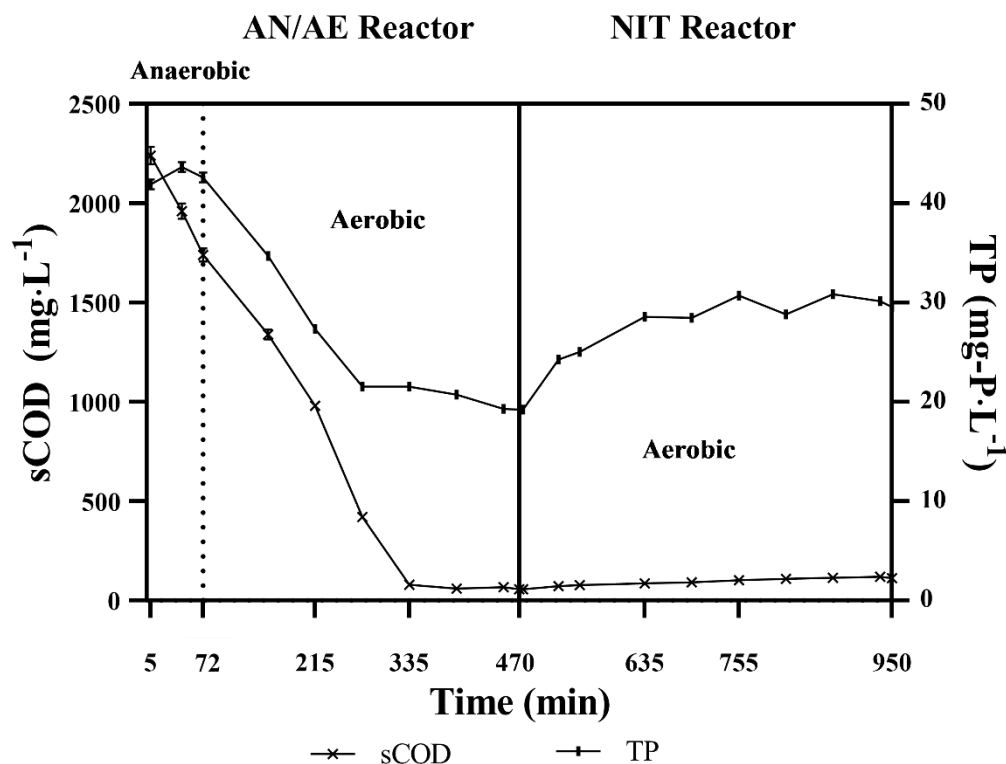


Figure 4.3 Average and 95% confidence interval of sCOD, and TP concentrations across the 16-hour cycle for the AN/AE reactor and the NIT reactors of one representative profile study following steady-state conditions. The vertical dotted line indicates the transition from the anaerobic to the aerobic stage in the AN/AE reactor. The vertical solid line indicates the transition from the AN/AE reactor to the NIT reactor. Error bars are small, and hence not evident for all points in the graph.

Table 4.3 Average and 95% confidence interval of the SALR, SARR, and removal efficiency for the AN/AE reactor, and for the total 16-hour cycle. The SARR in the AN/AE reactor are calculated to when the constituent degradation is complete, while the SARR in the NIT reactor is calculated to when the TAN degradation is complete.

Reactor	Constituent	Time (min)	SALR ($\text{g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$)	SARR ($\text{g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$)	Removal Efficiency (%)
AN/AE	sCOD ¹	323 ¹	25.5±0.5	24.8±0.5	97.3±1.8
AN/AE	TP	270 ¹	0.56±0.01	0.27±0.01	49±1

¹Calculated to when the removal of the constituent is complete.

TP was also monitored in the two reactors in series (AN/AE and NIT) across the 16-hour cycle (Figure 4.2). The kinetics of the AN/AE reactor are again presented as SALR and SARR (Table 4.3). TP is loaded in the AN/AE reactor at an SALR of $0.56\pm0.01 \text{ g}\cdot\text{P}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$, and removed at an SARR of $0.27\pm0.01 \text{ g}\cdot\text{P}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$, corresponding to a removal efficiency of $49\pm1\%$ (Table 4.3). The TP reaches a final concentration of $19.2\pm0.4 \text{ mg}\cdot\text{P}\cdot\text{L}^{-1}$ in the AN/AE reactor, and then increases to a final concentration of $29.5\pm0.6 \text{ mg}\cdot\text{P}\cdot\text{L}^{-1}$ in the NIT reactor (Figure 4.2). Similar net phosphorous removals were reported in previous studies performing EBPR for dairy wastewater treatment with suspended growth technology, however with up-stream fermentation and hydraulic retention times ranging from 12 hours to 2.5 days (Comeau et al., 1996; Rezaee et al., 2012; Gil-Pulido et al., 2018). With cheese production wastewater, TP has been treated with aerobic MBBRs through cellular assimilation, with only $6.3 \text{ mg}\cdot\text{P}\cdot\text{L}^{-1}$ and $10 \text{ mg}\cdot\text{P}\cdot\text{L}^{-1}$ being treated (Andreottola et al., 2002; Zkeri et al., 2020) (Table 4.1). Although slight EBPR has been detected in intermittently aerated MBBRs, with sequencing batch operation (Luostarinen et al., 2006). Thus the anaerobic and aerobic cycling, along with the sequencing batch operation appears to permit EBPR, and improves the overall TP removal compared to aerobic operation of MBBRs.

A release of TP is observed in this study at a concentration of $1.7 \pm 0.04 \text{ mg-P} \cdot \text{L}^{-1}$ in the anaerobic stage of the AN/AE reactor, which is statistically significant compared to the influent concentration ($p = 0.01$) (Figure 4.2). This release of TP is lower than previous reports where phosphorous release up to $30 \text{ mg-PO}_4^{3-} \cdot \text{L}^{-1}$ with SB-MBBRs treating municipal wastewater (Pastorelli et al., 1999; Helness and Ødegaard, 2001; Humbert et al., 2018; Torresi et al., 2019). Possibly, the lower phosphorous release in this study may be explained by assimilation of phosphorous in the anaerobic stage masking the phosphorous release; or there may be PAOs present that perform fermentation, and may not release phosphorous (Bickers et al., 2003). Another possibility is that the PAOs in the SB-MBBR AN/AE reactor exist in the suspended growth phase and wash out into the NIT reactor at the end of the AN/AE cycle, limiting the release of phosphorous from PAOs, and in turn limiting the overall PAO activity in the AN/AE reactor. This is supported by the increase in TP concentrations in the NIT reactor, which may be a result of the PAOs transported from the AN/AE reactor to the NIT reactor and then lysed in the NIT reactor, causing a release of TP and increase in concentration. Although there is likely an assimilation of TP in the NIT reactor likely due to growth of nitrifiers, the autotrophic nitrifying population have an approximate yield of $0.12 \text{ g-VSS} \cdot \text{g-NH}_4^{-1}$ (Metcalf&Eddy, 2014) with an estimated phosphorous assimilation of 2% as VSS (Gujer et al., 1995; Fagerbakke et al., 1996). As such, the removal of TP through assimilation by the nitrifiers would represent approximately $0.2 \text{ mg-P} \cdot \text{L}^{-1}$, which is negligible compared to the observed TP release by the suspended growth PAOs. **3.2**

Nitrogen removal

The TN, TAN, $\text{NO}_3^- \text{-N}$, and $\text{NO}_2^- \text{-N}$ concentrations were monitored across the 16-hour cycle of the two reactors in series (AN/AE and NIT), and the kinetics were calculated as SALR and SARR (Figure 4.3, Table 4.4). The TN is loaded in the AN/AE reactor at an SALR of $2.34 \pm 0.20 \text{ g-N} \cdot \text{m}^{-3}$

$^2\text{d}^{-1}$, and removed at an SARR of $1.38 \pm 0.12 \text{ g-N} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$, with a removal efficiency of $58.8 \pm 5.0\%$ (Table 4.4). The TN reaches a final concentration of $101 \pm 9 \text{ mg-N} \cdot \text{L}^{-1}$ in the AN/AE reactor, and the change in TN concentration is not statically significant in the NIT reactor, with a final concentration of $105 \pm 9 \text{ mg} \cdot \text{L}^{-1}$ being observed (Figure 4.3) (Verstraete and Focht, 1977; Castignetti and Hollocher, 1984; Robertson and Kuenen, 1990; Stein, 2011; Tsitouras et al., 2020). The TN removal in the AN/AE reactor of this study is higher than previous work that shows lower TN removal rates of $0.47 \text{ g-N} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ for similar inlet TN concentrations with a horizontal-flow biofilm reactor (Marques et al., 2017). The TN was removed in the AN/AE reactor at a carbon, calculated from sCOD measurements, to TN ratio of 100:53, which is equivalent to the carbon to nitrogen ratio observed for anoxic heterotrophic growth (Metcalf&Eddy, 2014). Considering that there was no source of NO_3^- or NO_2^- in the synthetic wastewater, and concentrations of NO_3^- and NO_2^- remain below $1.2 \text{ mg-N} \cdot \text{L}^{-1}$ in the AN/AE reactor, the TN is likely removed through heterotrophic nitrification. Heterotrophic nitrifiers simultaneously perform nitrification and denitrification (nitrifier denitrification) under aerobic conditions (Verstraete and Focht, 1977; Castignetti and Hollocher, 1984; Robertson and Kuenen, 1990; Stein, 2011; Tsitouras et al., 2020), without accumulation of NO_2^- and NO_3^- in the bulk liquid. Heterotrophic nitrifiers have been observed in MBBRs (Mannina et al., 2017), and are known to withstand high concentrations of carbon, and high C:N ratios (Li et al., 2015). Furthermore, the TN removal ceases after 335 minutes (Figure 4.3), which is the same timepoint when sCOD removal ceases (Figure 4.2), thus the TN removal is likely associated with heterotrophic growth and not autotrophic growth.

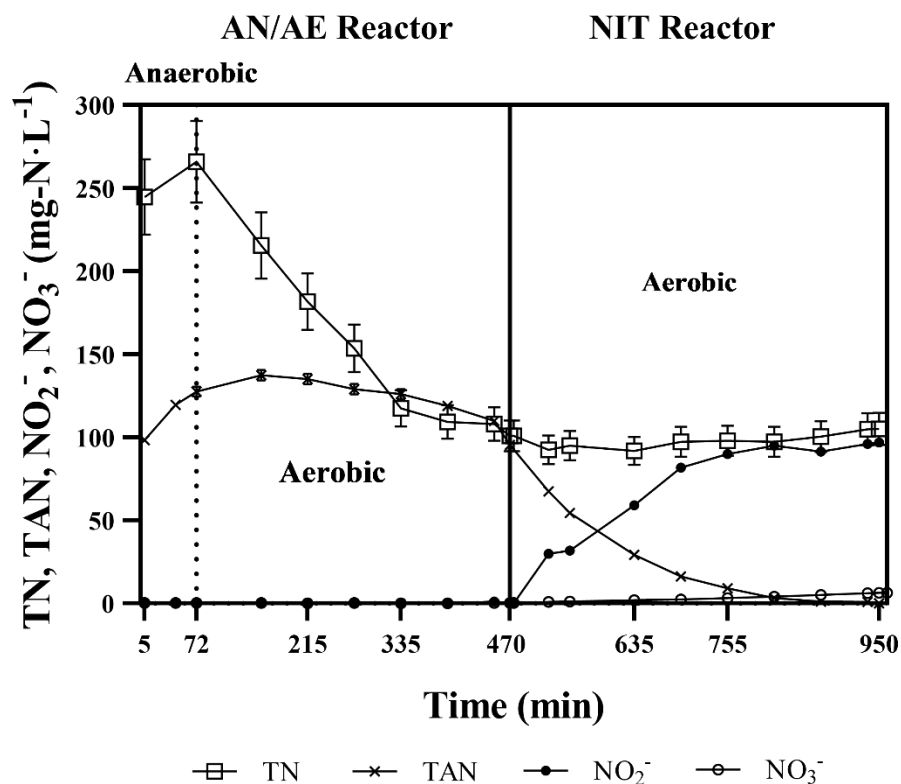


Figure 4.4 Average and 95% confidence interval of TN, TAN, nitrate, and nitrite concentrations across the 16-hour cycle for the AN/AE reactor and the NIT reactors of one representative profile study following steady-state conditions. The vertical dotted line indicates the transition from the anaerobic to the aerobic stage in the AN/AE reactor. The vertical solid line indicates the transition from the AN/AE reactor to the NIT reactor. The vertical solid line indicates the transition from the AN/AE reactor to the NIT reactor. Error bars are small, and hence not evident for all points in the graph.

Table 4.4 Average and 95% confidence interval of the SALR, SARR, and removal efficiency for nitrogen. Dominant pathway of TN removal in the AN/AE reactor is through heterotrophic growth and dominant pathway of TAN removal in the NIT reactor is partial nitrification.

System	Constituent	Time (min)	SALR (g·m ⁻² ·d ⁻¹)	SARR (g·m ⁻² ·d ⁻¹)	Removal Efficiency (%)
AN/AE ¹	TN	335 ¹	2.34±0.20	1.38±0.12	58.8±5.0
NIT ¹	TAN	405 ¹	1.09±0.03	1.07±0.05	98.7±2.4
Total ²	TN	875	1.26±0.11	0.718±0.061	57.1±4.8
Total ²	TAN	875	0.506±0.014	0.500±0.014	98.7±2.7

¹Calculated to when the removal of the constituent is complete.

²Calculated to when the TAN removal is complete.

The TAN concentration increases in the anaerobic stage of the AN/AE reactor from 98.3±2.4 mg-N·L⁻¹ to 138±33 mg-N·L⁻¹, likely due to ammonification (Tsitouras et al., 2020), and then decreases to 95.5±2.2 mg-N·L⁻¹ at the end of the aerobic stage (Figure 4.3). The TAN is then loaded in the NIT reactor at an SALR of 1.09±0.05 g-N·m⁻²·d⁻¹, and removed at an SARR of 1.07±0.05 g-N·m⁻²·d⁻¹, corresponding to a removal efficiency of 98.7±2.4%, where a final concentration of 0.23±0.01 mg-N·L⁻¹ exiting the NIT reactor is reached (Table 4.4). With the addition of a second reactor (NIT reactor), thereby separating the heterotrophs and autotrophs, TAN oxidation is achieved in the NIT reactor. These findings support that the autotrophic nitrifying populations are limited in the AN/AE reactor, due to competition with heterotrophs given the high C:N ratio in the AN/AE reactor (Torkaman et al., 2015; Yadu et al., 2018). Following removal of sCOD, after 335 minutes, the TAN decreased by 16.0±0.4 mg-N·L⁻¹, thus a small autotrophic nitrifying population may be present in the AN/AE reactor, however the population or activity of these bacteria are not sufficient to achieve significant TAN removal, and thus the second reactor in series is necessary to establish sufficient nitrifying activity. The TAN removal rates in this study compare favourably to TAN removal rates for treatment of dairy

wastewater with constructed wetlands ($0.83 \text{ g-NH}_4\text{-N}\cdot\text{m}^{-2}\text{d}^{-1}$) (Geary and Moore, 1999). In aerobic MBBRs treating industrial wastewater, TN is removed through cellular assimilation, with removal efficiencies ranging from 13-96% (Andreottola et al., 2002; Luostarinen et al., 2006) (Table 4.1), however the TN removal is dependent on organic loading rate and C:N ratio, since cellular assimilation of nitrogen is only removed at a C:N ratio of 12:1 (Metcalf&Eddy, 2014). Nitrification has been achieved in anaerobic and aerobic MBBRs operated in-series, with TAN removal efficiencies of 97%, however with retention times of over 1 day (Chen et al., 2008; Zkeri et al., 2020). Thus the configuration herein, with the upstream MBBR operating with anaerobic and aerobic cycling permits similar TAN removal to previous studies, however with lower retention times.

NO_2^- -N concentrations increase in the NIT reactor from $0 \text{ mg-N}\cdot\text{L}^{-1}$ to $96.8\pm0.1 \text{ mg-N}\cdot\text{L}^{-1}$ throughout the cycle, while NO_3^- -N concentrations remained low at concentrations less than $6.30 \text{ mg-N}\cdot\text{L}^{-1}$ (Figure 4.3). Therefore, following the 16-hour cycle the TN exists mainly as NO_2^- -N, which can be treated through downstream denitrification or can be recirculated back to the AN/AE reactor. The average pH and DO concentration in the NIT reactor is 7.6 ± 0.2 and $8.4\pm0.2 \text{ mg-O}_2\cdot\text{L}^{-1}$ respectively. The pH in the NIT reactor is in range that is required to select for ammonia oxidizing bacteria (AOB) over nitrite oxidizing bacteria (NOB) (Villaverde et al., 1997). Although the DO is not limited in the system, the accumulation of NO_2^- -N in the system is likely due to elevated TAN concentrations that suppresses nitrite oxidation (Schopf et al., 2019; Rong et al., 2020). High influent TAN concentrations is known to suppress NOB in biofilms, by creating competition for DO between the AOB and NOB populations within the biofilm, and partial nitrification has been achieved in MBBRs with high TAN loading (Rong et al., 2020). Therefore this configuration can be incorporated as a partial nitrification system, which permits downstream anaerobic ammonia

oxidation, reducing energy requirements compared to nitrification denitrification processes (Deng et al., 2020; Dimitrova et al., 2020). Microbiome analysis of the biofilm is required to confirm the suppression of the nitrite oxidizing bacteria.

4.4.3 VSS, TN and TP concentration change

The change in influent and effluent VSS, TN, and TP concentrations, the solids, nitrogen and phosphorous mass balances, were calculated and analyzed for the aerobic stage of the AN/AE reactor and the NIT reactor; and subsequently used to calculate the TN and TP content of the VSS (Table 4.5). The VSS increases in concentration by $544 \pm 35 \text{ mg-VSS} \cdot \text{L}^{-1}$ in the AN/AE reactor, while the TN and TP concentrations decrease in the AN/AE reactor by $165 \pm 15 \text{ mg-N} \cdot \text{L}^{-1}$, and $23 \pm 1 \text{ mg-P} \cdot \text{L}^{-1}$, respectively. The decrease in VSS and increase in TN and TP concentrations in the NIT reactor is estimated to be a result of cells from the AN/AE reactor that were fed into the NIT reactor and lyse during the 8 hours of exposure to aeration in the NIT reactor. Although exposed to approximately 2.25 hours of aeration in the AN/AE reactor at low sCOD removal and lower sCOD concentrations, the significantly longer exposure to aeration in the NIT reactor demonstrates likely PAO lysing. The following discussion of the TN content, and TP content in the NIT reactor refers to the microbial activity occurring in suspension in the AN/AE reactor that is fed to the NIT reactor.

Table 4.5 Changes in VSS, TN and TP concentrations in the aerobic stage of the AN/AE reactor and across the complete cycle of the NIT reactor. Change in TN and TP relative to the change in the VSS also presented. Brackets designate a decrease in values. Samples were acquired at the beginning (72 min) and end of the aerobic stage (456 min) in the AN/AE reactor. Samples were acquired at the beginning (470 min) and end of the nitrification cycle (945 min) for the NIT reactor.

Reactor	ΔVSS (mg-VSS·L⁻¹)	ΔTN (mg-N·L⁻¹)	ΔTP (mg-P·L⁻¹)	TN content (g-N·g-VSS⁻¹)	TP content (g-P·g-VSS⁻¹)
AN/AE	544±35	(165±15)	(23±1)	0.303±0.028	0.045±0.002
NIT	(66.7±3.3)	4.16±0.36	10±0.2	0.062±0.005	0.150±0.003

The decrease in TN and TP concentrations relative to the increase in VSS concentration in the aerobic stage of the AN/AE reactor is 0.303±0.028 g-N·g-VSS⁻¹ and 0.045±0.002 g-P·g-VSS⁻¹, respectively. The increase in TN and TP concentrations relative to the decrease in VSS concentration in the NIT reactor is 0.062±0.005 g-N·g-VSS⁻¹ and 0.150±0.003 g-P·g-VSS⁻¹, respectively (Table 4.5).

For the AN/AE reactor, the TP content is greater than would be expected for heterotrophic bacteria (0.02 g-P·g-VSS⁻¹) (Gujer et al., 1995; Fagerbakke et al., 1996), and considering the TP content of ordinary heterotrophs, 11±0.5 mg-P·L⁻¹ is likely removed by cellular assimilation of TP, and 12±0.5 mg-P·L⁻¹ is removed through EBPR. A previous study of a continuous aerobic MBBR treating dairy wastewater with the influent organic concentrations similar to this study achieved approximately 10±0.4 mg-P·L⁻¹ through heterotrophic assimilation. Thus, the anaerobic and aerobic cycling in the AN/AE reactor of this study demonstrates EBPR, effectively doubling the TP removal compared to an aerobic continuous MBBR system (Zkeri et al., 2020). Considering that 7-11 g of rb-COD is required to remove 1 g of TP (Metcalf & Eddy, 2014), only 85.4 g-rbCOD

was likely up taken and stored by PAOs. Considering 500 ± 9 g-sCOD is removed during the anaerobic stage, 415 ± 8 g-sCOD was likely removed through anaerobic degradation.

The TP concentration increases by 10 ± 0.4 mg·L⁻¹ in the NIT reactor, which is similar to the concentration of TP affiliated to PAO uptake in the AN/AE reactor. This supports the inference that the increase in TP in the NIT reactor is a result of the cell lysis of PAOs from the AN/AE reactor. Therefore, PAO cells are likely present and active in the AN/AE reactor, in suspension. Although there is a net growth in the NIT reactor, likely through nitrifiers, the nitrifier yield is only 0.12 g-VSS·g-NH₄⁻¹ (Metcalf & Eddy, 2014), and TP typically represents 2% of cells represented as VSS (Gujer et al., 1995; Fagerbakke et al., 1996), thus the removal of TP through assimilation from nitrifiers would only be 0.2 mg-P·L⁻¹. Microbiome analysis is required to confirm the presence of PAOs in the suspended biomass of the AN/AE reactor. Finally, given the lysis of cells and the corresponding increase in sCOD, TN and TP in the NIT reactor, for subsequent pilot and full-scale application a solids removal in between the AN/AE and NIT reactor is suggested to remove the cells entering the NIT reactor. Since the sludge from the AN/AE reactor is rich in phosphorous, the configuration herein presents the possibility for phosphorous recovery, providing a source of this non-renewable resource, which can be utilized as fertilizers (AR, 2009; Yuan et al., 2012; Nielsen et al., 2019).

4.4.4 Biofilm characteristics

The biofilm morphology was analyzed for the AN/AE and NIT reactor. The biofilm appears to have a rougher surface, as defined by the biofilm surface showing more undulations and heterogeneity, in the AN/AE reactor compared to the NIT reactor (Figures 4.4 a-d.). Differences in the biofilm morphology are likely due to differences in the microbiome communities. Both the higher carbon loading, and the presence of the anaerobic stage in the AN/AE reactor compared to

the NIT reactor likely selects for differing microbial communities in the two reactors, since operational conditions are known to affect the composition of the microbiome community (Cydzik-Kwiatkowska and Zielińska, 2016). Furthermore, the rougher biofilm surface may also be a result of the lower shear force applied to the AN/AE reactor, since the NIT reactor was aerated throughout the entire cycle, and increased shear force is linked to a smoother biofilm morphology (Van Loosdrecht et al., 1995). Although the reactors were mixed during the anaerobic stage, the mixing was limited to prevent DO entrainment to the bulk liquid. Therefore the shear force was lower during the anaerobic stage compared to the aerobic stage.

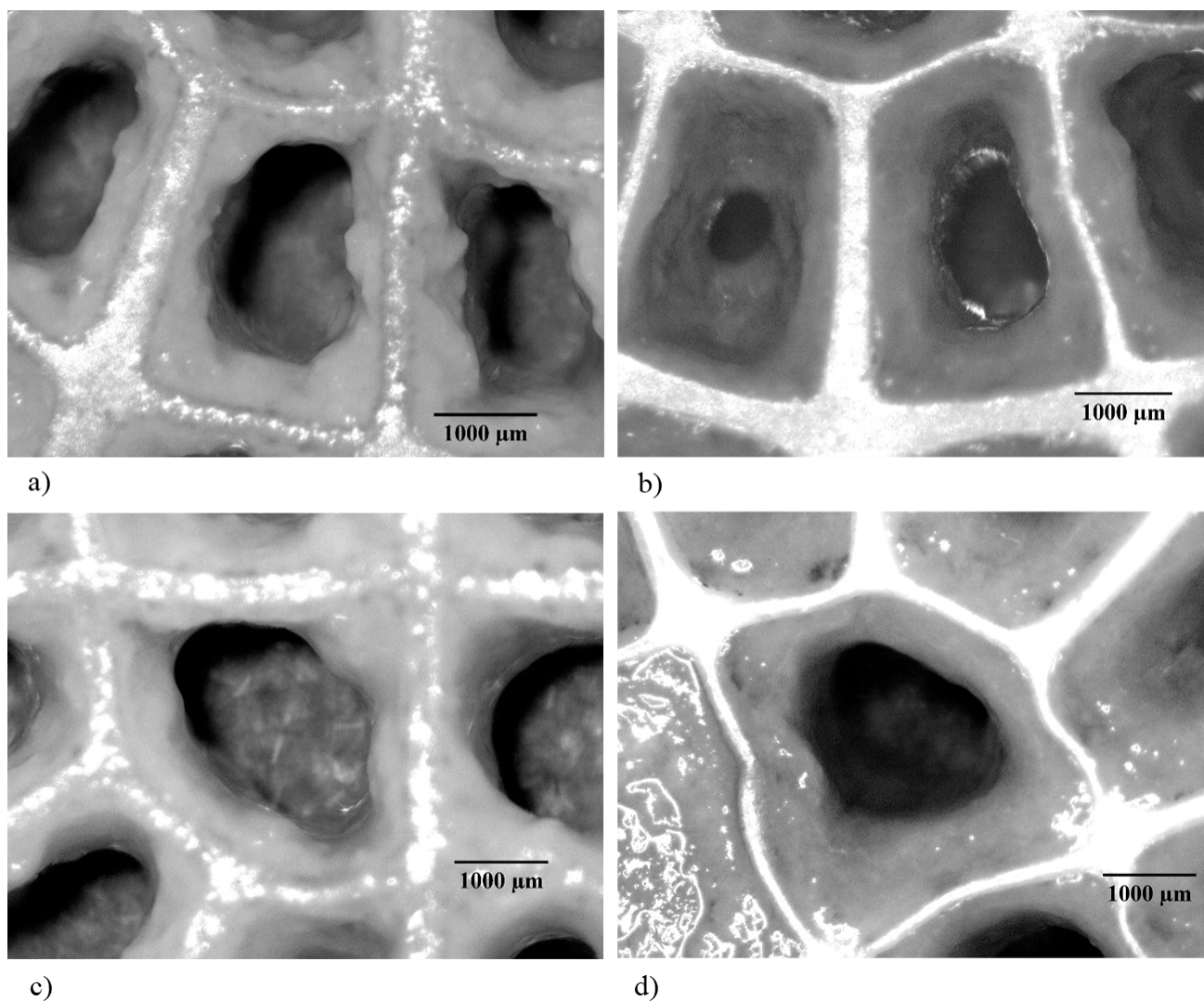


Figure 4.5 Stereoscope images, with a $\times 2$ magnification, of biofilm attached to two carriers a), c) from the AN/AE reactor, and two carriers b), d) from the NIT reactor.

The biofilm characteristics were analyzed by measuring biofilm thickness, dry-mass, and dry-density. The biofilm dry-density is calculated based on the biofilm dry-mass and the biofilm thickness measurements of this study; therefore a total of 5 biofilm dry-density measurements were calculated per reactor. The biofilm thickness is $338 \pm 17 \mu\text{m}$ and $432 \pm 38 \mu\text{m}$ in the AN/AE reactor and the NIT reactor respectively; the biofilm dry-mass is $75.6 \pm 7.5 \text{ mg} \cdot \text{carrier}^{-1}$ and $61.4 \pm 8.0 \text{ mg} \cdot \text{carrier}^{-1}$ in the AN/AE reactor and NIT reactor, respectively. The biofilm dry-density is

$72.7 \pm 7.2 \text{ kg} \cdot \text{m}^{-3}$ and $46.3 \pm 6.1 \text{ kg} \cdot \text{m}^{-3}$ in the AN/AE and the NIT reactor respectively (Figure 4.5). The biofilm thickness in both reactors happened to be similar to that of the biofilm of the MBBR operated at the Hawkesbury IFAS treatment system ($400 \text{ } \mu\text{m}$), where the carriers in this study were harvested (Young et al., 2017a). The biofilm dry-density of the carriers operated at the IFAS system was $45 \text{ kg} \cdot \text{m}^{-3}$, which is similar to that of the NIT reactor. However, the biofilm dry-density of $72.7 \pm 7.2 \text{ kg} \cdot \text{m}^{-3}$ in the AN/AE reactor is greater, with the operation of the AN/AE reactor and the high organic and nitrogen concentrations selecting for a greater biofilm dry-density. Finally, the biofilm dry-mass is greater in both the AN/AE and NIT reactor compared to the biofilm dry-mass of the carriers at the Hawkesbury IFAS system, indicating that operation of both reactors resulted in acclimatization of the biofilm to specific reactor conditions. For both the AN/AE and the NIT reactor the biofilms dry-mass and thickness were greater than previous reports for MBBRs performing nitrification (Rezaee et al., 2012; Hoang et al., 2014; Young et al., 2017b), and in the upper range for MBBRs performing partial nitritation (Schopf et al., 2019).

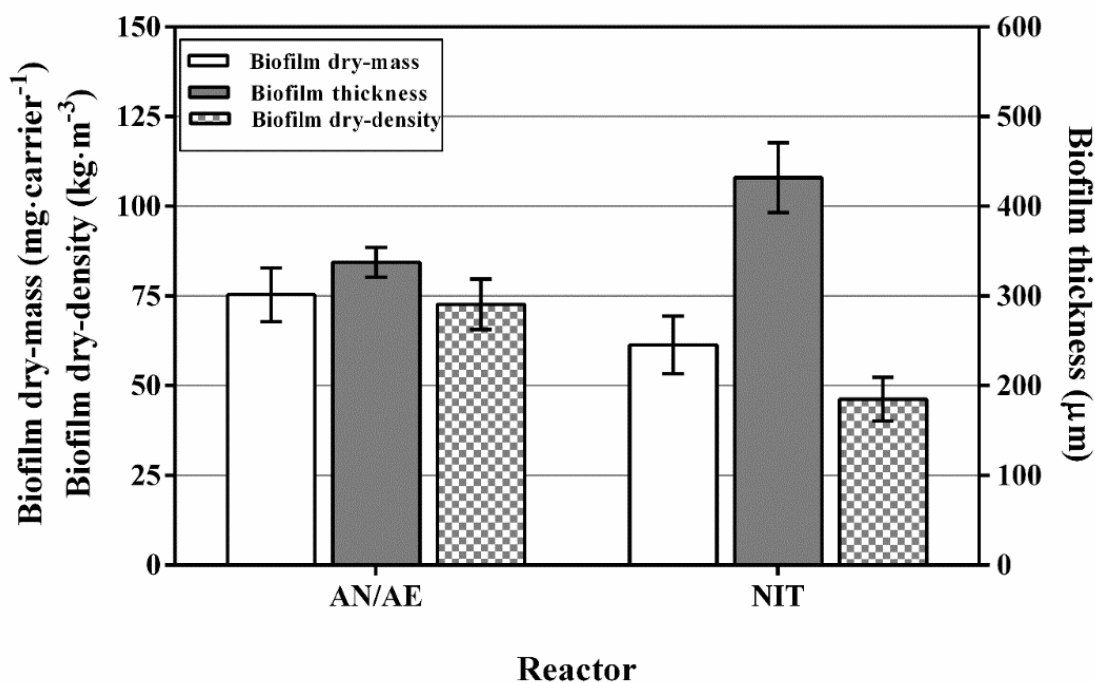


Figure 4.6 Average and 95% confidence interval of the biofilm dry-mass, biofilm thickness, and biofilm dry-density.

4.4.5 Implications of the study

The study herein presents a compact treatment system for the on-site treatment of carbon, nitrogen, and phosphorous from cheese production wastewater. The findings of this research demonstrate the feasibility to achieve EBPR for the treatment of cheese production wastewater. The anaerobic and aerobic operation permit the selection of PAOs, and allow to double the removal of TP compared to a continuously aerated MBBR treating wastewaters at similar organic loadings. This implies that less chemical treatment of phosphorous would be required to treat TP from cheese production wastewaters, therefore providing a potentially more cost effective solution. It is noted that approximately half the TP is removed through EBPR, while half of the TP is removed through cellular assimilation by heterotrophs, and therefore there is potential for optimization and increased TP removal of the system. Finally, the observation that NOBs are suppressed in the system

indicates that the SB-MBBR system presented in this research has the potential to be implemented in a deammonification system if combined with an ANAMMOX system.

4.5 Conclusion

The two SB-MBBR reactors operated in series achieves sCOD removal at an SARR of $24.8 \pm 0.5 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$, and TAN oxidation at an SARR of $1.07 \pm 0.05 \text{ g-N} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ with a $98.7 \pm 2.4\%$ removal efficiency, concurrent with nitrite buildup. With the degradation of sCOD in the first reactor, the AOB communities thrive in the NIT reactor where partial nitrification occurs likely due to the elevated TAN loading of the reactor, effectively suppressing the NOB population. TP is removed, in part through EBPR, with an SARR of $0.56 \pm 0.01 \text{ g-P} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$, is shown within the herein configured MBBR system, with evidence of PAOs existing in the system, and likely in suspension in the AN/AE reactor. Piloting of the SB-MBBR is required to determine the footprint requirement for the full-scale SB-MBBR, and this work is being planned at this time. This study presents a small land footprint, energy efficient configured technology applied to the treatment of elevated carbonaceous wastewaters to achieve complete removal of organics, partial nitrification and EBPR.

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5 Article 3 – Biofilm morphology and microbiome of sequencing batch moving bed biofilm reactors treating cheese production wastewater

5.1 Abstract

With increasing government regulations, food and beverage industries are required to treat their wastewaters on-site, creating a demand for compact and efficient treatment technologies. This study involved the microbial analyses of a single sequencing batch moving bed biofilm reactor (SB-MBBR) system treating cheese production wastewater, operating with anaerobic and aerobic stages. Specifically, this research studied the effects of anaerobic staging times and aeration rates on the biofilm morphology, dry-mass, thickness, dry-density, embedded biomass viability, and the microbiome community. Increasing anaerobic staging time improved organic carbon removal rates to $41.6 \pm 1.3 \text{ g} \cdot \text{m}^{-2} \text{ d}^{-1}$, which was observed with a rougher biofilm surface, and a lower dry-density, and therefore a biofilm with enhanced mass transport. Furthermore, the microbiome community contains a significant relative abundance of taxa capable of denitrification and possibly enhanced biological phosphorous removal (EBPR), *Brachymonas*, *Dechloromonas*, and *Rhodoferax*, and a lower abundance of fermenting bacteria with increasing anaerobic staging time, and with lower aeration. This study herein is the first to provide evidence of possible polyphosphate accumulating organisms (PAOs) in a biofilm of the SB-MBBR treating cheese production wastewater. Thus, this study demonstrates the feasibility of the SB-MBBR for the on-site biological treatment of carbon nitrogen and phosphorous from cheese production wastewater.

5.2 Background

The production of cheese from raw milk generates wastewater volumes up to 4 times that of the milk product (Prazeres et al., 2012; Carvalho et al., 2013; Tatoulis et al., 2015). The resultant wastewater typically contains high concentrations of chemical oxygen demand (COD) (0.8–102

$\text{g}\cdot\text{L}^{-1}$), total nitrogen (TN) ($0.01\text{-}1.7\text{ g}\cdot\text{L}^{-1}$), and total phosphorous (TP) ($0.006\text{-}0.5\text{ g}\cdot\text{L}^{-1}$) (Carvalho et al., 2013), with volumes and characteristics varying largely among production facilities (Kolev Slavov, 2017). Due to the high organic and nutrient content, discharging cheese production wastewater induces excessive oxygen depletion and eutrophication in receiving water bodies (Hutchinson, 1973; Boesch et al., 2006). As such, to prevent environmental degradation of receiving waters, many governing agencies are implementing increasingly stringent discharge regulations, thereby requiring enhanced treatment through municipal wastewater resource recovery facilities (WRRFs) (Gazette, 2012; Román-Sánchez et al., 2015; Hendriks and Langeveld, 2017). However, sewage discharge of cheese production wastewaters can overload municipal WRRFs (Wang et al., 2005; Kolev Slavov, 2017), therefore, municipalities are also limiting cheese production wastewater concentrations discharged to sewer collectors through financial and regulatory industrial discharge bylaws. Hence, there is a demand for on-site treatment of cheese production wastewaters with compact treatment technologies (Rusten et al., 1996; Tatoulis et al., 2015; Van Lier et al., 2015).

Cheese production wastewater is commonly treated through chemical, physical, or biological processes, with biological processes often being the more economical treatment for organic and nutrient removal (Vidal et al., 2000; Sathasivan, 2009). Biological treatment of cheese production wastewater has been achieved via anaerobic, or aerobic processes, or a combination of the two. Examples of anaerobic treatment technologies for cheese production wastewater include: anaerobic digestion (Plan et al., 1995), upflow anaerobic film (Strydom et al., 1995), and membrane reactor systems (Li and Corrado, 1985). Aerobic treatment technologies include: conventional activated sludge (CAS) (Donkin and Russell, 1997; Lateef et al., 2013), sequencing batch reactors (Torrijos et al., 2001; Li and Zhang, 2002; Asadi et al., 2012), membrane bioreactors

(Fraga et al., 2017), and the moving bed biofilm reactor (MBBR) (Rusten et al., 1996; Andreottola et al., 2002; Tatoulis et al., 2015). Anaerobic degradation of cheese production wastewater is limited, since complete organic degradation is often not achieved, and nutrient removal is not possible via anaerobic treatment. On the other hand, aerobic treatment requires larger land foot prints, high energy inputs and elevated operational intensity compared to anaerobic treatment, and results in excessive sludge production again compared to anaerobic treatment (Keller et al., 1997; Sirianuntapiboon and Tondee, 2000). To meet regulatory requirements, within the space limitation for on-site treatment of cheese production wastewater, there is an urgent need for compact treatment technologies, such as the MBBR.

The MBBR is an attached-growth wastewater treatment technology, whereby biofilms grow attached to high density polyethylene carriers (Ødegaard et al., 1994). When operated under sequencing batch mode, referred to as the SB-MBBR, this technology can maintain anaerobic and aerobic conditions in a single reactor, permitting the proliferation of PAOs (Kolev Slavov, 2017). The SB-MBBR has been shown to treat the elevated organic loading requirements of cheese production wastewater (Rusten et al., 1996; Andreottola et al., 2002; Tatoulis et al., 2015), and is therefore a feasible technology for treating cheese production wastewater. Combined carbon and nitrogen treatment have also been achieved with SB-MBBRs, whereby nitrogen was removed with SB-MBBRs following anaerobic treatment of carbon (Andreottola et al., 2002).

Studying the biofilm morphology and microbiome communities can allow for improvements in the design and operation of the SB-MBBR technology, by identifying operational conditions that enhance the survival and performance of key microorganisms in SB-MBBR systems. The operational parameters influence the formation and kinetics of the complex microbial community structures (Cyzdik-Kwiatkowska and Zielińska, 2016). Specifically, wastewater

characteristics and system operation, such as substrate type, pH, hydraulic retention time (HRT), substrate loading rate (Mohan et al., 2010), and aeration rate (Yuan et al., 2020) have been known to affect the microbiome community of biofilms, and thus the kinetics and removal efficiency of the SB-MBBR technology. Furthermore, identifying the presence of certain functional groups through sequencing technologies can support the feasibility of biological nutrient removal with the SB-MBBR technology.

Early investigations of wastewater microbial communities utilized culture-techniques; however, the inability to culture wastewater microbes limited these studies. With the discovery of polymerase chain reaction (PCR) technology, key microorganisms in wastewater treatment have been identified through sequencing the most abundant organisms within wastewater microbial samples. Specifically, microbiome analyses have been conducted for anaerobic treatment of dairy wastewater with denaturing gradient gel electrophoresis (DGGE) (Liu et al., 2002; Mohan et al., 2010; Cammarota et al., 2013). Dominant bacterial phyla reported in anaerobic MBBR systems treating cheese production wastewaters are *Synergistetes*, *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*, which are also known to colonize already formed aerobic MBBR biofilms over time (Liu et al., 2010). To date, cheese production wastewater microbiome studies have been limited to DGGE, while high-throughput technologies have yet to be implemented to study cheese production wastewater treatment microbiomes. Furthermore, the microbiome of SB-MBBRs treating industrial wastewater has yet to be investigated, and to date, EBPR microbiota has only been studied in suspended growth treatment systems (Coats et al., 2017; Yang et al., 2017; Gil-Pulido et al., 2018).

This research will investigate an anaerobic and aerobic staged single SB-MBBR system, and will specifically study the effects of anaerobic staging duration and aeration rates on the

biofilm morphology, dry-mass, thickness, and dry-density, as well as the biofilm embedded cell viability and microbiome community. Investigation of the microbiome will focus on the fermenters, heterotrophs, and PAO communities. Finally, this study aims to use the biofilm microbial data to explain the improved soluble chemical oxygen demand (sCOD) surface area removal rate (SARR) observed in a previous study with increased anaerobic staging times (Tsitouras et al., 2020).

5.3 Materials and methods

5.3.1 Experimental set-up

The research was conducted in three laboratory-scale SB-MBBR systems with a 2.3 L operating volume. The SB-MBBRs were filled to a 47% fill with K5 AnoxKTM carriers (AnoxKaldnes, Lund, Sweden), which have a protected surface area of 800 m²·m⁻³. The cycle was initiated with a fill stage, where each SB-MBBR was filled with 2 L of synthetic wastewater over a 5 minute period using a peristaltic pump drawing the influent from a feed reservoir. Next was an anaerobic stage, and an aerobic stage. The air pumps were shut off 14 minutes prior to decanting, termed the anoxic stage, to permit denitrification in the case of nitrate formation. Mixing was provided by mechanical mixers during the anaerobic stage, while both mixing and aeration were provided through air pumps and air diffusers from the bottom of the reactors during the aerobic stage. The final stage was the decant stage, where the entire bulk liquid volume from each reactor was decanted with a peristaltic pump over a 5 minute period (Figure 5.1). To test the effects of anaerobic staging times, the three SB-MBBRs were operated in parallel with varying anaerobic staging times: 72 minutes (reactor 1), 120 minutes (reactor 2), and 168 minutes (reactor 3). The anaerobic stage was followed by aerobic staging times of 384 minutes (reactor 1), 331 minutes (reactor 2), and 288 minutes

(reactor 3), with an overall 8-hour cycle for each reactor (Table 5.1). The reactors were operated at an average temperature of $22\pm0.2^{\circ}\text{C}$ throughout the experiment.

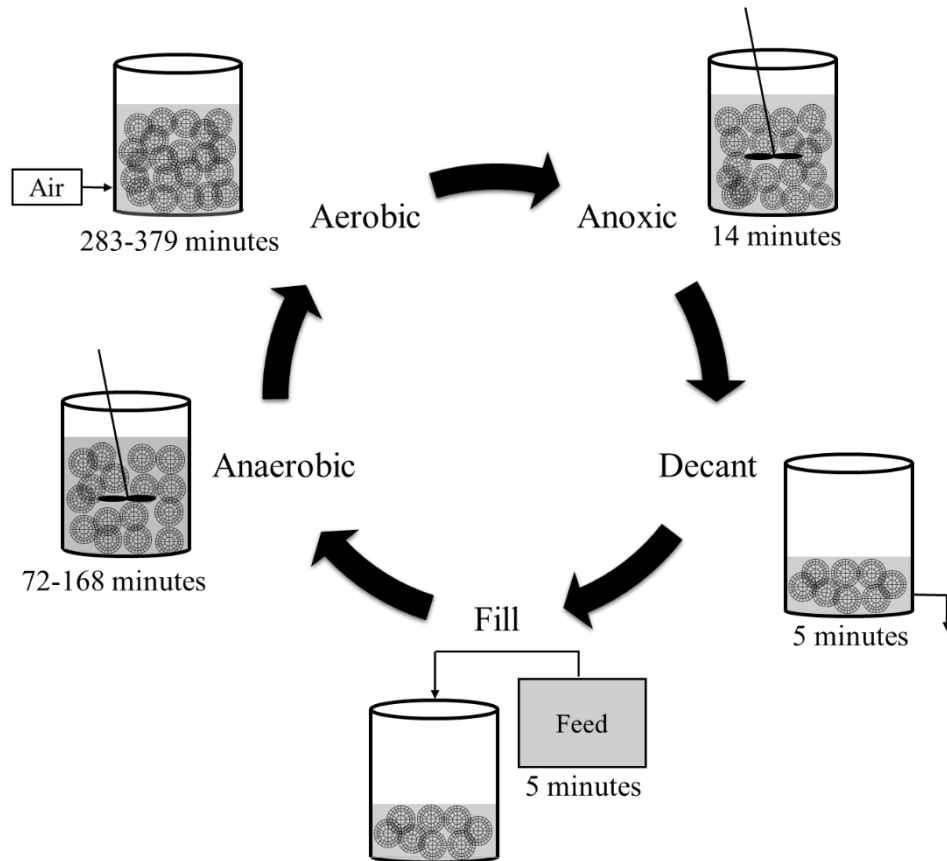


Figure 5.2 Operation, stages and ranges of the staging time for the SB-MBBRs.

Table 5.1 Reactor staging times for the three SB-MBBRs.

Stage	Reactor 1 (min)	Reactor 2 (min)	Reactor 3 (min)
Fill	5	5	5
Anaerobic	72	120	168
Aerobic	379	331	283
Anoxic	14	14	14
Decant	5	5	5
Idle	5	5	5

To test the effects of aeration, at approximately half way through the experimental phase of the study, a second air pump was added to the second reactor doubling the aeration rate from $2.4 \text{ L} \cdot \text{min}^{-1}$ to $4.8 \text{ L} \cdot \text{min}^{-1}$ in the system. The second pump was operated for only the first 2 hours of the aerobic stage. The enhanced aeration allowed for the maintenance of the dissolved oxygen (DO) concentration above $2 \text{ mg} \cdot \text{L}^{-1}$ throughout the entire aerobic stage.

5.3.2 System start-up

The K5 carriers were harvested from the Hawkesbury integrated fixed film activated sludge WRRF in Ontario, Canada, and were previously shown to contain a low percent abundance of nitrifying bacteria, and are dominated by heterotrophs (Young et al., 2017a). The reactors were operated at stable conditions for 5 months. Steady state conditions were subsequently determined by 6 consecutive cycles operated with steady performance, with steady state being defined as $\pm 10\%$ variance in the removal of sCOD, TN and TP along with pH values and DO concentrations in the SB-MBBRs. Following the addition of the second air pump, the SB-MBBRs were operated for an additional 7 months, until steady state conditions were achieved in reactor 2.

5.3.3 Wastewater source

The synthetic wastewater in this study was designed to simulate the carbon, nitrogen, and phosphorous content of St. Albert Cheese Factory wastewater (Ontario, Canada). The concentration of the synthetic wastewater was as follows: sCOD: $2500 \text{ mg} \cdot \text{L}^{-1}$, TN: $250 \text{ mg} \cdot \text{L}^{-1}$, total ammonia nitrogen (TAN): $100 \text{ mg} \cdot \text{L}^{-1}$, and TP: $40 \text{ mg} \cdot \text{L}^{-1}$. Specific composition of the synthetic wastewater was: NH_4Cl : $89 \text{ mg} \cdot \text{L}^{-1}$, KH_2PO_4 : $69 \text{ mg} \cdot \text{L}^{-1}$, NaHCO_3 : $304 \text{ mg} \cdot \text{L}^{-1}$, $\text{MgCl}_2 \cdot \text{H}_2\text{O}$: $58 \text{ mg} \cdot \text{L}^{-1}$, $\text{CaCl}_2 \cdot \text{H}_2\text{O}$: $29 \text{ mg} \cdot \text{L}^{-1}$, and FeCl_3 : $5 \text{ mg} \cdot \text{L}^{-1}$ (Delatolla et al., 2009). The carbon sources of the synthetic wastewater were: dextrose: $510 \text{ mg} \cdot \text{L}^{-1}$, sodium acetate: $340 \text{ mg} \cdot \text{L}^{-1}$, and peptone: $510 \text{ mg} \cdot \text{L}^{-1}$, selected to mimic the readily and non-readily degradable carbon

characteristic of cheese effluent. The biological oxygen demand (BOD) to COD ratio was 0.7 with approximately $86 \text{ mg} \cdot \text{L}^{-1}$ of the soluble COD (sCOD) being slowly-biodegradable or recalcitrant.

5.3.4 Constituent analysis

The sCOD, TP, TN, and volatile suspended solids (VSS) were analyzed to assess the reactor kinetics and metabolic activity of the microbes in this study. Samples were taken through the cycle of each reactor, and triplicate samples were collected from the influent, at the end of the anaerobic, aerobic, and anoxic stages, and from the decanted effluent. The errors from the triplicate samples were averaged and applied to the other measurements to indicate sampling, analytical and human error. The samples were filtered through a $0.45 \text{ }\mu\text{m}$ -pore sized filter before testing. HACH methods 8000-sCOD, 10072-TN and 1012-TP were used to test bulk liquid samples of sCOD, TN, and TP respectively. Triplicate samples were also collected at the end of the anaerobic, aerobic, and anoxic stages to measure the VSS. Standard method 2450 E-(VSS ignited at 550°C) was used to test the solids.

5.3.5 Biofilm dry-mass

The biofilm dry-mass was analyzed using a protocol adopted from Delatolla et al. (2009). Following steady state conditions, five carriers were harvested from each reactor, and for each operational condition. The carriers were incubated overnight at 105°C , dried in a desiccator for 20 minutes, and then weighed. Next, the biofilm was removed from the carrier with warm water and a stiff-bristled brush. The carriers were again dried overnight, cooled in a desiccator for 20 minutes, and weighed once more. The biofilm dry-mass was defined by the difference between these two masses.

5.3.6 Biofilm morphology and thickness

The biofilm morphology and thickness were analyzed using stereoscope images (Zeiss Stemi, 305, US, VA). Four carriers were harvested from each reactor, and for each operational condition following steady state conditions. Five images were taken at random locations, at $\times 2$ magnification, from each of the 4 replicate samples. Measurements were recorded from the edge of the carrier to the edge of the biofilm; 1000 measurements were taken per carrier, with 200 measurements taken per image (Schopf et al., 2018). The images were analyzed using the software Digimizer V.4.6.1 (Belgium, Ostend). Images presented were converted from RGB to a grey-scale, and the brightness and contrast were adjusted using the FIJI software V.2.0.0 (US, NY) (Rueden et al., 2017).

5.3.7 Cell viability

The viability of the cells encapsulated in the biofilm was detected with a 510/AxioImager Confocal laser scanning microscope (CLSM) (Zeiss, US, VA), and a $\times 63$ water objective. The embedded cells were stained with the Film TracerTM LIVE/DEAD® Biofilm viability kit (FilmTracer, 2009) (Life Technologies, US, CA), and the biofilm was stained with the calcofluor white stain. One carrier was harvested from each reactor, and for each operational condition. Five images were taken, at 5 μm intervals through the depth of the biofilm, at five different locations for each carrier (Zen, 2009). The FIJI software V.2.0.0 (US, NY) was used to numerate the live and dead cells in the biofilm (Rueden et al., 2017). The biofilm, represented as the calcofluor white stain, was outlined, and the viable and non-viable cells were identified through the observation of green and red fluorescence. The biofilm area and live and dead cell counts were used to calculate the biofilm cell coverage, and live fraction of cells.

5.3.8. Sequencing analysis of the 16S rRNA gene

DNA was extracted from the biofilm from 5 carriers for each reactor, and for each operational condition. The biofilm was separated from the carrier by cutting the center piece of the carrier, which was then placed in 750 µl of nuclease free water in a 1.5 µl microcentrifuge tube. The tube was vortexed, then the carrier piece was removed, and the microcentrifuge tube was centrifuged at 14 000 x g for ten minutes. The supernatant was removed, and then the protocol for the MPBio FastDNA® SPIN Kit was followed according to the manufacturer. Finally, the DNA was stored at -80°C.

To analyze the microbiome community, a two-step PCR targeting the V6-region of the 16S gene was followed according to the protocol previously described by Young et al. (2016). In brief, the first PCR targeted the V6 region of the 16S gene and attached 4-6 nucleotides and the Illumina (US, San Diego) adapters. The second PCR amplified the first PCR product, while attaching the Illumina flow cell adaptor. Then, the amplicons were verified with a 2% agarose gel, and were cleaned with Montage PCR96 cleanup kit (EMD Millipore, US, Billerica). The amplicons were quantified with the Quant-iT dsDNA HS Assay Kit (Life Technologies, Canada, Burlington), where 50 ng of DNA was pooled from each sample, and sequenced with the Illumina HiSeq2500 at the Centre for Applied Genomics (TCGA, Canada, ON).

Sequencing generated paired-end reads of 2×100 base pairs, which were analyzed with BioLinux operated on a Dell Precision T7610 workshop. The base paired-end reads were assembled through the Fast Length Adjustment of Short reads (FLASH) software (Magoc and Salzberg, 2011). The sequences were filtered to a minimum quality score of 20 over 90% of the sequences with Fastx toolkit. The filtered reads were then demultiplexed, and the barcodes were trimmed with Novobarcode (Goecks et al., 2010). Operational taxonomical units (OTUs) were

clustered with a closed reference strategy of 97% sequence similarity with the Quantitative Insights Into Microbial Ecology (QIIME) software, version 1.8 (Caporaso et al., 2011). The OTUs were aligned to the MiDAS database (Nierychlo et al., 2019) through the UCLUST algorithm in QIIME.

The sequences were further analyzed using the pyloseq package, in R (3.4.3). The sequences were rarefied to an even depth of 170 000 sequences, with a seed set to 28132 prior to measuring alpha diversity. The alpha diversity was measured through Chao1, and Simpson diversity (Hughes et al., 2001; Kim et al., 2017) (Simpson, 1949). Finally, the beta diversities were plotted using the Principal Coordinate Analysis (PCoA) with weighted-unifrac distances (Lozupone et al., 2007).

5.3.9. Statistical methods

The statistical significance of the kinetics was assessed using the t-test, where a p -value < 0.05 was used to identify significance. The statistical significance of the biofilm dry-mass, thickness, and dry-density, and for the microbiome analysis was calculated using a one-way analysis of variance (ANOVA) with the non-parametric test, Tukey's method, to perform multiple comparisons. A p -value < 0.05 was used to identify significance for the biofilm dry-mass, thickness, and dry-density, and for the microbiome analysis. Linear correlation was used to compare the effects of air supplied to the system on biofilm thickness. Analysis of similarities (ANOSIM) was used to test statistical significance between microbiome communities (Chapman and Underwood, 1999). The statistical analysis was performed in Prism 9.1.0.

5.4 Results and discussion

5.4.1 Reactor kinetics

sCOD, TN, and TP removal efficiencies; sCOD SARR; TP content of the VSS; and the C:N:P ratios were calculated for each operational condition (Table 5.2). The sCOD removal efficiencies

are $97.3 \pm 1.8\%$, $97.9 \pm 2.0\%$, and $98.1 \pm 2.9\%$ with 72, 120, and 168 minute anaerobic staging times; and $96.3 \pm 5.8\%$ with enhanced aeration. The TN removal efficiencies are $58.8 \pm 5.0\%$, $69.1 \pm 10.4\%$, and $50.3 \pm 0.5\%$ with 72, 120, and 168 minute anaerobic staging times; and $50.4 \pm 2.0\%$ with enhanced aeration. The TP removal efficiencies are $49 \pm 1\%$, $59 \pm 1\%$, and $62 \pm 2\%$ with 72, 120, and 168 minute anaerobic staging times; and $51 \pm 1\%$ with enhanced aeration. The sCOD SARR is calculated considering the duration of time for sCOD to be degraded to a final concentration of $47.5 \pm 1.8 \text{ mg} \cdot \text{L}^{-1}$, and not to the end of the cycle, with the remaining sCOD being most likely recalcitrant carbon. The sCOD SARRs are $24.8 \pm 0.5 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ ($97.3 \pm 1.8\%$), $29.6 \pm 0.6 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ ($98.3 \pm 2.0\%$), and $41.6 \pm 1.3 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ ($98.1 \pm 2.9\%$) with 72, 120, and 168 minute anaerobic staging times; and $35.5 \pm 2.1 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ ($97.8 \pm 5.9\%$) with enhanced aeration. The sCOD SARR increases significantly with increasing anaerobic staging time from 72 to 168 minutes ($p < 0.0001$). However, the sCOD SARR does not increase significantly with enhanced aeration ($p = 0.5$).

The C:N:P ratios are calculated based on the sCOD, TN, and TP removed/consumed in the aerobic stage. The C:N:P ratios are 100:53.4:7.9, 100:49.3:7.0, 100:38.4:7.6, for the reactors operated with anaerobic staging times of 72, 120, and 168; and 100:44.8:6.1 with enhanced aeration (Table 5.2). The C:N ratios in the aerobic stage for each operational condition is similar to that which would be expected for anoxic growth (100:53) (McCarty, 1965) (Table 5.2). However, the high carbon loading (Figueroa and Silverstein, 1992; Bassin et al., 2015) and C:N ratios (Tijhuis et al., 1994; Van Benthum et al., 1997; Bassin et al., 2012) of the synthetic wastewater being fed to the SB-MBBRs likely limited conventional nitrification, with autotrophic nitrifiers likely being outcompeted by the faster growing heterotrophs. Moreover, there was no source of $\text{NO}_3^-/\text{NO}_2^-$ in the synthetic wastewater to support anoxic growth, hence heterotrophic nitrification likely occurred in the SB-MBBRs for each condition. This finding is in line with

previous reports of heterotrophic nitrification observed in wastewater treatment (Schmidt, 1960; Robertson and Kuenen, 1990); specifically with MBBRs (Mannina et al., 2017), and in treatment of high-carbon loaded wastewater (Li et al., 2015).

The TP content is estimated as a ratio of TP removed/consumed to the VSS produced in the aerobic stage. The TP content is $4.5 \pm 0.2\%$, $4.1 \pm 0.2\%$, and 4.3 ± 0.2 with 72, 120, and 168 minute anaerobic staging times; and $2.3 \pm 0.1\%$ with enhanced aeration (Table 5.2). The TP content does not differ significantly with increasing anaerobic staging times ($p = 0.1$), however decreases significantly with increasing aeration rate ($p = 0.0001$). In fact, the TP content with $4.8 \text{ L} \cdot \text{min}^{-1}$ is equivalent to the TP content expected from heterotrophic cells, therefore the TP was likely removed through assimilation with the higher aeration. Thus, considering the significantly higher TP content with $2.4 \text{ L} \cdot \text{min}^{-1}$ aeration rate, EBPR likely occurs with the lower aeration rate. These findings suggest that low aeration is required to achieve EBPR with SB-MBBRs treating cheese-production wastewater, which is supported by previous studies which showed that low DO concentrations favour the selection of PAOs over non-PAO heterotrophs (Oehmen et al., 2007).

Table 5.2 Average and 95% confidence interval of the sCOD SARR, Δ TP, and TP content of the SB-MBBRs.

Anaerobic staging (min)	sCOD SARR ($\text{g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$)	Δ TP ($\text{mg-P} \cdot \text{L}^{-1}$)	TP content (%)	C:N:P
72	24.8 ± 0.5	23.4 ± 1.30	4.5 ± 0.2	100:53.4:7.9
120	29.6 ± 0.6	20.8 ± 0.4	4.1 ± 0.2	100:49.3:7.0
120*	35.5 ± 2.1	18.3 ± 0.2	2.3 ± 0.1	100:44.8:6.1
168	41.6 ± 1.3	21.8 ± 0.7	4.3 ± 0.2	100:38.4:7.6

*Operated at an aeration rate of $4.8 \text{ L} \cdot \text{min}^{-1}$, all other conditions operated at an aeration rate $2.4 \text{ L} \cdot \text{min}^{-1}$.

5.4.2 Biofilm morphology, dry-mass, thickness and dry-density

The biofilm morphology of the SB-MBBR was analyzed through stereoscope images acquired for each operational condition. The biofilm surface appears to increase in roughness with increasing anaerobic staging times from 72 minutes to 120 minutes, to 168 minutes (Figures 5.2. a, b, d), and when decreasing the aeration rate from $4.8 \text{ L} \cdot \text{min}^{-1}$ to $2.4 \text{ L} \cdot \text{min}^{-1}$ (Figures 5.2. b, c). The increase in roughness may be attributed to the decreased shear force applied to the biofilm with decreasing aerobic staging times, associated with longer anaerobic staging times, and the reduced shearing attributed to lower aeration. The increase in biofilm surface roughness increases the biofilm surface area, thereby enhancing the mass transport to the biofilm (de Beer and Stoodley, 1995), and mass transport of substrate into the biofilm is considered a limiting factor in the kinetics of the MBBR technology (Liu et al., 2009; Guimerà et al., 2015, Torresi et al., 2016). Hence, the increased surface roughness, and therefore enhanced mass transport through the biofilm with longer anaerobic staging may contribute to the increase in sCOD SARR with increasing anaerobic staging times (Table 5.2).

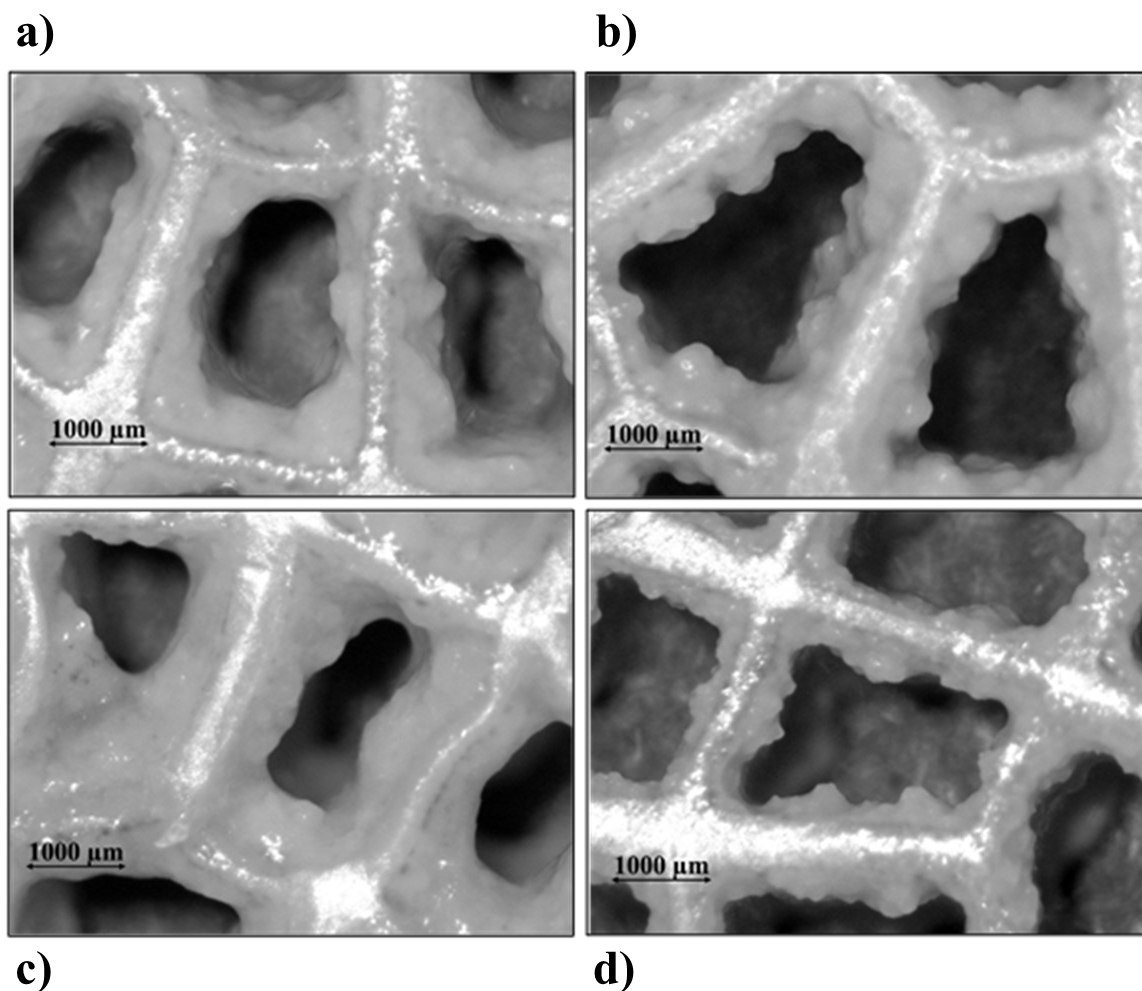
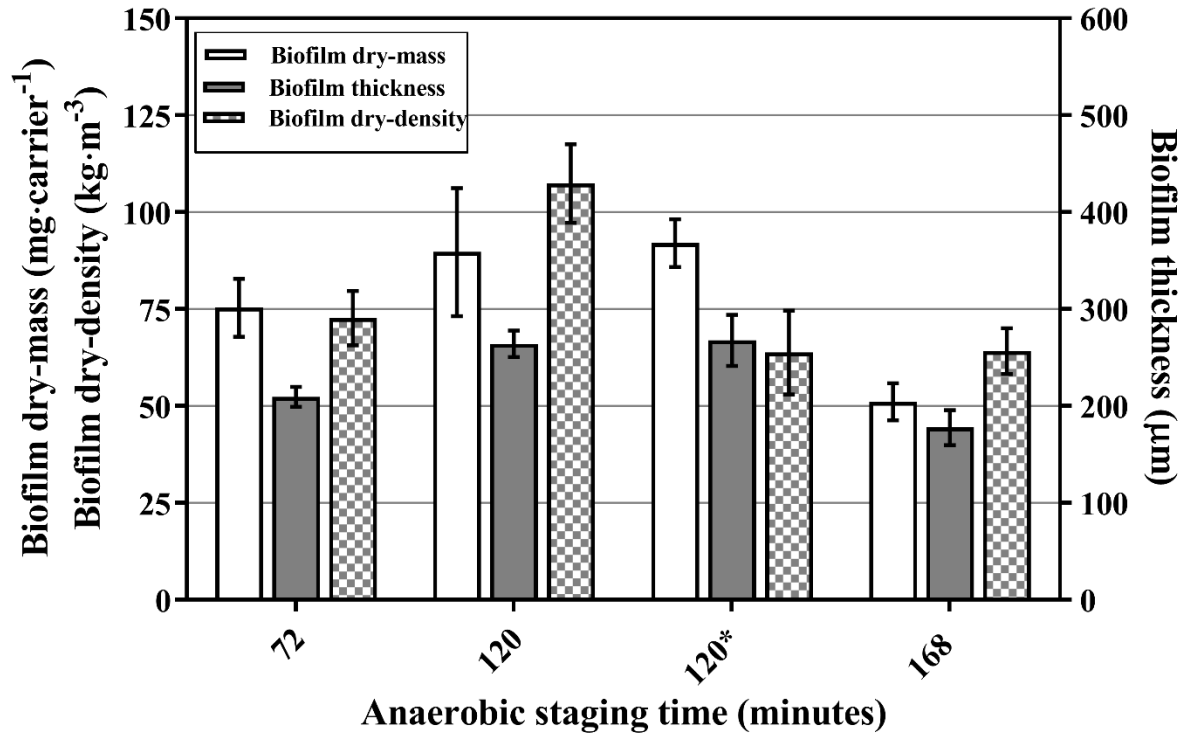


Figure 5.2 Stereoscope images of biofilm from the SBMBBRs operated under anaerobic stages of 72 minutes (a), 120 minutes (b, c), and 168 minutes (d), and with double the aeration was applied in (c).

The biofilm dry-mass, and dry-density are presented for each reactor with various anaerobic staging times and aeration rates (Figure 5.3). The biofilm dry-densities are calculated with the biofilm dry-mass, biofilm thickness, and surface area of the carriers. The biofilm dry-masses are $75.36 \pm 7.47 \text{ mg} \cdot \text{carrier}^{-1}$, $89.70 \pm 9.90 \text{ mg} \cdot \text{carrier}^{-1}$, and $51.60 \pm 5.44 \text{ mg} \cdot \text{carrier}^{-1}$ with 72, 120, and 168 minute anaerobic staging times; and $92.02 \pm 6.13 \text{ mg} \cdot \text{carrier}^{-1}$ with enhanced aeration. The biofilm dry-densities are $72.27 \pm 7.20 \text{ kg} \cdot \text{m}^{-3}$, $107.4 \pm 10.1 \text{ kg} \cdot \text{m}^{-3}$, and $64.18 \pm 6.00 \text{ kg} \cdot \text{m}^{-3}$ with

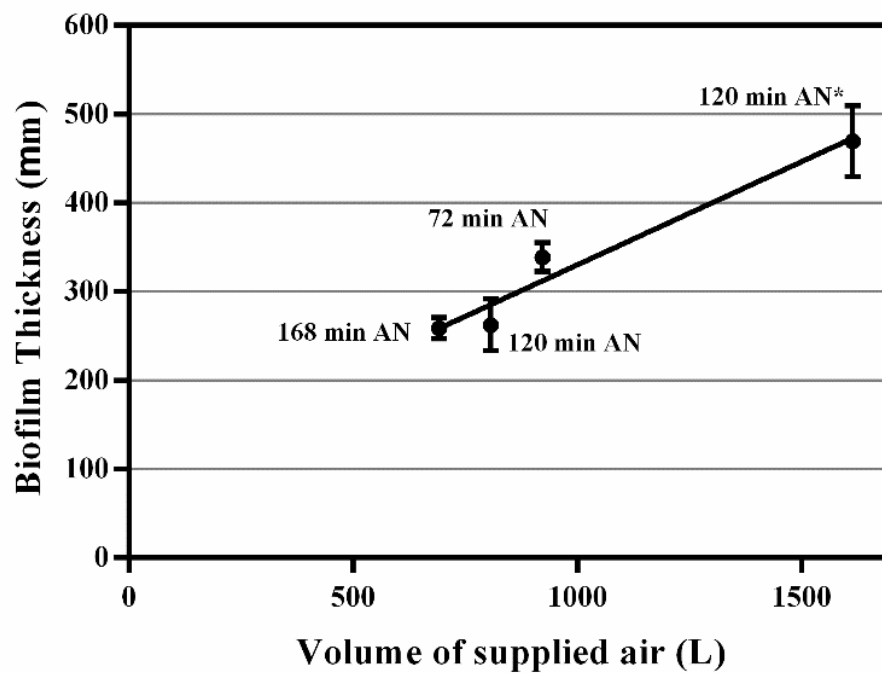
72, 120, and 168 minute anaerobic staging times; and $63.83 \pm 4.25 \text{ kg} \cdot \text{m}^{-3}$ with enhanced aeration. The biofilm dry-mass decreases significantly with the 168-minute anaerobic staging time compared to all other operating conditions ($p < 0.02$), while the biofilms dry-mass does not change significantly between all other operating conditions ($p > 0.1$). The biofilm dry-density increases significantly with increasing anaerobic staging times from 72 and from 120 minutes, and decreases significantly from 120 to 168 minutes ($p < 0.0001$). The biofilm dry-density decreases significantly with enhanced aeration ($p < 0.0001$). Lower biofilm dry-densities may be indicative of increased biofilm porosity, which is associated with enhanced mass transfer through the biofilm, and therefore increased SARRs (Guimerà et al., 2015). Thus, the decrease in biofilm dry-density with the longest anaerobic staging time may also explain the increase in SARR with increasing anaerobic staging time to 168 minutes (Table 5.2).



*Operated at an aeration rate of $4.8 \text{ L} \cdot \text{min}^{-1}$, all other conditions operated at an aeration rate $2.4 \text{ L} \cdot \text{min}^{-1}$.

Figure 5.3 Average and 95% confidence interval of biofilm dry-mass, thickness, and dry-density.

The biofilm thickness is compared to the volume of air supplied to each reactor, and for each operational condition, calculated with respect to the aeration rate and the duration of the aerobic stage (Figure 5.4). The biofilm thickness increases linearly with the total volume of air supplied to the system ($R^2 = 0.96$). The biofilm thickness is $469.3 \pm 39.9 \mu\text{m}$, $337.0 \pm 16.66 \mu\text{m}$, $271.9 \pm 25.7 \mu\text{m}$, and $258.97 \pm 11.9 \mu\text{m}$ with 1613 L, 910 L, 806 L, and 679 L of supplied air. The biofilm thickness changes significantly with total volume of air supplied to the system ($p < 0.0001$). MBBRs with thinner biofilms are considered to be more efficient compared to those with thicker biofilms, likely due to lower mass transport limitations associated with thin biofilms (Liu and Capdeville, 1994; Ødegaard, 1999; Laspidou and Rittmann, 2004; Gapes and Keller, 2009; Piculell et al., 2016; Torresi et al., 2016). Thus, the increasing sCOD SARR with increasing anaerobic staging times (Table 5.2) may be partially attributed to the lower biofilm thickness with the lower volume of air supplied to the system. However, considering the biofilm thickness does not differ significantly between 806 L, and 679 L (120 and 168 minute anaerobic staging times), likely the effects of increased biofilm surface roughness, lower biofilm dry-mass and dry-density explain the higher SARR with 168 minute compared to the 120 minute anaerobic staging time.



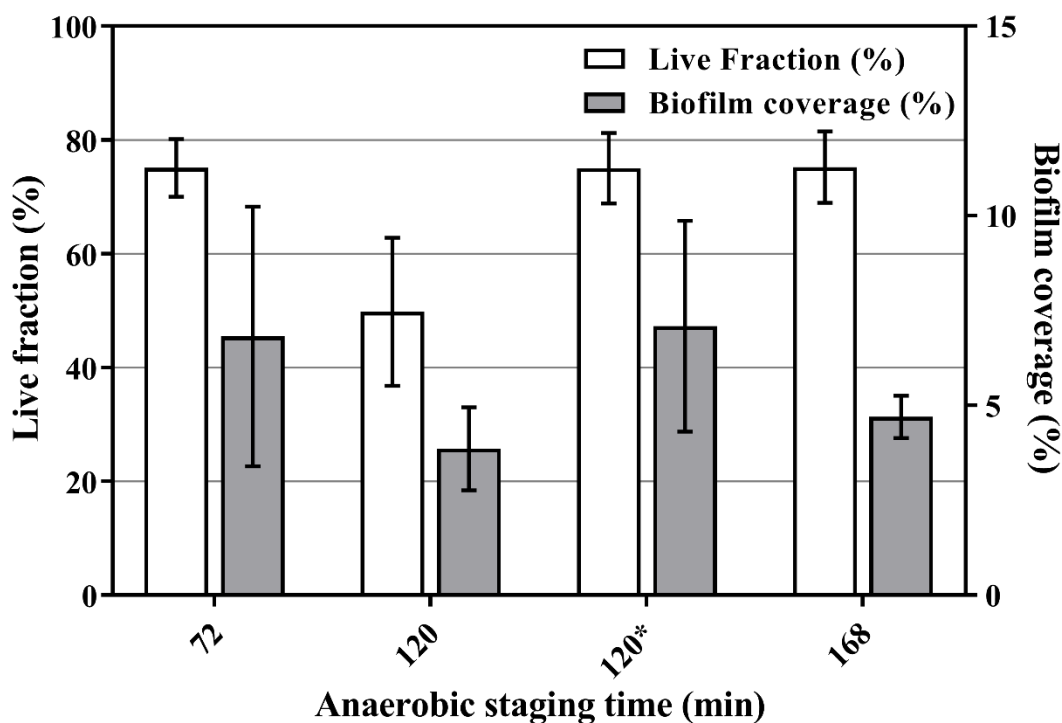
*Operated at an aeration rate of $4.8 \text{ L} \cdot \text{min}^{-1}$, all other conditions operated at an aeration rate $2.4 \text{ L} \cdot \text{min}^{-1}$.

Figure 5.4. Average and 95% confidence interval of biofilm thickness with the volume of air supplied to the reactors during the aerobic stage.

5.4.3 Cell viability

Cell viability is measured by staining and illuminating the embedded biomass in the biofilm. Viability measurements are maintained to the upper layer, and likely the active layer of the biofilm (Tian and Delatolla, 2019). The cell coverage is $6.82 \pm 3.42\%$, $3.86 \pm 1.09\%$, and 4.70 ± 0.56 with 72, 120, and 168 minute anaerobic staging times; and $7.09 \pm 2.78\%$ with enhanced aeration. The live fraction of the biofilm is $75.1 \pm 5.1\%$, $49.3 \pm 12.4\%$, and $75.2 \pm 6.3\%$ with 72, 120, and 168 minute anaerobic staging times; and $75.0 \pm 6.1\%$ with enhanced aeration (Figure 5.5). The cell coverage decreases significantly with increasing the anaerobic staging time from 72 to 120 minutes ($p = 0.003$), but does not differ significantly with increasing the anaerobic staging time from 120 to 168 minutes ($p = 0.6$). The cell coverage also increases significantly with enhanced aeration ($p < 0.0001$). The live fraction is significantly lower with 120 minute anaerobic staging time compared

to the 168 minute anaerobic staging time, and with enhanced aeration ($p < 0.002$). The live fraction of the embedded biomass does not change significantly for each other operational condition ($p = 1.0$) (Figure 5.5). Lower sCOD SARR with 120 minutes compared to 168 minute anaerobic staging time (Table 5. 2) may in part be due to a lower live fraction of embedded cells in the biofilm with 120 minute anaerobic staging time.



*Operated at an aeration rate of $4.8 \text{ L} \cdot \text{min}^{-1}$, all other conditions operated at an aeration rate $2.4 \text{ L} \cdot \text{min}^{-1}$.

Figure 5.5 Average and 95% confidence interval of the cell coverage and live fraction of the embedded biomass in the biofilm.

5.4.4 Microbial community diversity

Alpha diversity metrics were applied to compare the microbiome diversity within the reactors with varying anaerobic staging times and with enhanced aeration. The Chao1 estimator compares the species richness for each operational condition (Hughes et al., 2001; Kim et al., 2017), while the Simpson diversity index compares the species richness and evenness for each operational

condition (Simpson, 1949). The number of observed species are 455.8 ± 24.8 , 462.0 ± 63.7 , and 425.8 ± 13.9 with 72, 120, and 168 minute anaerobic staging times; and 468.6 ± 77.8 with enhanced aeration (Table 5.3). The Chao1 estimators are 635.5 ± 68.6 , 705.0 ± 153.1 , and 579.9 ± 31.9 with 72, 120, and 168 minute anaerobic staging times; and 659.8 ± 71.7 with enhanced aeration (Table 5.3). The number of observed species does not differ significantly with each operational condition ($p = 0.6$) and the Chao1 estimators also do not differ significantly with each operational condition ($p = 0.3$).

Table 5.3 Average and 95% confidence interval of the alpha diversity of the microbiome communities.

Anaerobic staging	Observed	Chao1 Estimator	Simpson Diversity
72	455.8 ± 24.8	635.5 ± 68.6	0.9192 ± 0.0173
120	462.0 ± 63.7	705.0 ± 153.1	0.9160 ± 0.0103
120*	468.6 ± 77.8	659.8 ± 71.7	0.9472 ± 0.0061
168	425.8 ± 13.9	579.9 ± 31.9	0.8465 ± 0.0325

*Operated at an aeration rate of $4.8 \text{ L} \cdot \text{min}^{-1}$, all other conditions operated at an aeration rate $2.4 \text{ L} \cdot \text{min}^{-1}$.

The Simpson diversity indices are 0.9192 ± 0.0173 , 0.9160 ± 0.0103 , and 0.8465 ± 0.0325 for the reactors operating with 72, 120, and 168 minute anaerobic stages; and 0.9472 ± 0.0061 with enhanced aeration. The Simpson diversity decreases significantly when increasing the anaerobic staging time to 168 minutes ($p = 0.01$). With a 168 minute anaerobic staging time the increased SARR is thought to be in part due to greater degradation of the slowly-degradable carbon, resulting in a greater proportion of readily-degradable carbon being available at the on-set of the aerobic stage (Tsitouras et al., 2020). Slowly-degradable carbon selects for a more diverse microbial community due to competition for substrate (Huston, 1994; Torresi et al., 2018), while easily degradable carbon will select for a more even population (Li et al., 2012; Li et al., 2013; Torresi

et al., 2018). Therefore, the higher proportion of readily degradable carbon being available at the onset of the aerobic stage may have permitted the selection of a less even microbiome population with 168 minute anaerobic staging time.

The Simpson diversity index increases significantly when increasing the aeration rate from $2.4 \text{ L} \cdot \text{min}^{-1}$ to $4.8 \text{ L} \cdot \text{min}^{-1}$ ($p = 0.02$) at an anaerobic staging time of 120 minutes (Table 5.3). The increase in Simpson diversity with enhanced aeration is associated with a smoother morphology (Figure 5.2), higher dry-mass, and greater thickness (Figure 5.3), and therefore a biofilm with a lower mass transfer rate. Lower aeration increases the likelihood of portions of the biofilm remaining anaerobic throughout the entire cycle, likely permitting a population of anaerobic bacteria as well as facultative bacteria, resulting in a more even population with enhanced aeration.

Microbiome diversity is thought to positively influence ecosystem functionality (Johnson et al., 2015; Torresi et al., 2018), considering communities with higher diversity are likely to have more functional traits and can occupy more microbial niches (Briones and Raskin, 2003; Stewart and Franklin, 2008). However, in the current study a less even population was associated with a higher sCOD SARR and the observation of EBPR activity; while the most even population, with enhanced aeration, did not achieve EBPR. Therefore, the less even microbiome community in this study could be indicative of a selection of the desired PAOs over competing organisms. Similarly, previous reports also found denitrification kinetics to be negatively associated with microbiome diversity (Johnson et al., 2015; Torresi et al., 2018).

Beta diversity is shown in this study using PCoA to depict differences in diversity with respect to anaerobic staging times and aeration rates (Figure 5.6). The beta diversity is calculated using weighted-unifrac distances, which calculate differences in diversity with respect to phylogenetic distances between taxa, and the relative abundances of each taxa (Chang et al., 2011).

ANOSIM analysis showed that the microbiome communities differ significantly with each operational condition ($p < 0.01$). The microbiome communities appear to cluster with respect to both anaerobic staging times and aeration rates. Furthermore, increasing the anaerobic staging time from 72 to 120 minutes, and decreasing the aeration rate from 4.8 to 2.4 $\text{L} \cdot \text{min}^{-1}$ causes a significant shift in the microbiome community. Likely the increase in SARR with anaerobic staging time, and the observed EBPR activity with lower aeration is a result of the shift in the microbiome community. The microbiome communities cluster closer together with 120 and 168 minute anaerobic staging times. Therefore, the effects of the biofilm roughness, dry-mass, dry-density and live cell fraction likely resulted in the higher SARR of sCOD with 168 minute anaerobic staging time compared to 120 minute anaerobic staging times, and differences in the microbiome community likely had less of an effect for these two anaerobic staging times.

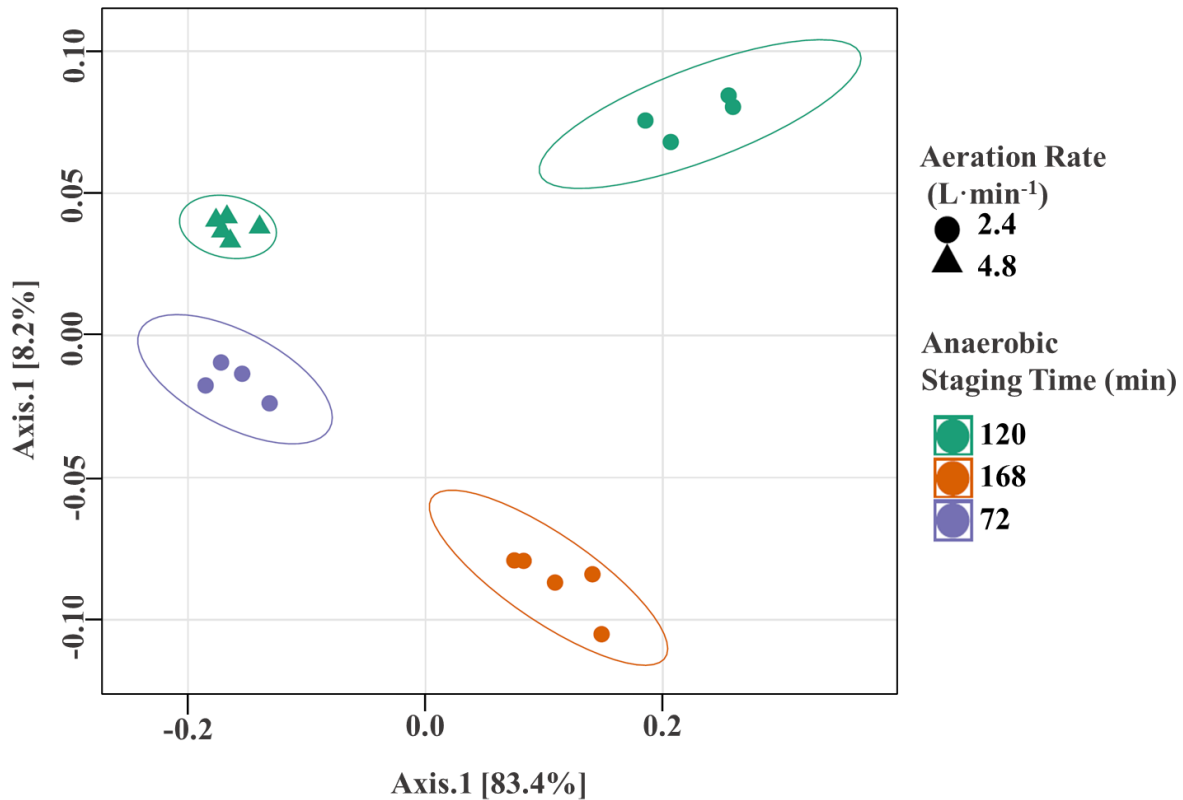
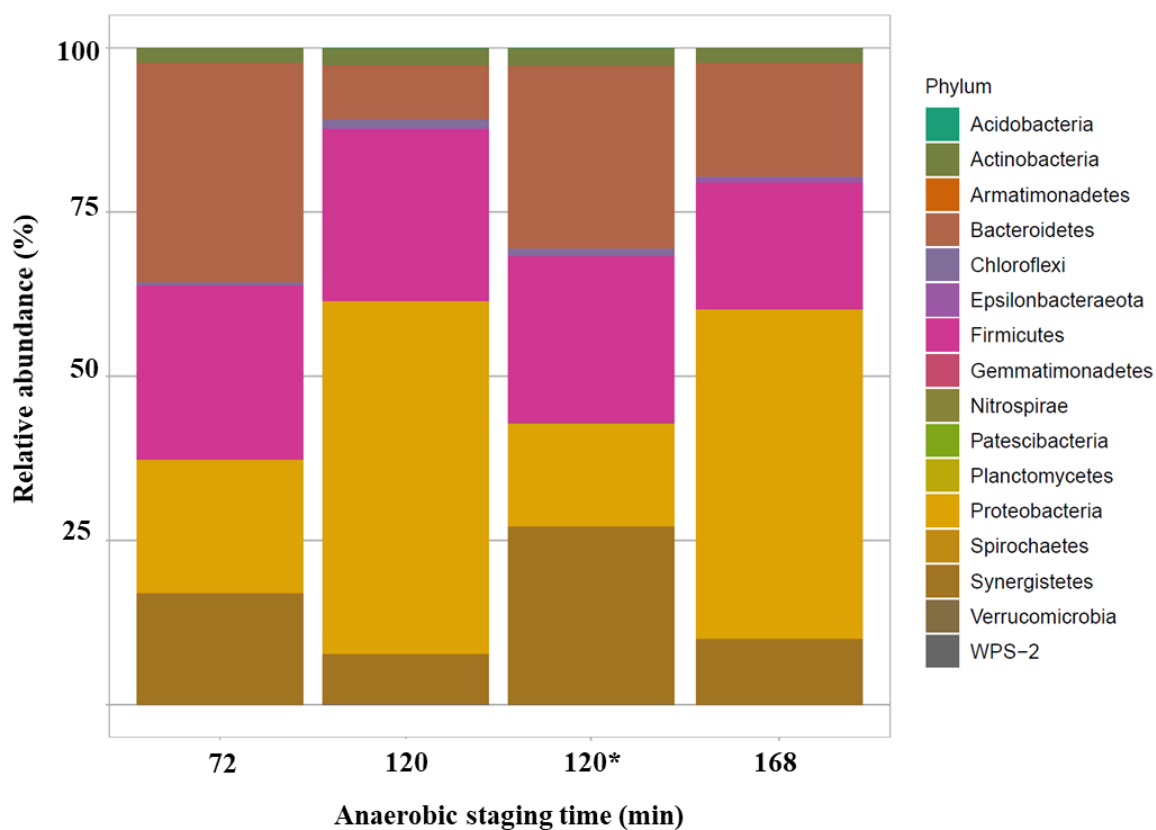


Figure 5.6 PCoA of microbiome populations, with weighted-unifrac distances.

5.4.5 Dominant taxa

For each operational condition, the taxa present at relative abundances $> 2\%$ are classified into 17 phyla (Figure 5.7). The top phyla are *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Synergistetes* for each operational condition, and each of these phyla have been previously observed in cheese production wastewater treatment systems (Liu et al., 2002; Liu et al., 2010; Mohan et al., 2010; Cammarota et al., 2013). The 19 most abundant taxa are also identified to the species level (Table 5.4). Specifically, fermenting bacteria from the *Rikenellaceae* and *Synergistaceae* family (Levén et al., 2007; Madigan et al., 2008; Cardinali-Rezende et al., 2009; Dong et al., 2016) are the most abundant taxa with 72 minute anaerobic staging time, and 120 minute anaerobic staging time with enhanced aeration. Species *midas_s_8005* from the *Rikenellaceae* family is present at relative abundances of $24.0 \pm 3.9\%$ and $11.4 \pm 1.6\%$ with 72 minute anaerobic staging time, and 120 minute anaerobic staging time with enhanced aeration respectively. Species *midas_s_2374* from the *Synergistaceae* family is present at relative abundances of $10.8 \pm 0.7\%$ and $17.2 \pm 1.9\%$ with 72 minute anaerobic staging time, and 120 minute anaerobic staging time with enhanced aeration respectively. These fermenting bacteria were each present at relative abundances below 6% in the reactors operated with 120 and 168 minute anaerobic staging times.



*Operated at an aeration rate of $4.8 \text{ L} \cdot \text{min}^{-1}$, all other conditions operated at an aeration rate $2.4 \text{ L} \cdot \text{min}^{-1}$.

Figure 5.7 Average relative abundance of phyla for each operational condition.

Table 5.4 Average and 95% confidence interval of the percent abundance of the 19 most dominant taxa identified.

Trophic Level			Anaerobic Staging Time			
Phylum	Family	Species	72 min	120 min	120 min*	168 min
<i>Bacteroidetes</i>	<i>Bacteroidaceae</i>	<i>Bacteroides_graminisolvans</i>	0.448±0.075	0.419±0.037	0.222±0.068	2.96±0.64
<i>Bacteroidetes</i>	<i>Dysgonomonadaceae</i>	<i>Petrimonas_sulfuriphila</i>	2.07±0.29	2.54±0.30	3.22±0.72	2.53±0.31
<i>Bacteroidetes</i>	<i>Rikenellaceae</i>	<i>midas_s_8005</i>	24.0±3.9	0.007±0.002	11.4±1.6	3.04±1.15
<i>Bacteroidetes</i>	<i>Rikenellaceae</i>	<i>midas_s_3329</i>	3.72±0.20	2.03±0.58	6.78±0.43	3.88±0.42
<i>Bacteroidetes</i>	<i>Rikenellaceae</i>	<i>midas_s_6724</i>	2.35±0.20	1.94±0.53	4.63±0.75	3.08±0.28
<i>Firmicutes</i>	<i>Christensenellaceae</i>	<i>midas_s_4201</i>	0.678±0.059	0.794±0.096	1.71±0.27	0.708±0.072
<i>Firmicutes</i>	<i>Clostridiaceae_1</i>	<i>midas_s_140</i>	6.51±1.07	4.21±0.74	4.01±0.40	4.42±0.72
<i>Firmicutes</i>	<i>Eubacteriaceae</i>	<i>midas_s_2229</i>	2.94±0.54	0.006±0.005	0.323±0.066	0.358±0.046
<i>Firmicutes</i>	<i>Family_XI</i>	<i>midas_s_6440</i>	4.83±0.63	2.77±0.53	2.54±0.28	0.866±0.152
<i>Firmicutes</i>	<i>Family_XI</i>	<i>midas_s_1214</i>	2.63±0.19	1.75±0.29	6.72±0.53	4.39±0.69
<i>Firmicutes</i>	<i>Lachnospiraceae</i>	<i>midas_s_2985</i>	2.97±1.17	0.712±0.068	-	3.22±0.47
<i>Proteobacteria</i>	<i>Comamonadaceae</i>	<i>Brachymonas_denitrificans</i>	3.75±0.37	20.6±3.1	1.54±0.565	6.83±0.907
<i>Proteobacteria</i>	<i>Burkholderiaceae</i>	<i>midas_s_482</i>	0.889±0.019	5.69±1.43	1.14±0.26	0.691±0.116
<i>Proteobacteria</i>	<i>Comamonadaceae</i>	<i>Rhodoferax_ferrireducens</i>	0.649±0.074	0.654±0.134	7.28±1.18	36.5±4.42
<i>Proteobacteria</i>	<i>Burkholderiaceae</i>	<i>Simplicispira_piscis</i>	1.04±0.11	6.46±0.40	1.18±0.15	1.36±0.19
<i>Proteobacteria</i>	<i>Rhodocyclaceae</i>	<i>midas_s_4927</i>	1.37±0.35	17.1±3.4	1.48±0.334	1.33±0.14
<i>Proteobacteria</i>	<i>Moraxellaceae</i>	<i>Acinetobacter_parvus</i>	3.06±1.00	0.045±0.008	0.156±0.076	0.460±0.152
<i>Synergistetes</i>	<i>Synergistaceae</i>	<i>midas_s_2374</i>	10.8±0.7	5.38±1.91	17.2±1.9	3.59±0.65
<i>Synergistetes</i>	<i>Synergistaceae</i>	<i>midas_s_249</i>	4.05±0.68	2.15±0.49	6.75±0.68	4.78±0.54

Denitrifier *Brachymonas denitrificans* (Leta et al., 2005), and *midas_s_4927* from the genus *Dechloromonas* (McIlroy et al., 2016) were the most abundant taxa observed in the reactor operating with a 120 minute anaerobic staging time, which were present at relative abundances of 20.6±3.1%, and 17.1±3.4% respectively (Table 5.4). The denitrifier *Rhodoferax ferrireducens*

(Finneran et al., 2003) was the most abundant taxa for the reactor operating with a 168 minute anaerobic staging time, and was present at a relative abundance of $36.5 \pm 4.42\%$. *Brachymonas denitrificans*, *midas_s_4927*, and *Rhodoferrax ferrireducens* were each present at relative abundances $< 8\%$ with the 72 minute anaerobic staging time, and the 120 minute anaerobic staging time and with enhanced aeration (Table 5.4). The dominance of the denitrifiers over fermenters with longer anaerobic staging times may contribute to the increase in SARR with longer anaerobic staging times, considering that denitrifying bacteria have a more efficient metabolism compared to fermenting bacteria (Metcalf & Eddy, 2014). These findings also support that the microbiome communities cluster more closely together with 120 and 168 minute anaerobic staging times, compared to 72 minute anaerobic staging time, and 120 minute anaerobic staging time with enhanced aeration. Likely the shift in microbiome community is from a community dominated by fermenters to a community of denitrifiers.

The presence of denitrifiers, and the C:N ratio removed in the aerobic stages that is in line with anoxic growth (Table 5.2), supports that denitrification occurred for each operational condition. The C:N ratios are greater with longer anaerobic staging times, which would suggest less denitrifier activity, although denitrifiers dominated the microbiome community with longer anaerobic staging times. Likely the longer anaerobic staging times allowed for more anaerobic uptake of carbon, whereby nitrogen is not assimilated, resulting in less carbon being available for denitrification. However, the longer anaerobic staging time may have provided an advantage to the denitrifiers, which exhibit facultative growth (Hirashi et al., 1991), to grow during the anaerobic stage as well as the aerobic stage over strictly anaerobic bacteria that would not be active during the aerobic stage.

5.4.6 Heterotrophic, fermenters, and PAO functional groups

The relative abundance of non-PAO heterotrophs, fermenters, and possible putative-PAOs are compared for each operational condition (Figure 5.8). The taxa defined as non-PAO heterotrophs in this study include the families: *Anaerolineaceae*, *Beijerinckiaceae*, *Burkholderiaceae*, *Dysgonomonadaceae*, *Enterococcaceae*, *Flavobacteriaceae*, *Moraxellaceae*, *Neisseriaceae*, *Propionibacteriaceae*, *Rhizobiaceae*, *Rhodobacteraceae*, *Rhodocyclaceae* (excluding *Accumulibacter* and *Dechloromonas*), *Weeksellaceae*; and the *Aminobacterium* genus from the *Synergistaceae* family. The taxa defined as fermenters include the families: *Acidaminococcaceae*, *Bacteroidaceae*, *Clostridiaceae*, *Eubacteriaceae*, *Family_XI*, *Lachnospiraceae*, *Rikenellaceae*, *Ruminococcaceae*, *Synergistaceae*, and *Veillonellaceae* (Nierychlo et al., 2019). The putative-PAOs include the genera: *Halomonas*, *Accumulibacter*, *Dechloromonas*, and *Tetrasphaera* (Nierycho et al., 2019), and *Brachymonas* (Shi and Lee, 2007). The *Comamonadaceae* family has been shown to store poly-phosphate (poly-P) (Ge et al., 2015), however the specific genera have yet to be defined as PAOs. Therefore the genera from *Comamonadaceae* family are also categorized as putative PAOs for this studies, and these include: *Comamonas*, *Acidovorax*, *Alicyclophilus*, *Diaphorobacter*, *Hydrogenophaga*, *Ottowia*, *Polaromonas*, *Rhodoferrax* (Coats et al., 2017). It should be noted, not all taxa within the *Comamonadaceae* family likely are PAOs, and the presence of PAO taxa does not confirm that EBPR is occurring, as these bacteria may be performing other metabolic pathways that do not include the uptake of phosphorous. Finally, each organism represents an abundance relative to the total population, and not as total counts.

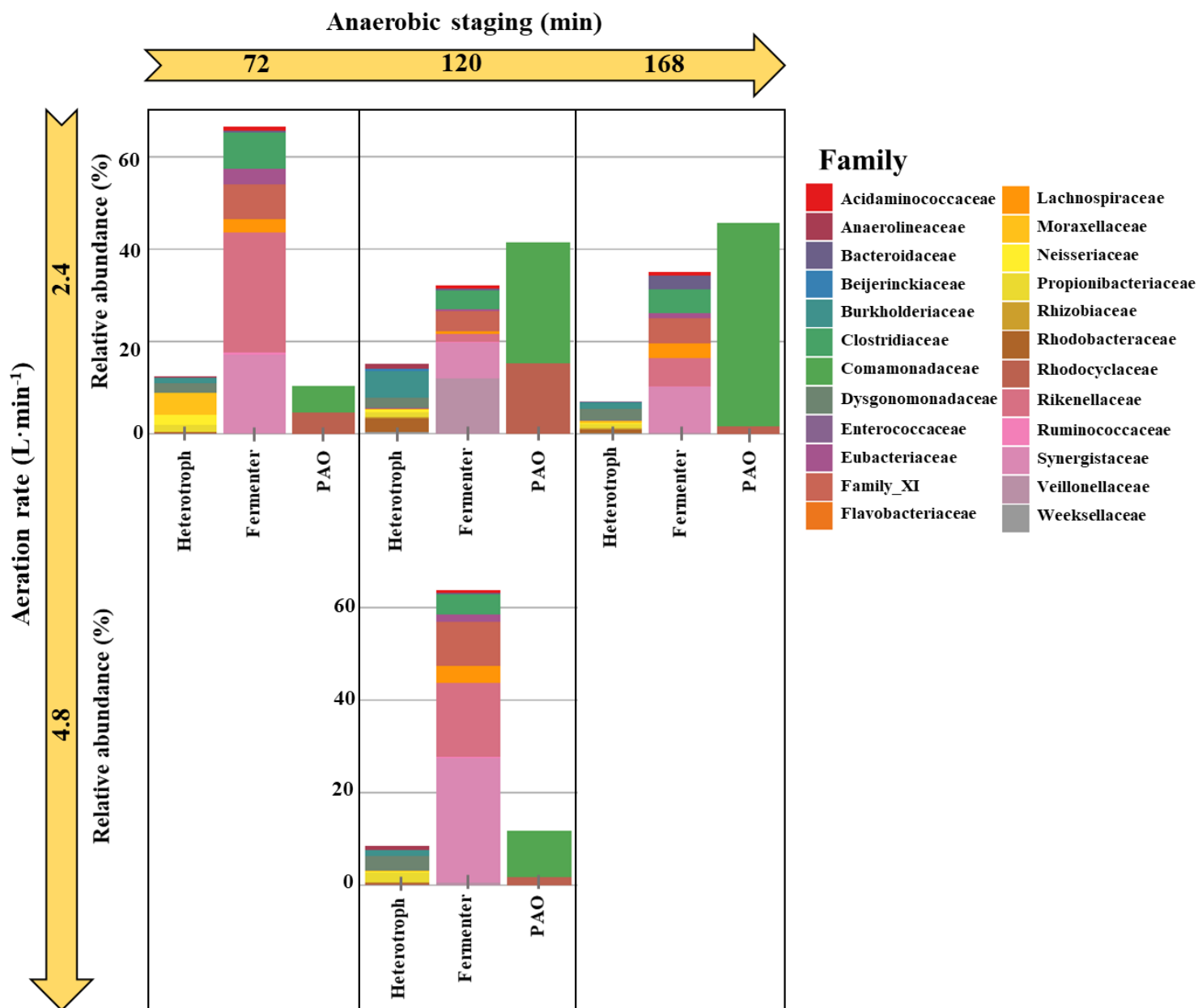


Figure 5.8 Average relative abundance of the heterotrophs, fermenters, and putative PAOs at the family level with respect to anaerobic staging times and aeration rates (MiDAS) (Nierychlo et al., 2019).

The relative abundances of non-PAO heterotrophs are $13.9 \pm 1.7\%$, $14.7 \pm 1.9\%$, and $9.09 \pm 3.1\%$ with 72, 120 minute, and 168 minute anaerobic staging times; and $14.8 \pm 2.0\%$ with enhanced aeration. The relative abundances of fermenters are $71.4 \pm 3.4\%$, $34.1 \pm 5.1\%$, and

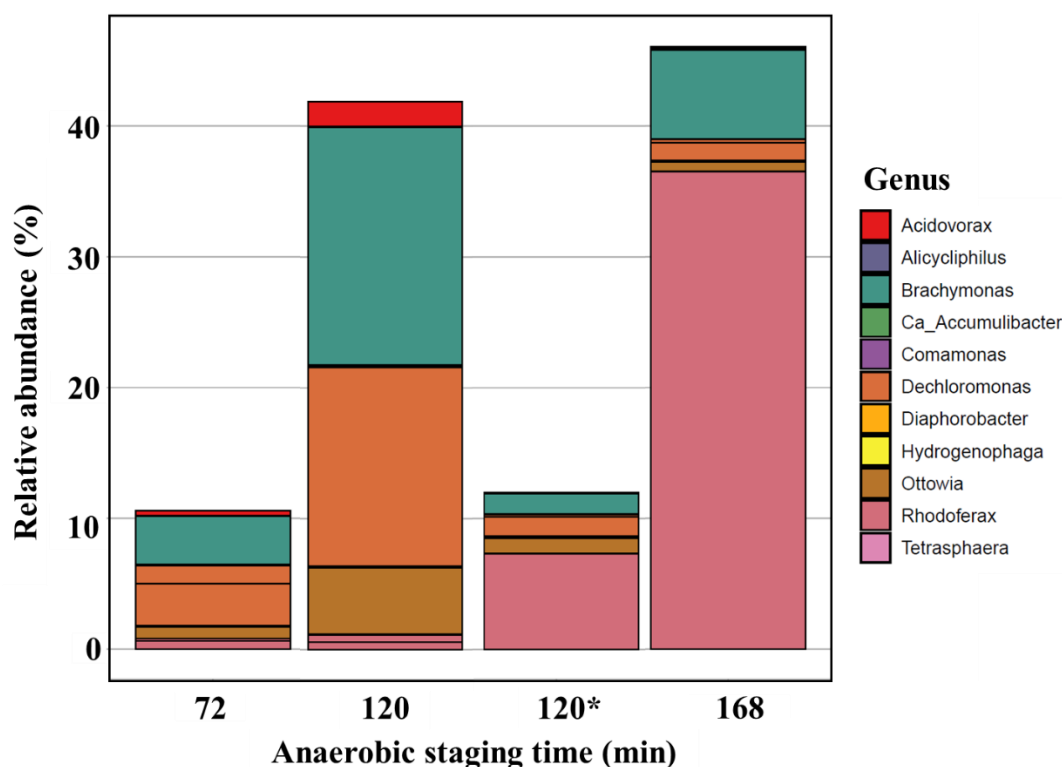
39.3±3.8% with 72, 120 minute, and 168 minute anaerobic staging times; and 71.1±2.7% with enhanced aeration. The relative abundances of possible putative PAOs are 10.6±0.1%, 41.8±9.1%, and 43.1±8.4% with 72, 120, and 168 minutes anaerobic staging times; and 8.88±1.11% with enhanced aeration. The relative abundance of the non-PAO heterotrophs does not change significantly with increasing anaerobic staging time from 72 to 120 minutes ($p > 0.08$), however decreases significantly when increasing the anaerobic staging time to 168 minutes ($p = 0.01$). The relative abundance of non-PAO heterotrophs does not differ significantly with enhanced aeration ($p = 1.0$). The relative abundance of fermenters decreases significantly with increasing anaerobic staging time from 72 to 120 minutes ($p < 0.0001$), and the relative abundance of fermenters does not change significantly when further increasing the anaerobic staging time to 168 minutes ($p = 0.2$). On the other hand, the possible putative PAO relative abundance increases significantly with increasing anaerobic staging time from 72 to 120 minutes ($p < 0.0001$), and does not change significantly when further increasing the anaerobic staging time to 168 minutes ($p = 1.0$). The relative abundance of fermenters increases significantly with enhanced aeration ($p < 0.0001$), while the possible putative-PAO relative abundance decreases significantly with enhanced aeration ($p < 0.0001$). These findings support that the microbiome shift observed with longer anaerobic staging times and lower aeration rates (Figure 5.7) is a result of the selection of putative PAOs over fermenters. Likely the thicker biofilm selected for with the 72 minute anaerobic staging time, and with enhanced aeration, permitted a higher relative abundance of fermenters, whereby mass transfer limitations limited the DO concentrations at portions of the biofilm (Torresi et al., 2016), permitting the dominance of a fermenting population. Therefore, thinner biofilms, below 262.4±29.2 μm , may be indicative of a population dominated by PAOs over fermenters. It should be noted that the relative abundance of PAOs has not been shown to correlate to TP removal

efficiency (Coats et al., 2017), which could explain the limited TP removal with a high relative abundance of possible putative PAOs. However, the dominance of possible putative PAOs could provide evidence of the potential of the system to achieve EBPR.

The significant decrease in relative abundance of possible putative PAOs with increasing the aeration rate from $2.4 \text{ L} \cdot \text{min}^{-1}$ to $4.8 \text{ L} \cdot \text{min}^{-1}$ supports that EBPR occurs with $2.4 \text{ L} \cdot \text{min}^{-1}$, and not with $4.8 \text{ L} \cdot \text{min}^{-1}$, as was hypothesized given the TP content above what would have been expected from heterotrophic assimilation with the lower aeration (Table 5.2). Although the relative abundance of possible putative PAOs was significantly lower with a 72 minute anaerobic stage than with 120 and 168 minute anaerobic stages, the TP content with a 72 minute anaerobic stage did not differ significantly from the reactors operated with 120 and 168 minute anaerobic staging times (Table 5.2). Thus, possibly the EBPR activity occurred in the suspended solids in the reactor operated with a 72 minute anaerobic staging time instead of in the biofilm. In a previous report, when operating a second SB-MBBR in series following the reactor with the 72 minute anaerobic staging time, evidence that PAOs are washed into the second reactor substantiates that PAOs exist in the bulk liquid in the SB-MBBR operated with the 72 minute anaerobic staging time (Tsitouras et al., 2021). The formation and colonization of biofilms by organisms requires the expression genes involved in EPS secretion, lipopolysaccharide synthesis genes, flagella biosynthesis and motility genes (Niba et al., 2007). Therefore, PAOs may express these genes in lower quantities with lower anaerobic staging times, below 120 minutes, compared to the higher anaerobic staging times. Also, if the PAOs are easily washed out from the biofilms, the lower shear force attributed to the anaerobic staging time (due to shorter duration of aeration), lower aeration rate, and anaerobic staging time, may have better permitted the PAOs to proliferate within the biofilm. Finally, increasing the anaerobic staging time from 72 to 120 minutes, and decreasing the aeration

rate from 4.8 to 2.4 L·min⁻¹ may have selected for a peripheral microbiome community, forming symbiotic relationships with the PAOs, that is required for the PAOs to proliferate in the biofilm.

The taxa defined as possible putative-PAOs were grouped for each operational condition and are presented down to the genus to level (Figure 5.9). The most abundant putative-PAO genera are *Dechloromonas* from the *Rhodocyclaceae* family with the 120 minute anaerobic staging time; and *Brachymonas* and *Rhodoferax* from the *Comamonadaceae* family with the 168 minute anaerobic staging time (Figure 5.9). *Brachymonas*, *Dechloromonas*, and *Rhodoferax* are also known to perform denitrification, and *Rhodoferax* is known to perform fermentation (Hirashi et al., 1991). Therefore, the selected PAOs may be fermenting PAOs and/or denitrifying PAOs. The relative abundance of *Accumulibacter*, frequently observed in EBPR treatment systems (McIllory et al., 2015) is not observed in high abundances in this study, therefore possibly *Accumulibacter* may not be selected for with cheese production wastewater treatment. Similarly, an investigation of intermittently aerated SBRs treating dairy waster, where EBPR was observed, observed *Comamonadaceae* up to 80% relative abundance, while *Accumulibacter* was not the dominant PAO (Gil-Pulido et al., 2018). Therefore *Dechloromonas*, *Rhodoferax*, and *Brachymonas* species may be more important PAOs for achieving EBPR in cheese production wastewater treatment compared to *Accumulibacter*.



*Operated at an aeration rate of 4.8 L·min⁻¹, all other conditions operated at an aeration rate 2.4 L·min⁻¹.

Figure 5.9 Average relative abundance of putative PAOs for each operational condition.

5.5 Conclusion

This study identified a link between removal rates, biofilm morphology, and microbiome community. Specifically, increased carbon removal rates were observed with longer anaerobic staging times, which improved the SARR of sCOD to $41.6 \pm 1.3 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$, and was associated with a rougher biofilm surface, lower dry-density, thinner biofilm, and higher live cell fraction. Furthermore, the most significant microbiome shift was observed with longer anaerobic staging times and lower aeration, which selected for a microbiome community dominated by denitrifiers *Brachymonas*, *Dechloromonas*, and *Rhodoferrax*, which may be putative PAOs, and with a lower abundance of fermenting bacteria. This research therefore presents the first microbiome study of EBPR in an SB-MBBR system treating cheese production wastewater, and presents supporting

evidence that EBPR is feasible with SB-MBBRs for on-site treatment of cheese production wastewater.

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6 Article 4 – Microbiome of two strategies for ammonia removal of the sequencing batch moving bed biofilm reactor treating cheese production wastewater

6.1 Abstract

On-site treatment of total ammonia nitrogen (TAN) from cheese production wastewater must abide by sewage discharge bylaws to prevent overloading of municipal water resource recovery facilities, eutrophication of receiving waters, and toxicity to aquatic life. The moving bed biofilm reactor (MBBR) is a compact technology established for treating TAN from wastewater via nitrification. The sequencing batch moving bed biofilm reactor (SB-MBBR) shows potential for on-site biological treatment of carbon nitrogen and phosphorous from cheese production wastewater. The competition between nitrifiers and heterotrophs limits TAN oxidation in SB-MBBRs treating cheese production wastewater. Thus, this study compares two strategies to achieve TAN oxidation using the SB-MBBR: extended aerobic operation of a single SB-MBBR, and operating two SB-MBBR systems in series. The extended aerobic operation required 810 hours before TAN oxidation was achieved, where a microbiome shift was required for a sufficient ammonia oxidizing bacteria (AOB) population to grow within the biofilm to achieve TAN oxidation. Thus, a single SB-MBBR is not feasible for achieving nitrification simultaneously with carbon and phosphorous removal when treating cheese production wastewater. Operating two SB-MBBRs in series achieved TAN removal, possibly through partial nitrification (PN), with a TAN surface area removal rate (SARR) of $1.07 \pm 0.05 \text{ g-N} \cdot \text{m}^{-2} \text{d}^{-1}$, and an AOB relative abundance of $3.55 \pm 0.68\%$. With two SB-MBBRs in series, TAN oxidation occurs in the second reactor where the C:N ratio is lowered to 0.5:1, thereby avoiding the competition between heterotrophs and autotrophs, and permitting the AOBs to thrive. This is the first study to analyze the microbiome of the SB-MBBR achieving TAN removal from cheese production wastewater, and

presents that two SB-MBBRs operated in series is required for TAN oxidation of the wastewater. Furthermore, this is the first study to show evidence that PN can be achieved in an SB-MBBR system treating cheese production wastewater, and demonstrates the potential for the SB-MBBR to be incorporated in a deammonification system.

6.2 Background

Cheese is one of the main agricultural goods produced worldwide, and the production of cheese is a major source of industrial effluent in Europe (Demirel et al., 2005). Specifically, the processing of cheese generates between 1 and 6 liters of wastewater per liter of processed milk (Guerreiro et al., 2020), and contains high nitrogen contents due to the presence of milk proteins, amino acids, and urea. Nitrogen exists in cheese production wastewaters in the forms: TAN, NO_2^- , NO_3^- ; (Guillen-Jimenez et al., 2000), and total nitrogen (TN) concentrations typically range between 0.01-1.7 $\text{g}\cdot\text{L}^{-1}$, and TAN concentrations between 60 to 270 $\text{g}\cdot\text{L}^{-1}$ (Carvalho et al., 2013, Farizoglu et al., 2004). Thus discharging cheese production wastewaters to natural water bodies leads to environmental degradation, and eutrophication (Cheng et al., 2016; Rong et al., 2020); while molecular or unionized forms of ammonia cause acute and chronic toxicity to fish and other aquatic life (EPA, 1993). Furthermore, discharging cheese production wastewaters to sewers can overload municipal water resource recovery facilities (WRRFs) (Janczukowicz et al., 2008). Thus, increasingly stringent regulations are being implemented by governing agencies to municipal WRRFs by implementing financial and regulatory industrial discharge bylaws (Gazette 2012; Román-Sánchez et al., 2015; Hendriks and Langeveld 2017). Therefore, on-site treatment of cheese production wastewater is needed (Rusten et al., 1996; Tatoulis et al., 2015; Van Lier et al., 2015), creating a demand for compact treatment systems.

Nitrogen is removed biologically from cheese production wastewater through nitrification and denitrification, which has been achieved with various suspended growth technologies, including conventional activated sludge (CAS) (Bortone and Piccinini 1991, Donkin and Russell 1997), membrane bioreactors (Fraga et al., 2017), and wetlands (Kasapgil et al., 1994). Nitrification is the process whereby TAN is oxidized to nitrite by AOB, and nitrite is subsequently oxidized to nitrate by nitrite oxidizing bacteria (NOB). Denitrification is the biological reduction of nitrate or nitrite to nitrogen gas performed by various anoxic bacteria (Pajares and Bohannan 2016). Deammonification can provide a more sustainable process, compared to nitrification/denitrification, whereby nitrogen oxidation is stopped at nitrite (PN), and anaerobic ammonia oxidation (ANAMMOX) is used in combination with PN, to oxidize TAN with nitrite (Laureni et al., 2016). PN requires the selection of AOB populations with a suppression NOB populations. Strategies in suppressing the NOB population include, control of temperature, free ammonia toxicity, free nitrite acid toxicity, dissolved oxygen (DO), and maintaining low sludge retention times (Hellings et al., 1998; Blackburne et al., 2008, Brockmann and Morgenroth 2008).

The MBBR is a compact treatment system that houses free-moving-polyethylene carriers, on which biofilms are maintained (Ødegaard et al., 1994). MBBRs are advantageous as the movement of the carriers minimizes the risk of clogging, thereby eliminating the need for backwashing, and permitting efficient mass transfer of substrate and DO into the biofilm (Rusten et al., 2006). The specific design of the many carriers allows for a high surface area and structure that protects the biofilm from abrasion. This structure permits the maintenance of a significant biofilm concentration, which allows for treatment with a small land footprint, while preventing the washout of slow-growing bacteria such as nitrifiers (Johnson et al., 2000; Ødegaard et al., 2000).

When MBBRs are operated under sequencing batch mode, as a SB-MBBR, the cycles of the system can be modified in real-time response to commonly observed variations in the cheese production wastewater characteristics and flow rates (Andreottola et al., 2002). Hence the SB-MBBR is an attractive technology for cheese production wastewater treatment. However, challenges in achieving nitrification in MBBR units for cheese production wastewater treatment arise due to competition between heterotrophs and nitrifiers for oxygen and space within the biofilm. The competition is exacerbated by the high organic loading, and the high C:N ratios of cheese production wastewaters (Carvalho et al., 2013) that is known to favour heterotrophs over nitrifiers, including AOBs (Fdz-Polanco et al., 2000; Xia et al., 2008). Thus, TAN oxidation has only been achieved in SB-MBBRs following anaerobic treatment (Kasapgil et al., 1994; Nadais et al., 2010), whereby nitrogen was removed with MBBRs downstream of anaerobic treatment of carbon (Andreottola et al., 2002). Furthermore, the biofilm morphology has not yet been assessed for the SB-MBBR technology, nor for TAN oxidizing biofilms treating cheese production wastewater with MBBRs. Thus, the design, operation, and the microbial and microbiome characteristics of SB-MBBR systems for carbon, and nitrogen treatment of cheese production wastewaters remains largely unknown.

By identifying conditions required for selecting key microorganisms and community structures for optimum treatment conditions (Cyzdik-Kwiatkowska and Zielińska 2016; Mohan et al., 2010), the knowledge of the microbiology can advance the understanding of the SB-MBBR making way for improved design of pilot- and full-scale SB-MBBR operations. Hence, the following study presents a novel investigation of the biofilm microbiology of two SB-MBBR operational strategies to identify the operation that will permit TAN oxidation to occur along with the removal of carbon and phosphorous from cheese production wastewater. Specifically, this study compares the

biomass viability and microbiome community of SB-MBBR systems operated under the two different strategies to achieve TAN oxidation: (i) extended aerobic operation beyond the depletion of organic-content in the single SB-MBBR reactor to achieve TAN oxidation, and (ii) two SB-MBBRs in series configured to separate carbon removal and TAN oxidation, thereby removing the competition for oxygen between the heterotrophs and the autotrophic nitrifiers.

6.3 Materials and methods

6.3.1 Reactor Set-up

This study compared two strategies to achieve TAN oxidation in SB-MBBR systems treating cheese production wastewater. The first strategy involved extending the aerobic operation of an SB-MBBR (AN/AE) system operated with anaerobic and aerobic stages that is designed to treat carbon and phosphorous (Tsitouras et al., 2020; Tsitouras et al., 2021). The first cycle of the AN/AE reactor was initiated using a peristaltic pump filling 2 L of synthetic wastewater from a feed reservoir into the AN/AE reactor; followed by an anaerobic stage of 72 minutes, and an aerobic stage of 398 minutes. Although a 168-minute anaerobic staging time was previously shown to result in higher SARR of sCOD (Tsitouras et al., 2020), a 72 minute anaerobic staging time was chosen to limit the exposure of anaerobic conditions to the nitrifiers, considering the KO_2 of nitrifiers (0.5) (Wett et al., 2011). Following the aerobic stage, the air pumps were shut off for 14 minutes during the anoxic stage prior to decanting. The anoxic stage was included in the cycle to permit denitrification in the case of nitrate formation. The AN/AE reactor was then decanted over a 5-minute period. To simulate an extended aerobic operation, which would be operated as an extension of the aerobic stage in the AN/AE reactor, a batch reactor (NIT_ExtendedAE) was operated with 10 carriers taken from the AN/AE reactor. The NIT_ExtendedAE reactor was

operated with a 280 mL volume, and was fed with the bulk liquid from the AN/AE reactor. The NIT_ExtendedAE reactor was aerated for 33 days (810 hours) in order to observe TAN oxidation.

The second strategy involved operating two SB-MBBRs in series, where a second reactor was added in series following the AN/AE reactor. After completion of the AN/AE reactor cycle (fill, anaerobic, aerobic, anoxic, decant), the AN/AE reactor effluent was fed into a second reactor (NIT_Series), which was operated with continuous aeration throughout the cycle to achieve TAN oxidation. The NIT_Series reactor was aerated for 465 minutes followed by the reactor being decanted over a 5 minute period, and the total cycle time for the NIT_Series reactor was 8 hours. The two reactors in series, as a complete system, were operated with a total cycle time of 16 hours (Figure 6.1).

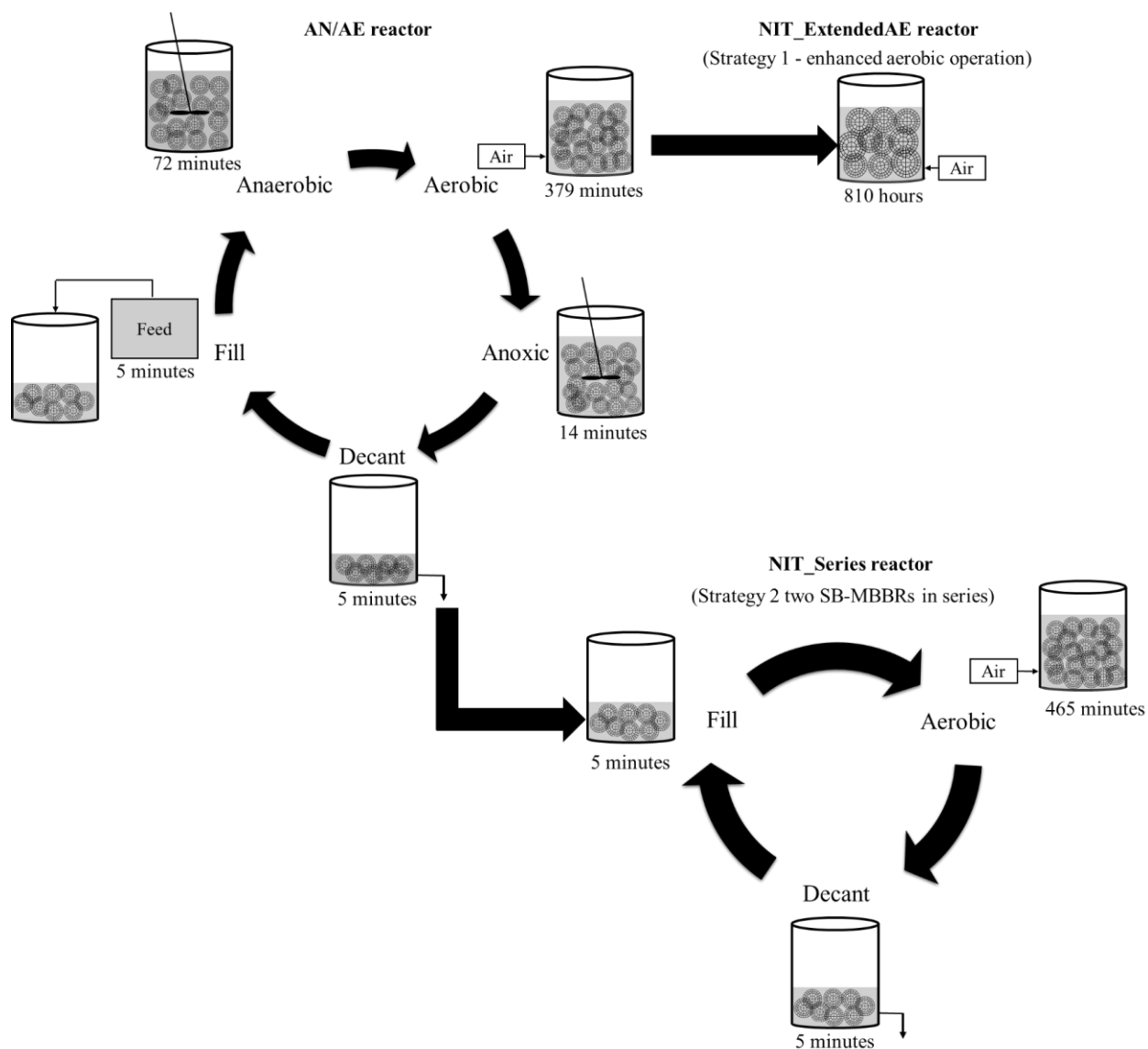


Figure 6.1 Operational strategies for achieving TAN oxidation with the SB-MBBR system.

6.3.2 Inoculation of reactors

The AN/AE and NIT_Series reactors were inoculated with K5 AnoxK™ carriers (AnoxKaldnes, Lund, Sweden), which have a protected surface area of $800 \text{ m}^2 \cdot \text{m}^{-3}$. The carriers were harvested from the Hawkesbury integrated fixed film activated sludge wastewater treatment plant in Ontario, Canada. The carriers harvested from the Hawkesbury facility contained a low relative abundance of nitrifying bacteria, and were dominated by heterotrophic bacteria (Young et al., 2017a). The AN/AE reactor

was operated for 12 months prior to the experiment, while the NIT_Series reactor was added in-series, and both reactors were operated for an additional 1 month before the NIT_Series reactor reached steady-state. The influent and effluent soluble chemical oxygen demand (sCOD), total phosphorous (TP), TAN, NO_3^- , and NO_2^- concentrations were analyzed six consecutive times across a period of 14 days for each reactor to determine steady state. The DO and pH was also monitored throughout the 14 days. For this study, steady state was defined as a variance in the sCOD and TAN removal rates within $\pm 10\%$. The NIT_ExtendedAE reactor was operated to simulate an extended aerobic stage of the AN/AE reactor, and therefore an acclimatization period was not required.

6.3.3 Wastewater source

For this study, a synthetic wastewater was fed to the AN/AE reactor, and the specific composition was as follows: NH_4Cl : $89 \text{ mg}\cdot\text{L}^{-1}$, KH_2PO_4 : $69 \text{ mg}\cdot\text{L}^{-1}$, NaHCO_3 : $304 \text{ mg}\cdot\text{L}^{-1}$, $\text{MgCl}_2\cdot\text{H}_2\text{O}$: $58 \text{ mg}\cdot\text{L}^{-1}$, $\text{CaCl}_2\cdot\text{H}_2\text{O}$: $29 \text{ mg}\cdot\text{L}^{-1}$, and FeCl_3 : $5 \text{ mg}\cdot\text{L}^{-1}$ (Delatolla et al., 2009), dextrose: $510 \text{ mg}\cdot\text{L}^{-1}$, sodium acetate: $340 \text{ mg}\cdot\text{L}^{-1}$, and peptone: $510 \text{ mg}\cdot\text{L}^{-1}$. The synthetic wastewater simulated the carbon, nitrogen, and phosphorous composition of the wastewater from the St. Albert Cheese Factory, located in Ontario, Canada. The carbon sources of the synthetic wastewater were selected to mimic the readily and non-readily degradable carbon characteristic of cheese production wastewater. The concentrations of the synthetic wastewater were as follows, sCOD: $2500 \text{ mg}\cdot\text{L}^{-1}$, TAN: $100 \text{ mg}\cdot\text{L}^{-1}$, TN: $250 \text{ mg}\cdot\text{L}^{-1}$, and TP: $40 \text{ mg}\cdot\text{L}^{-1}$. The synthetic wastewater consisted of a biological oxygen demand (BOD) to chemical oxygen demand (COD) ratio of 0.7 with approximately $86 \text{ mg}\cdot\text{L}^{-1}$ of the sCOD being slowly-biodegradable or recalcitrant.

6.3.4 Constituent analysis

The following parameters were analyzed: sCOD, TP, TAN, NO_3^- , NO_2^- , DO, and pH. Bulk-liquid samples were taken throughout the operational cycle for each reactor, and were filtered through a

0.45 μm -pore sized filter before testing. Triplicate samples were taken a minimum of 3 times for each reactor; the errors from the triplicate samples were averaged and applied to all other measurements to indicate sampling, analytical and human error. HACH methods 8000-sCOD, 1012-TP, 10205-TAN, and 835- NO_3^- were used to test the concentrations in the bulk liquid samples of sCOD, TP, TAN, and NO_3^- respectively. While standard methods 5210-BOD, 4500B- NO_2^- were used to test the BOD, and NO_2^- concentrations in the bulk liquid (APHA, 2005). DO and pH measurements were acquired using a HACH multi-meter with a DO and pH probe (HACH, USA, Colorado).

6.3.5 Cell viability

The embedded biomass was analyzed through images taken with a 510/AxiolImager confocal laser scanning microscope (CLSM) (Zeiss, US, VA), with a $\times 63$ water objective. For each reactor, one carrier was harvested, and the center piece of the carrier was cut with a scalpel exposing the protected biofilm. The biofilm was stained with the Film Tracer™ LIVE/DEAD® Biofilm viability kit (Life Technologies, US, CA), following the FilmTracer protocol (2009). These stains differentiate between live and dead cells since they differentiate in their ability to penetrate cell membranes. Finally, the calcofluor white stain was used to identify the biofilm.

The embedded biomass was visualized with the CLSM, and with the Zen2009 software using the Z-stack function. 5 images were taken every 5 μm through the depth of the biofilm, at 5 different locations for each carrier. The gain was adjusted to prevent oversaturation of the image. The viable and non-viable cells were identified by measuring the green and red fluorescence. FIJI software V.2.0.0 (FIJI, US, NY) was utilized to numerate the live and dead cells in the biofilm, and to outline the biofilm to measure the biofilm area (Schindelin et al., 2012). The viable and non-viable cells were used to calculate the biofilm cell coverage, and live fraction of cells.

6.3.6 Sequencing analysis of the 16S rRNA gene

The microbiome was studied from the DNA, which was extracted from the biofilm of five carriers harvested from each reactor. First, the biofilm was separated from the carrier by cutting the center piece of the carrier with a scalpel, and placing the piece in a 1.5 µl microcentrifuge tube with 750 µl of nuclease-free water. The microcentrifuge tube was vortexed allowing the biofilm to fall off the carrier, after which the carrier piece was removed, and the microcentrifuge tube was centrifuged at 14 000 x g for ten minutes. The supernatant was removed from the microcentrifuge tube, and the protocol for the MPBio FastDNA® SPIN Kit was then followed according to the manufacturer.

The microbiome community was analyzed through a two-step polymerase chain reaction (PCR) that targeted the V6-region of the 16S gene, following the protocol previously described (Young et al., 2016). Briefly, the first PCR targeted the V6 region of the 16S gene and attached 4-6 nucleotides and the Illumina adapters (Illumina, US, San Diego). The second PCR amplified the first PCR product, while attaching the Illumina flow cell adaptors. The resulting amplicons were verified with a 2% agarose gel and cleaned with the Montage PCR96 cleanup kit (EMD Millipore, Billerica, MA). The amplicons were pooled, whereby 50 ng from each sample was quantified with the Quant-iT dsDNA HS Assay Kit (Life Technologies, Burlington, Canada), and sequenced with the Illumina HiSeq2500 at the Centre for Applied Genomics (TCGA, Canada, ON).

6.3.7 Sequencing and sequence analysis

The MiSeq sequencing generated paired-end reads of 2x100 base pairs (Young et al., 2016), and were analyzed through BioLinux operated on a Dell Precision T7610 workshop. The base paired-end reads were then assembled with the Fast Length Adjustment of Short reads (FLASH) software (Magoč and Salzberg 2011). The sequences were filtered to a minimum quality score of 20 over 90% of the sequences with the `fast_quality_filter` command from the Fastx toolkit. The filtered sequences were

then demultiplexed, and the barcodes were trimmed with Novobarcode (Goecks et al., 2010). Operational taxonomical units (OTUs) were clustered with a closed reference strategy of 97% sequence similarity using the Quantitative Insights Into Microbial Ecology (QIIME) software, version 1.8 (Caporaso et al., 2011). The OTUs were aligned to the MiDAS database (Nierychlo et al., 2019) through the UCLUST algorithm in QIIME.

The alpha and beta diversity, and the taxonomy of the sequences were further analyzed using the phyloseq package in R studio (3.4.3). The sequences were rarefied to an even depth of 170 000 sequences, with a seed set to 28132. The alpha diversity was analyzed by defining the number of observed species, the Chao1 estimator, and Simpson diversity. The diversities between the biofilm populations in each reactor were compared through beta-diversity metrics, which were plotted using the Principal Coordinate Analysis (PCoA) with weighted-unifrac distances.

6.3.8 Statistical methods

The statistical significance was calculated using a one-way analysis of variance (ANOVA) with the non-parametric test, Tukey's method, to perform multiple comparisons. A p -value < 0.05 was used to identify significance for the kinetics analysis, biofilm dry-mass, thickness, and dry-density, and for the microbiome analysis. Analysis of similarities (ANOSIM) was used to test statistical significance between microbiome communities (Chapman and Underwood, 1999). The statistical significance of the diversity between microbiome communities was assessed through the analysis of similarities (ANOSIM) metric (Chapman and Underwood 1999). The statistical analysis was performed in Prism 9.1.0.

6.4 Results and discussion

6.4.1 Kinetic and metabolic analysis

The sCOD, TP, and TAN concentrations are analyzed in the AN/AE reactor, and the pH, and DO measurements are acquired throughout the cycle of the AN/AE reactor. The sCOD is removed with a SARR of $24.8 \pm 0.5 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$, corresponding to a removal efficiency of $97.3 \pm 1.8\%$, where the final sCOD concentration is $57.0 \pm 1.1 \text{ mg} \cdot \text{L}^{-1}$ in the AN/AE reactor. The TP is removed with a SARR of $0.27 \pm 0.01 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$, corresponding to a removal efficiency of $49 \pm 1\%$, where the final TP concentration is $11 \pm 0.2 \text{ mg} \cdot \text{P} \cdot \text{L}^{-1}$ in the AN/AE reactor. The TAN is not significantly removed in the AN/AE reactor, and the final concentration of TAN is $95.0 \pm 2.6 \text{ mg} \cdot \text{N} \cdot \text{L}^{-1}$. The pH ranges between 5.97-7.44 in the anaerobic stage, and 7.23-8.48 in the aerobic stage in the AN/AE reactor. The DO concentrations range between 0.09-0.92 $\text{mg} \cdot \text{L}^{-1}$ in the anaerobic stage, and 0.06-6.11 $\text{mg} \cdot \text{L}^{-1}$ in the aerobic stage in the AN/AE reactor.

The TAN, NO_3^- , and NO_2^- concentrations were monitored across the cycles in the NIT_ExtendedAE and NIT_Series reactors, and the kinetics of the TAN removal are presented as SARRs (Table 6.1). TAN is removed with a SARR of $0.0024 \pm 0.0001 \text{ g} \cdot \text{N} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$, corresponding to a removal efficiency of $89.5 \pm 2.0\%$ in the NIT_ExtendedAE reactor. TAN is removed with a SARR of $1.07 \pm 0.05 \text{ g} \cdot \text{N} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$, corresponding to a removal efficiency of $98.7 \pm 2.4\%$ in the NIT_Series reactor. 810 hours is required before TAN oxidation is observed in the NIT_ExtendedAE reactor, while TAN oxidation is observed after operating the SB-MBBR for 30 hours, following approximately 2 cycles, in the NIT_Series reactor. NO_2^- concentrations accumulated to $77.5 \pm 6.0 \text{ mg} \cdot \text{N} \cdot \text{L}^{-1}$ and $96.8 \pm 0.1 \text{ mg} \cdot \text{N} \cdot \text{L}^{-1}$ in the NIT_ExtendedAE and NIT_Series reactors respectively, while NO_3^- was not detected in either NIT reactors, suggesting that TAN was oxidized through PN in both reactors. The pH in the NIT reactor is in range that is required to select for ammonia oxidizing

bacteria (AOB) over nitrite oxidizing bacteria (NOB) (Villaverde et al., 1997). Given the high TAN concentrations in the NIT reactors, likely competition occurs between the AOB and NOB for oxygen, with the NOB population being outcompeted (Schopf et al., 2019; Rong et al., 2020). Therefore this configuration has the potential to be incorporated as a PN system, which could permit downstream anaerobic ammonia oxidation, reducing energy requirements compared to nitrification denitrification processes (Deng et al., 2020; Dimitrova et al., 2020)..

Table 6.1 Average and 95% confidence interval of the nitrogen kinetics in the NIT reactors.

Reactor	TAN SARR (g-N·m ⁻² d ⁻¹)	TAN removal efficiency (%)	Time to achieve nitrification (h)	NO ₂ ⁻ build-up (mg-N·L ⁻¹)
NIT_ExtendedAE	0.0024±0.0001	89.5±2.0	810	77.5±6.0
NIT_Series	1.07±0.05	98.7±2.4	30	96.8±0.1

TAN SARR and removal efficiency was significantly higher in the NIT_Series reactor compared to the NIT_ExtendedAE reactor ($p < 0.04$). Considering that the NIT_ExtendedAE reactor requires 810 hours before TAN oxidation is observed; this implies that the microbiome is acclimatizing to the lower C:N ratio, and the autotrophic nitrifying taxa are growing to a significant abundance in the biofilm, and only then achieving TAN oxidation. Similarly in a previous report, MBBR carriers were transferred from an IFAS system with a C:N ratio of 3:1, to a low carbon system, where a microbiome shift was observed and an acclimatization period of 28 days was required for a significant abundance of nitrifiers to establish in the biofilm (Young et al., 2017a). The high carbon loading (Figuerola and Silverstein, 1992; Bassin et al., 2015), and the high C:N ratio (Tijhuis et al., 1994; Van Benthum et al., 1997; Bassin et al., 2012) in the AN/AE reactor likely resulted in the autotrophs being out competed by heterotrophs within the AN/AE reactor. Also, given the low KO₂ of the nitrifiers (0.5) (Wett et al., 2011), the anaerobic stage may have prevented the growth of nitrifiers within the AN/AE reactor. Therefore the extended aerobic

operation strategy is not feasible when attempting to achieve EBPR and nitrification simultaneously in a single SB-MBBR treating cheese production wastewater. With the addition of a second SB-MBBR, the C:N ratio is lowered in this second SB-MBBR to a more ideal C:N ratio for nitrifier growth (0.5:1), and separates the heterotrophs from the autotrophic nitrifiers. As such, TAN oxidation is achieved in this study in the second SB-MBBR (NIT_Series reactor), thus two SB-MBBRs operated in series is demonstrated to be required to achieve carbon, phosphorous and TAN oxidation when treating cheese production wastewater using the SB-MBBR technology.

6.4.2 Embedded biomass

Cell viability is measured by staining and illuminating the embedded biomass in the biofilm (Figure 6.2). Viability measurements are maintained to the upper layer, and likely the active layer of the biofilm (Tian and Delatolla, 2019). The cell coverage of the biofilm (area of the cells per biofilm area) is $6.82 \pm 3.42\%$, 7.13 ± 0.61 , and $8.47 \pm 2.31\%$ for the AN/AE reactor, the NIT_ExtendedAE reactor and the NIT_Series reactor, which do not differ significantly ($p = 0.6$). The live fraction percentage (live cells per total cells) of the cells embedded in the biofilm is $75.1 \pm 5.1\%$, $73.0 \pm 6.8\%$, and $69.2 \pm 18.7\%$ for the AN/AE reactor, the NIT_ExtendedAE reactor, and the NIT_Series reactor, which do not differ significantly ($p = 0.8$). Differences in the SARR between the NIT_ExtendedAE reactor and the NIT_Series reactor were therefore not likely due to differences in biofilm cell coverage or viability of the embedded cells.

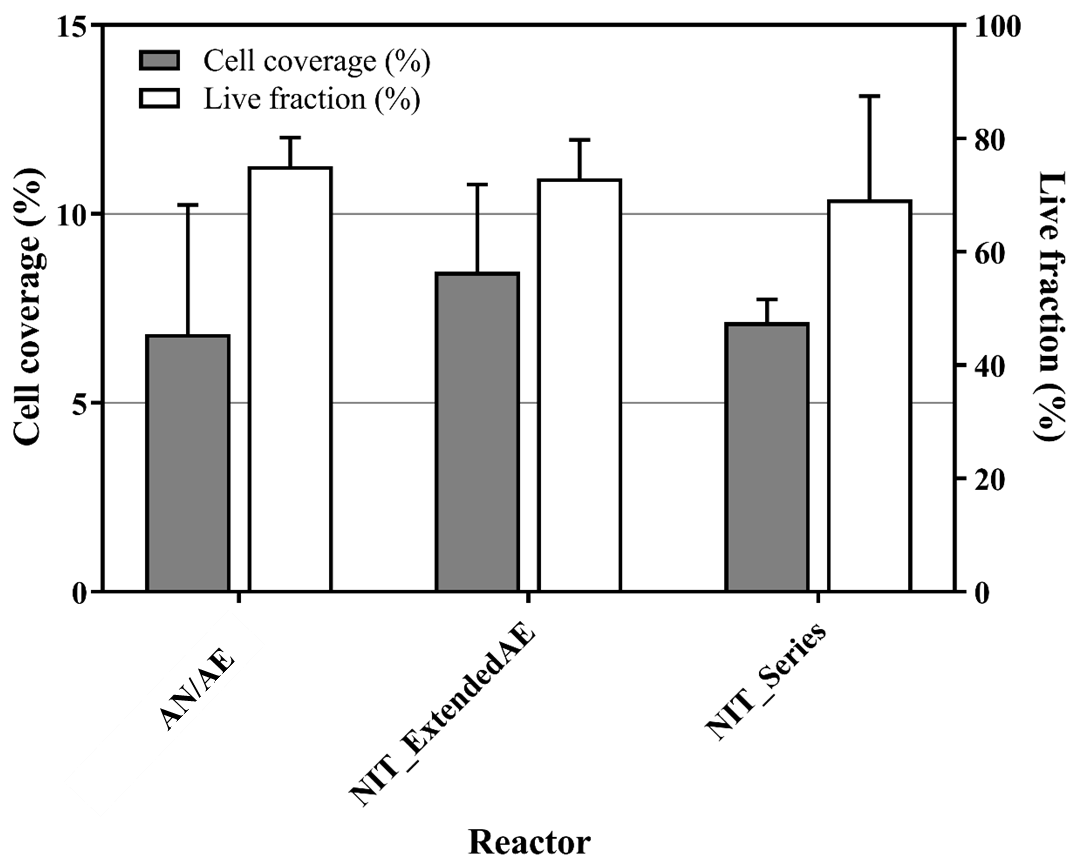


Figure 6.2 Average and 95% confidence interval of the cell coverage and the live fraction in the biofilm of each reactor.

6.4.3 Microbiome diversity

The diversity of the microbiome community in each reactor is measured using alpha diversity metrics (Table 6.2). The Chao1 estimator analyzes the species richness for each reactor (Hughes et al., 2001; Kim et al., 2017), while the Simpson diversity index analyzes the species richness and evenness for each reactor (Simpson 1949). The number of observed species are 464 ± 31 , 981 ± 233 , and 830 ± 47 , for the AN/AE reactor, the NIT_ExtendedAE reactor, and the NIT_Series reactor; the Chao1 estimators are 635.5 ± 68.6 , 980.8 ± 232.7 , and 830.0 ± 53.9 for the AN/AE reactor, the NIT_ExtendedAE reactor, and the NIT_Series reactor. Finally, the Simpson diversity indices are 0.9192 ± 0.0173 , 0.9625 ± 0.0035 , and 0.9549 ± 0.0069 for the AN/AE reactor, the NIT_ExtendedAE

reactor, and the NIT_Series reactor. The Chao 1 and Simpson diversities do not differ significantly between the NIT reactors ($p > 0.3$). However, both the NIT reactors are more significantly diverse compared to the AN/AE reactor for the number of observed species and the Simpson diversity ($p < 0.001$). The Chao1 diversity does not differ significantly in the NIT_ExtendedAE reactor compared to the AN/AE reactor ($p = 0.3$). However the Chao1 diversity is significantly greater in the NIT_Series reactor compared to the AN/AE reactor ($p = 0.03$). Although reactors with higher C:N ratios have been shown to exhibit higher alpha diversities in biofilm reactors (Xia et al., 2008), likely the anaerobic conditions in the AN/AE reactor limited the diversity of the microbiome community in the biofilm. The number of observed species in both NIT reactors were two times lower than previous reports of nitrifying MBBR microbiomes with similar nitrogen loading (Young et al., 2017b). The Chao 1 estimators in this study were 1-3 orders of magnitude lower than previous reports for MBBRs performing simultaneous nitrification denitrification (SND) for municipal wastewater treatment, while the Simpson diversities were in range of previous reports (Liu et al., 2020).

Table 6.2 Average and 95% confidence interval of the alpha diversities for each reactor.

Reactor	Observed	Chao1	Simpson
AN/AE	463.75±42.0	643.3±42.0	0.9190±0.017
NIT_ExtendedAE	709.3±53.9	980.9±237.5	0.9625±0.0035
NIT_Series	625.7±232.7	830.0±47.6	0.9549±0.0069

The beta diversity between each reactor is analyzed using weighted-unifrac distances, and is depicted through PCoA (Figure 6.3). The populations appear to cluster with respect to the reactor operation, and the maximum distance is 78.1%. The NIT_ExtendedAE and NIT_Series reactors cluster more closely compared to the AN/AE reactor, with the NIT reactors only differing by a maximum of 11.6%. Furthermore, ANOSIM analysis indicates the microbiome community is

significantly different between each reactor ($p = 0.002$). Therefore, the different operating strategies resulted in different microbiome communities between the NIT reactors. As expected the reactors performing PN were more similar compared to the AN/AE reactor performing carbon and phosphorous removal, likely due to the selection of nitrogen removing bacteria in the NIT reactors, which were not selected for in the AN/AE reactor. These findings support that the nitrifying bacteria are not present in the biofilm of the AN/AE reactor, and are likely being outcompeted by the heterotrophic organisms due to the higher carbon loading and the high C:N ratio of the AN/AE reactor. Young et al., (2017a) compared the microbiome of nitrifying MBBR carriers when transferred from a reactor treating high carbon wastewater to low carbon wastewater, and the two communities differed by only a maximum of 19%. The carbon loading in the Young et al., study was lower than the study herein, even with the high carbon wastewater. Hence, the high carbon loading, and the anaerobic conditions of the study herein are likely important factors contributing to the diversity between the microbiome communities (up to 78.1%) of the AN/AE reactor and the NIT reactors.

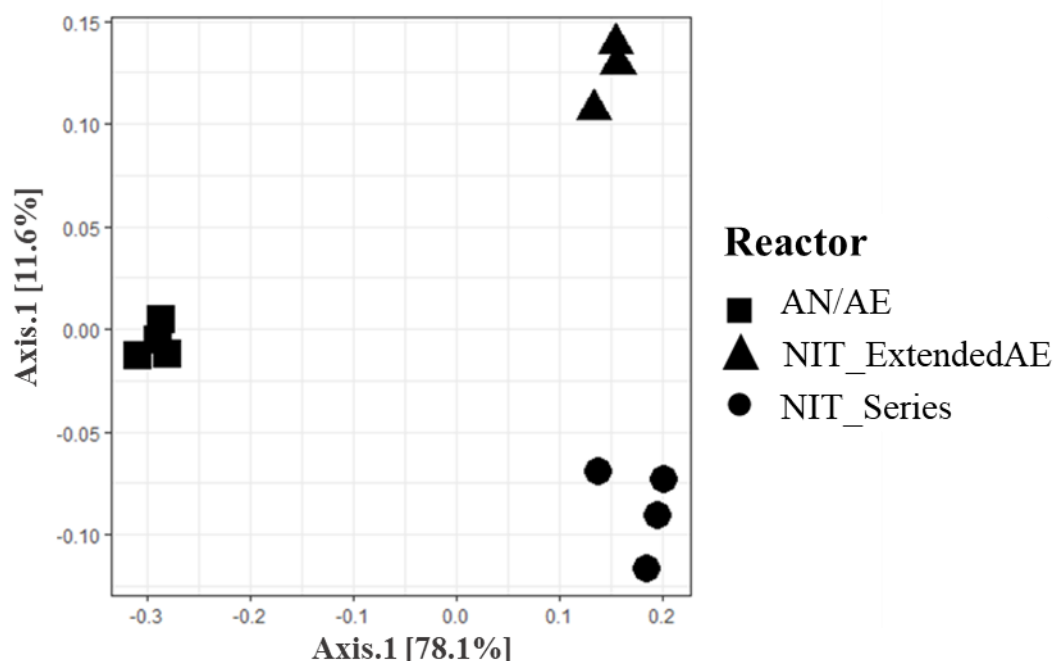


Figure 6.3 PCoA of microbiome populations, with weighted-unifrac distances between each reactor.

6.4.4 Dominant taxa

The average relative abundances of the taxa are presented at the phylum-level for each reactor (Figure 6.4), and the top ten taxa are presented down to the species level for each reactor (Table 6.3). The top phyla observed are *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Synergistetes* for the AN/AE reactor, while *Proteobacteria* dominated both the NIT_ExtendedAE and NIT_Series reactors. Previous reports of MBBRs treating carbon and TAN were dominated by *Bacteroidetes* and *Proteobacteria*, while nitrifying MBBRs have been shown to be dominated primarily by *Proteobacteria*, similar to the NIT reactors in this study. *Firmicutes* and *Synergistetes* were not observed to be dominant taxa in MBBRs treating carbon and TAN (Xia et al., 2008; Young et al., 2017a). Thus, the anaerobic stage likely permitted the dominance of *Firmicutes* and *Synergistetes* in the AN/AE reactor, which are mainly anaerobic taxa (Madigan et al., 2008).

An organism from the anaerobic *Rikenellaceae* family ($23.7 \pm 3.9\%$), and the anaerobic genus *Proteiniclasticum* from the *Clostridiaceae* family ($6.50 \pm 1.08\%$) are the most abundant taxa in the AN/AE reactor. The aerobic denitrifier *Dokdonella* ($11.7 \pm 2.7\%$); the AOB *Nitrosomonas europaea* are the most abundant taxa in the NIT_ExtendedAE reactor; and *Dokdonella* (8.23 ± 0.70), and the aerobic heterotroph *Ideonella* (9.10 ± 3.77) are the most abundant taxa in the NIT_Series reactor. The dominant bacteria shift from anaerobic bacteria to aerobic and nitrogen removing bacteria when comparing the AN/AE reactor and the NIT reactors, supporting the microbiome shift observed, between the AN/AE reactor and the NIT reactors through beta diversity analysis (Figure 6.3). Since a microbiome shift is required for the NIT_ExtendedAE to achieve TAN oxidation, from being dominated by anaerobic bacteria to being dominated by nitrogen removing bacteria, the extended aerobic operation is not feasible to achieve TAN oxidation in a single SB-MBBR treating cheese production wastewater. The most abundant taxa in the NIT_ExtendedAE reactor were nitrogen removing bacteria, while the most abundant taxa were aerobic heterotrophs in the NIT_Series reactor; despite the NIT_Series reactor having a higher TAN SARR and removal efficiency. The dominance of aerobic heterotrophs in the NIT_Series reactor substantiates that the peripheral heterotrophic community supports the nitrifiers in the biofilms of MBBRs performing TAN oxidation (Bae et al., 2015; Young et al., 2017b).

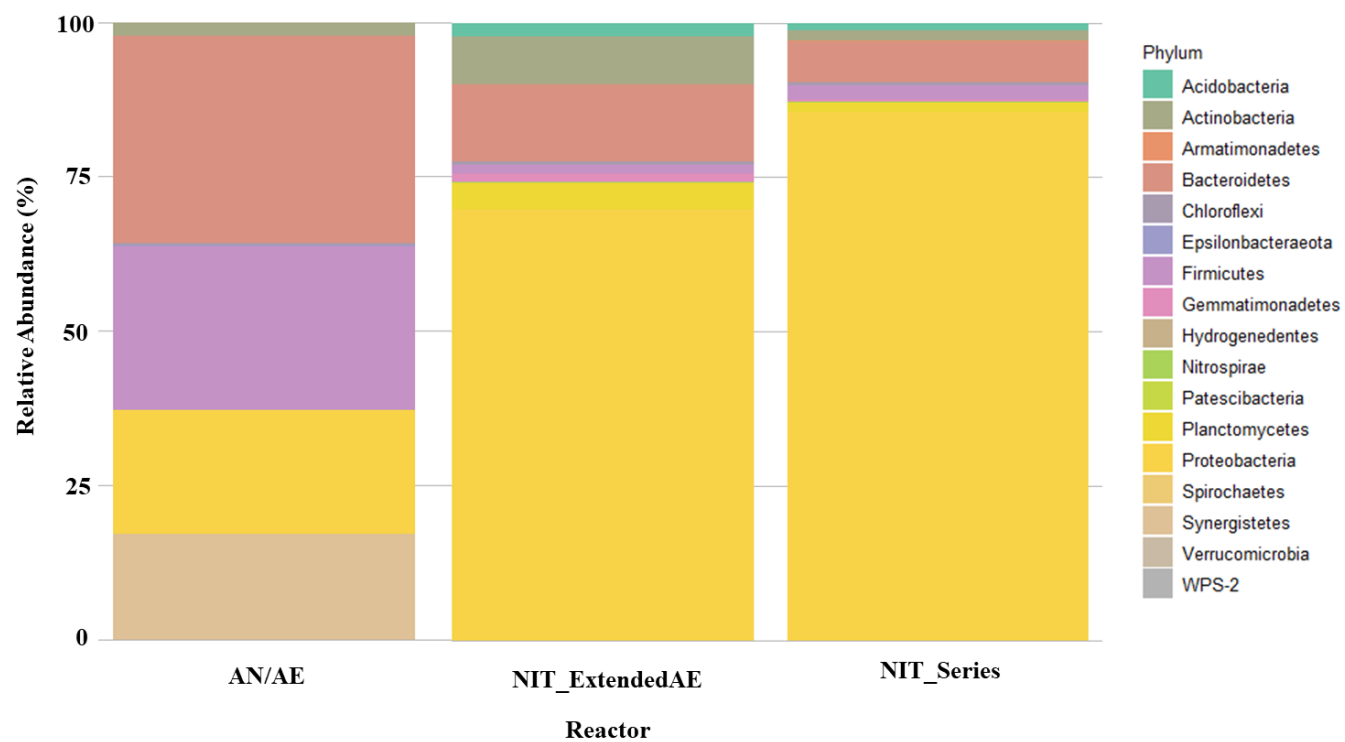


Figure 6.4 Average relative abundances of the taxa at the phylum level for each reactor.

Table 6.3 Average and 95% confidence interval of the percent abundance of the 10 most abundant taxa identified for each reactor.

Trophic level				Reactor		
Phylum	Family	Genus	Species	AN/AE	NIT_ExtendedAE	NIT_Series
<i>Actinobacteria</i>	<i>Dermatophilaceae</i>	<i>midas_g_724</i>	<i>midas_s_725</i>	0.565±0.090	4.79±0.72	0.737±0.298
<i>Bacteroidetes</i>	<i>Rikenellaceae</i>	<i>dgA-11_gut_group</i>	<i>midas_s_8005</i>	23.7±3.9	0.001±0.001	-
<i>Bacteroidetes</i>	<i>Rikenellaceae</i>	<i>DMER64</i>	<i>midas_s_3329</i>	3.66±0.19	0.006±0.002	0.015±0.003
<i>Bacteroidetes</i>	<i>env.OPS_17</i>	<i>midas_g_3838</i>	<i>midas_s_3838</i>	-	3.87±0.57	0.003±0.002
<i>Firmicutes</i>	<i>Clostridiaceae_1</i>	<i>Proteiniclasticum</i>	<i>midas_s_140</i>	6.50±1.08	0.330±0.100	0.503±0.205
<i>Firmicutes</i>	<i>Family_XI</i>	<i>Sedimentibacter</i>	<i>midas_s_6440</i>	4.78±0.63	0.014±0.005	0.001±0.001
<i>Firmicutes</i>	<i>Lachnospiraceae</i>	<i>XBB1006</i>	<i>midas_s_2985</i>	2.96±1.13	0.002±0.002	0.0003±0.0003
<i>Proteobacteria</i>	<i>Acetobacteraceae</i>	<i>Roseomonas</i>	<i>Roseomonas_lacus</i>	-	2.16±0.27	0.647±0.251
<i>Proteobacteria</i>	<i>Rhodobacteraceae</i>	<i>Defluviimonas</i>	<i>midas_s_337</i>	0.009±0.0001	1.85±0.08	5.61±1.01
<i>Proteobacteria</i>	<i>Rhodobacteraceae</i>	<i>Gemmobacter</i>	<i>Gemmobacter_aquaticus</i>	0.058±0.010	2.46±0.21	2.95±0.423
<i>Proteobacteria</i>	<i>Burkholderiaceae</i>	<i>Brachymonas</i>	<i>Brachymonas_denitrificans</i>	3.74±0.36	1.98±0.35	11.4±2.74
<i>Proteobacteria</i>	<i>Burkholderiaceae</i>	<i>Comamonas</i>	<i>midas_s_3904</i>	0.000	0.086±0.013	3.44±1.47
<i>Proteobacteria</i>	<i>Burkholderiaceae</i>	<i>Ideonella</i>	<i>midas_s_1694</i>	0.000	0.077±0.004	9.10±3.77
<i>Proteobacteria</i>	<i>Burkholderiaceae</i>	<i>midas_g_33</i>	<i>midas_s_264</i>	0.000	2.23±0.34	0.010±0.003
<i>Proteobacteria</i>	<i>Burkholderiaceae</i>	<i>Ottowia</i>	<i>midas_s_482</i>	0.874±0.027	5.33±0.72	2.06±0.24
<i>Proteobacteria</i>	<i>Burkholderiaceae</i>	<i>Simplicispira</i>	<i>Simplicispira_piscis</i>	1.02±0.11	6.30±0.80	4.96±1.05
<i>Proteobacteria</i>	<i>Nitrosomonadaceae</i>	<i>Nitrosomonas</i>	<i>Nitrosomonas_europaea</i>	0.004±0.002	6.93±1.61	1.85±0.38
<i>Proteobacteria</i>	<i>Rhodocyclaceae</i>	<i>Dechloromonas</i>	<i>midas_s_96</i>	3.23±1.03	0.643±0.813	0.008±0.004
<i>Proteobacteria</i>	<i>Moraxellaceae</i>	<i>Acinetobacter</i>	<i>Acinetobacter_parvus</i>	3.07±1.00	0.019±0.003	0.113±0.033
<i>Proteobacteria</i>	<i>Rhodanobacteraceae</i>	<i>Dokdonella</i>	<i>midas_s_124</i>	0.039±0.015	11.7±2.7	8.23±0.70
<i>Proteobacteria</i>	<i>Rhodanobacteraceae</i>	<i>midas_g_116</i>	<i>midas_s_3216</i>	0.009±0.002	0.006±0.002	2.42±0.96
<i>Proteobacteria</i>	<i>Xanthomonadaceae</i>	<i>Stenotrophomonas</i>	<i>midas_s_7952</i>	0.011±0.004	4.01±0.74	4.92±1.09
<i>Synergistetes</i>	<i>Synergistaceae</i>	<i>midas_g_249</i>	<i>midas_s_2374</i>	3.56±2.61	0.032±0.022	0.007±0.005
<i>Synergistetes</i>	<i>Synergistaceae</i>	<i>midas_g_249</i>	<i>midas_s_249</i>	4.78±0.54	0.015±0.005	0.005±0.004

6.4.5 AOB and NOB populations

The AOB and NOB taxa are grouped for each reactor to support that TAN oxidation occurs through biological activity in the NIT reactors (Figure 6.5). The AOB genus detected in this study is *Nitrosomonas*; the NOB genera detected are: *Nitrobacter*, *Nitrospira*, and *Nitroga*. Each organism is represented by an abundance relative to the total population, and not as total counts. The AOB populations are present at relative abundances of 0.0207±0.0029%, 7.01±2.44%, and 3.55±0.68% for the AN/AE reactor, the NIT_ExtendedAE reactor, and the NIT_Series reactor. The NOB relative abundances are 0.366±0.0675%, 0.707±0.236%, and 0.278±0.045% for the AN/AE reactor, the NIT_ExtendedAE reactor, and the NIT_Series reactor. The AOB relative abundances

are significantly higher in the NIT_ExtendedAE and the NIT_Series reactors compared to the AN/AE reactor ($p < 0.04$). The AOB relative abundances in the NIT reactors are in range of previous reports of nitrifying MBBRs, however the NOB population is lower than previous reports of nitrifying MBBRs (Xia et al., 2008; Young et al., 2017a; Young et al., 2017b). These findings support that TAN is oxidized to nitrite in both the NIT reactors through PN, considering AOBs are selected for and NOBs are not. PN is likely due to elevated TAN concentrations that suppresses nitrite oxidation (Schopf et al., 2019; Rong et al., 2020). With a high influent TAN concentration, competition for DO between AOB and NOB populations arise within the biofilm, with NOB populations being outcompeted (Rong et al., 2020). Thus, PN is feasible with SB-MBBRs treating cheese production wastewater, and therefore can be incorporated in a deammonification system. Quantitative assessment of the AOB and NOB activity through quantitative PCR should be performed to verify that PN is being achieved in the SB-MBBR. *Nitrosomonas* is an AOB frequently observed in nitrifying MBBRs (Xia et al., 2008; Bae et al., 2015; Young et al., 2017a). *Nitrospira* is an NOB commonly observed in nitrifying MBBRs (Xia et al., 2008; Bae et al., 2015; Young et al., 2017a), however *Nitrobacter* is not typically observed in wastewater treatment systems (Kelly et al., 2005). Possibly the cycling between anaerobic and aerobic conditions in the SB-MBBRs, and the components of the cheese production wastewater may have permitted the selection of *Nitrobacter*, which is not typically observed with MBBRs treating lower carbon wastewaters, with continuous and solely aerobic conditions.

The AOB population being only present at $0.0207 \pm 0.0029\%$ in the AN/AE reactor substantiates that a significant population of AOBs was not present in the biofilm of the AN/AE reactor to permit nitrification. The relative abundance of AOBs is lower than previous reports of an IFAS system operating with a high C:N ratio (3:1), and achieving TAN removal of only $27 \pm 1\%$,

where the AOB relative abundance was $2.0 \pm 1.5\%$ (Young et al., 2017a). These findings indicate that the significant beta diversity between the AN/AE reactor and the NIT reactors (Figure 6.3) is in part due to the selection of an AOB population in the NIT reactors, that is not present in the AN/AE reactors. Therefore, in the NIT_ExtendedAE, a significant microbiome shift is required to develop a sufficient AOB population to achieve TAN oxidation. This finding supports that the extended aerobic operation is not a feasible strategy to achieve TAN oxidation, and therefore a single SB-MBBR cannot be operated to achieve simultaneous carbon, phosphorous and TAN removal when treating cheese production wastewater. By operating the two SB-MBBRs in series, the C:N nitrogen ratio in the second reactor (NIT_Series) is lowered to 0.5:1, permitting the AOBs to proliferate in the SB-MBBR_Series reactor by averting competition from the heterotrophic organisms. Therefore, operating two SB-MBBRs in series is the suggested operational strategy to achieve carbon, phosphorous and TAN oxidation with the SB-MBBR technology treating cheese production wastewater.

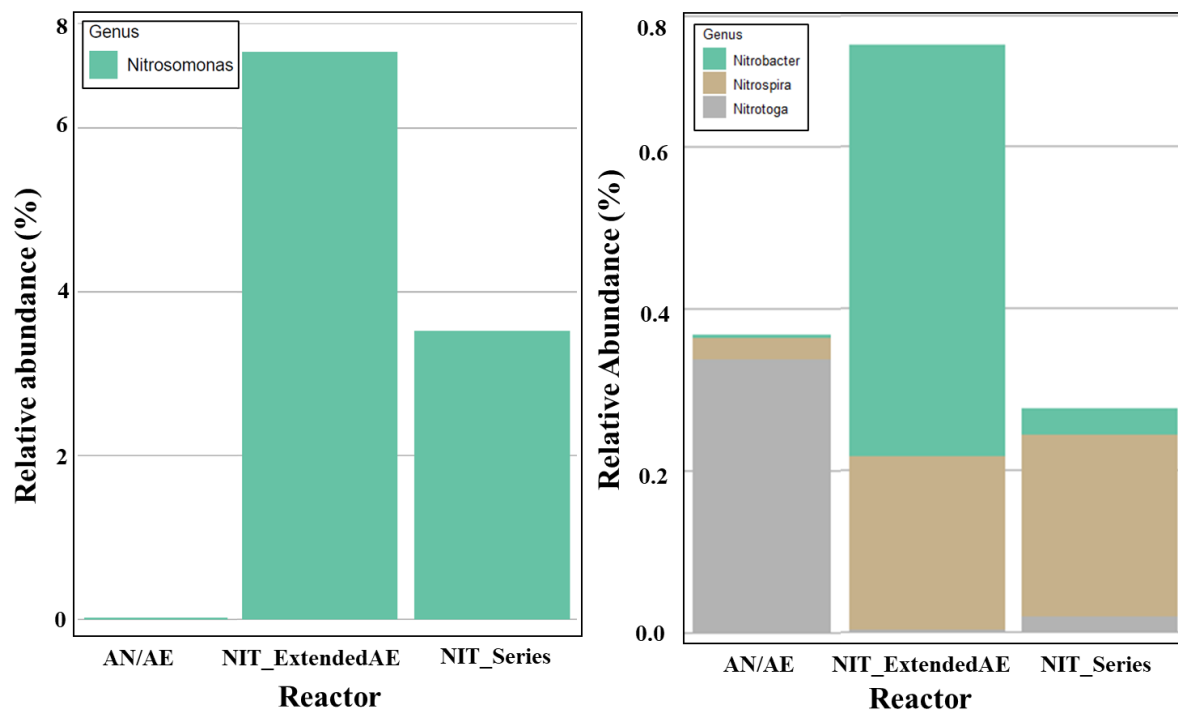


Figure 6.5 Average relative abundance of AOB (left) and NOB (right) genera for each reactor.

6.5 Conclusion

Achieving TAN oxidation with the NIT_ExtendedAE reactor requires 810 hours before TAN oxidation is achieved, since sufficient AOB population is not present in the AN/AE reactor to permit TAN oxidation, even after carbon is depleted. Thus, two SB-MBBRs should be operated in series, whereby the autotrophic nitrifying population can thrive by separating the autotrophic nitrifiers for the heterotrophs, thereby averting competition for oxygen. The NIT_Series reactor achieves a TAN removal efficiency of $98.7 \pm 2.4\%$, and a TAN SARR of $1.05 \pm 0.03 \text{ g-N} \cdot \text{m}^{-2} \text{ d}^{-1}$. The AOB population is present at a relative abundance of $8.49 \pm 2.24\%$ in the NIT_Series reactors, while an NOB population is not selected for. Thus, the SB-MBBR technology can be implemented in a deammonification system for the sustainable on-site treatment of TAN from cheese production wastewater. By operating with two SB-MBBRs in series, an AOB population can be selected for in the second SB-MBBR permitting the on-site treatment of TAN from cheese production wastewater.

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7 Conclusion, Discussion & Future Studies

7.1 Design and operation of sequencing batch moving bed biofilm reactor for treating carbon, nitrogen, and phosphorous from cheese production wastewater

This thesis investigates the potential along with the design and operation of the sequencing batch moving bed biofilm reactor (SB-MBBR) technology for the biological removal of carbon, nitrogen, and phosphorous from cheese production wastewater. The first investigated design in this dissertation is a single SB-MBBR reactor operating with anaerobic and aerobic staging. The dissertation ascertains that increasing anaerobic staging times increases the soluble chemical oxygen demand (sCOD) surface area removal rate (SARR) to $92.5 \pm 2.8 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ in the aerobic stage, beyond previous reports of the moving bed biofilm reactor (MBBR) technology. The sCOD SARR, however, does not increase with increasing aeration rate. The sCOD SARR increases with the longer anaerobic staging, and therefore with shorter aerobic staging, resulting in a more cost- and energy-efficient system, which is in-line with the design and operational initiatives of modern wastewater treatment facilities (Ødegaard, 2016; Henriques and Catarino, 2017). Furthermore, this research extends beyond the macro-scale and provides new and fundamental knowledge at the meso-, micro-, and molecular-scale. These analyses substantiate that with increasing anaerobic staging time, a biofilm is selected for with a rougher biofilm surface, a lower biofilm dry-density, a lower biofilm thickness, and therefore likely an enhanced biomass transport, which likely leads to the increase in sCOD SARR.

The TN SARR decreases with anaerobic staging time, contrary to the increase in sCOD SARR with increasing anaerobic staging times. Hence, a suggested anaerobic staging time of 130 minutes is identified for the SB-MBBR configuration in this dissertation, where an overall sCOD

SARR of $31.1 \text{ g}\cdot\text{m}^{-2}\text{d}^{-1}$ and TN SARR of $1.3 \text{ g}\cdot\text{m}^{-2}\text{d}^{-1}$ would be achieved. Increasing the aeration rate does not affect the TN removal kinetics. TN likely is removed through heterotrophic nitrification and denitrification in the SB-MBBR operated under each condition, corroborated by the C:N ratios being removed in each reactor, which range from 100:38 to 100:53, and the dominance of denitrifying organisms *Brachymonas*, *Dechloromonas*, and *Rhodoferax* (Leta et al., 2005; McIlroy et al., 2016) in the biofilm of the SB-MBBRs.

This thesis presents the first study to achieve enhanced biological phosphorous removal (EBPR) with the SB-MBBR technology treating cheese production wastewater. EBPR occurs with lower aeration ($2.4 \text{ L}\cdot\text{min}^{-1}$), whereby total phosphorous (TP) contents ($4.1\pm0.2\%$ – $4.5\pm0.2\%$) are greater than what would be expected from assimilation of phosphorous by ordinary heterotrophs for each tested anaerobic stage (Gujer et al., 1995; Fagerbakke et al., 1996). Both increasing the anaerobic staging time and decreasing the aeration rate improved the SARR of TP. Furthermore, increasing the anaerobic staging time above 72 minutes, and operating with the lower aeration selects for a higher abundance of putative denitrifying PAOs, including *Brachymonas* and *Dechloromonas* up to $43.1\pm8.4\%$ in biofilms of the SB-MBBRs. With both increasing anaerobic staging, and lower aeration, there is a microbiome shift from a community dominated by fermenting bacteria to a community dominated by putative PAOs.

7.1.1 Future studies

For each configuration the constituents are depleted following approximately 6 hours of operation. Therefore, future studies should investigate operating the system with a shorter aerobic stage. However, the duration of the aerobic stage, in particular the duration of time under “starvation” when carbon is depleted, will likely affect the poly-phosphate (poly-P) accumulating organism (PAO) population (WEF, 2011). Thus, future studies should look to optimize the aerobic stage, to

lower the overall cycle time, without impairing the PAO activity. The *Dechloromonas* (Petriglieri et al., 2020) and *Brachymonas* (Shi and Lee, 2007) have been shown to store poly-P, on the other hand, *Rhodoferrax* has yet to be observed to store poly-P. However, *Rhodoferrax* is a member of the *Comamonadaceae* family, from which taxa have been shown to store poly-P (Ge et al., 2015), and the *Comamonadaceae* family has been shown to dominate sequencing batch reactors performing EBPR from dairy waster (Gil-Pulido et al., 2018). Therefore *Rhodoferrax* may also be a putative PAO. Future studies should further investigate the metabolism of *Brachymonas*, *Dechloromonas*, and *Rhodoferrax* to improve the understanding of the PAO metabolism.

This dissertation demonstrated that controlling the operational conditions permitted control of the biofilm morphology and microbiome, which lead to improvements of the SB-MBBR performance. Given the correlation between aeration supplied to the system and biofilm thickness, future studies should further investigate the optimum aeration to achieve the optimum mass transfer into the biofilm. Furthermore, the studies with the z-carriers could be implemented to control the biofilm thickness, and in turn control the mass transfer of the MBBR. Also, future studies can evaluate the effects of increasing the anaerobic staging time further, and potentially select for a more specialized microbiome community to achieve EBPR. Further investigation into the microbiome community, including whole genome sequencing, transcriptomics, and proteomics could provide more accurate information about the microbiome community. While metabolomics and quantitative assessment of the metabolic activity could give insight to the EBPR activity and partial nitrification (PN), which could allow for improved design of the SB-MBBR treating phosphorous and nitrogen.

The thesis herein demonstrates the feasibility of EBPR with SB-MBBRs for on-site treatment of cheese production wastewater at the laboratory scale, with synthetic wastewater, and

at room temperature. However, prior to full-scale implementations of the SB-MBBR for on-site treatment, investigations of the operation with real cheese production wastewater, and scaled-up to pilot- and full-scale is required. Furthermore, the SB-MBBR operation should be evaluated at cold temperatures, before the SB-MBBR can be implemented for Canadian cheese production facilities, and for other cold climates. For EBPR, previous studies noted that PAOs have an advantage over competing organisms at cold temperatures as low as 10°C (Panswad et al., 2003; Lopez-Vazquez et al., 2009). Although Canadian wastewater reach temperatures of 1°C, EBPR may be an appropriate treatment regime for phosphorous given the advantage at cold temperatures.

7.2 Design and operational optimization strategies to achieve total ammonia nitrogen oxidation of cheese production wastewaters using the sequencing batch moving bed biofilm reactor technology

The dissertation investigates various design and operation strategies, including a single SB-MBBR and two SB-MBBRs operated in-series to achieve total ammonia nitrogen (TAN) removal from cheese production wastewater. The research concludes that the extended aerobic operation is not feasible for achieving TAN oxidation with carbon and phosphorous removal in a single SB-MBBR treating cheese production wastewater. With a single SB-MBBR, autotrophic nitrifiers are outcompeted by heterotrophs given the high carbon loading, and high C:N ration of the cheese production wastewater. Instead, with two SB-MBBRs operated in series, a TAN removal efficiency of $98.7 \pm 2.4\%$, and TAN SARR of $1.07 \pm 0.05 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ is achieved. The two SB-MBBRs operating in series achieves TAN oxidation by averting competition for oxygen between heterotrophs and autotrophic nitrifiers. The two strategies for achieving TAN removal with SB-MBBRs were studied at the meso-, micro-, and molecule scale, and provided new and fundamental information of a high-loaded SB-MBBR treatment system. With the two SB-MBBRs operated in

series, the ammonia oxidizing bacteria (AOB) populations were present at relative abundances above $8.49 \pm 2.24\%$, and nitrite oxidizing bacteria (NOB) are not selected for. Thus, TAN oxidation is likely achieved through PN with the two SB-MBBRs in series. Therefore, this study is the first to show the potential of PN with SB-MBBRs treating cheese production wastewater.

7.2.1 Future studies

Since the thesis demonstrates the achievement of PN in the SB-MBBR, deammonification processes are therefore feasible for on-site treatment of cheese production wastewater. Thus, future studies should investigate incorporating anaerobic ammonia oxidation (ANAMMOX) with PN to achieve the treatment of nitrogen with lower oxygen inputs, and without the need for carbon (Blackburne et al., 2008; Zeng et al., 2013). The SB-MBBR with extended aerobic operation, and the two SB-MBBRs operated in series were both operated at the laboratory scale, with synthetic wastewater, and at room temperature. As previously stated, investigations of the operation with real cheese production wastewater, scaled-up to pilot- and full-scale, and at cold temperatures is required prior to implementation of the SB-MBBR for on-site treatment of cheese production wastewater in Canada, and in other cold climates. Nitrification with the MBBR technology is known to be feasible for cold climates (Hoang et al., 2014), and as such the SB-MBBR is likely an appropriate technology for treating TAN from cheese production at cold temperatures. A previous report demonstrated that PN and anammox is stable in MBBRs at temperatures as low as 15°C . However, anammox activity was suppressed when the MBBRs were operated below 11°C , and thus operating deammonification at low temperatures may be a challenge during the winter in cold climates (Laureni et al., 2016).

With the two SB-MBBRs operating in series, PAO cells are passed from the first SB-MBBR, where EBPR is achieved, to the second reactor and lyse, as evidenced by the decrease in

volatile suspended solids (VSS) with an increase in TP, at a ratio of $0.150 \pm 0.003 \text{ g-P} \cdot \text{g-VSS}^{-1}$. This observation supports that PAO activity in the first SB-MBBR occurs, however given the increase in TP concentrations in the second SB-MBBR, future applications of this system should introduce a solids separation strategy in-between the two SB-MBBRs in series. Various technologies can be applied for solids separation, which include sedimentation, filtering, centrifugation, chemical processes, and flotation (Metcalf & Eddy, 2014). Specifically for on-site treatment of industrial wastewater the upflow anaerobic sludge blanket combined with a septic tank, as well as the anaerobic baffled reactors are examples of solids separation technologies that have been incorporated (Elmitwalli et al., 2003; Luostarinen, 2005). Since the excess sludge from the SB-MBBRs is rich in phosphorous, the configuration herein presents the possibility for phosphorous recovery, providing a source of this non-renewable resource, which can be utilized as fertilizers (AR, 2009; Yuan et al., 2012; Nielsen et al., 2019).

7.3 Synthesis

Current on-site treatment of cheese production wastewater employs lagoons of which have retention times in the order of months (Metcalf & Eddy, 2014). Given the retention time of the SB-MBBR treatment demonstrated in this thesis is only 16 hours, the present study shows that a significant reduction in size would be attributed to upgrading treatment to the SB-MBBR technology. Furthermore, these systems can be easily upgraded with the SB-MBBR technology in existing tanks at cheese production facilities (Andreottola et al., 2003). Pilot-scale studies should be implemented however to determine the precise sizing of the SB-MBBR for the treatment of cheese production wastewater for specific sites.

Energy inputs are in the order of 0.3-0.6 kwh for nutrient removal (Ødegaard, 2016), and a large portion is derived from the energy required for aeration. With the main findings of this

study presenting a significant increase in carbon removal efficiency with less aeration, the findings present a more cost and energy efficient system. The increase in carbon removal rates was associated with a clear shift in the microbiology of the SB-MBBR. Specifically, changes in operational conditions was associated with a shift in the biofilm morphology and microbiome, thus this dissertation demonstrates the knowledge of the microbiology of the SB-MBBR can lead to improved designs of the system.

The potential for the SB-MBBR to be incorporated into a deammonification system presents yet another opportunity for reducing aeration requirements for removal in the system. Also, the achievement of EBPR removal with the SB-MBBR demonstrates a more economical means of removing TP could be achieved from cheese production wastewater compared to chemical treatment. Furthermore, this dissertation presents the potential for phosphorous recovery given the phosphorous-rich solids derived from the SB-MBBR. Overall, this dissertation supports that the SB-MBBR technology can be operated to achieve biological removal of carbon, nitrogen, and phosphorous, and in such a way that is in line with modern wastewater treatment goals, which include a compact technology, that is energy efficient, and has the potential for resource recovery.

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